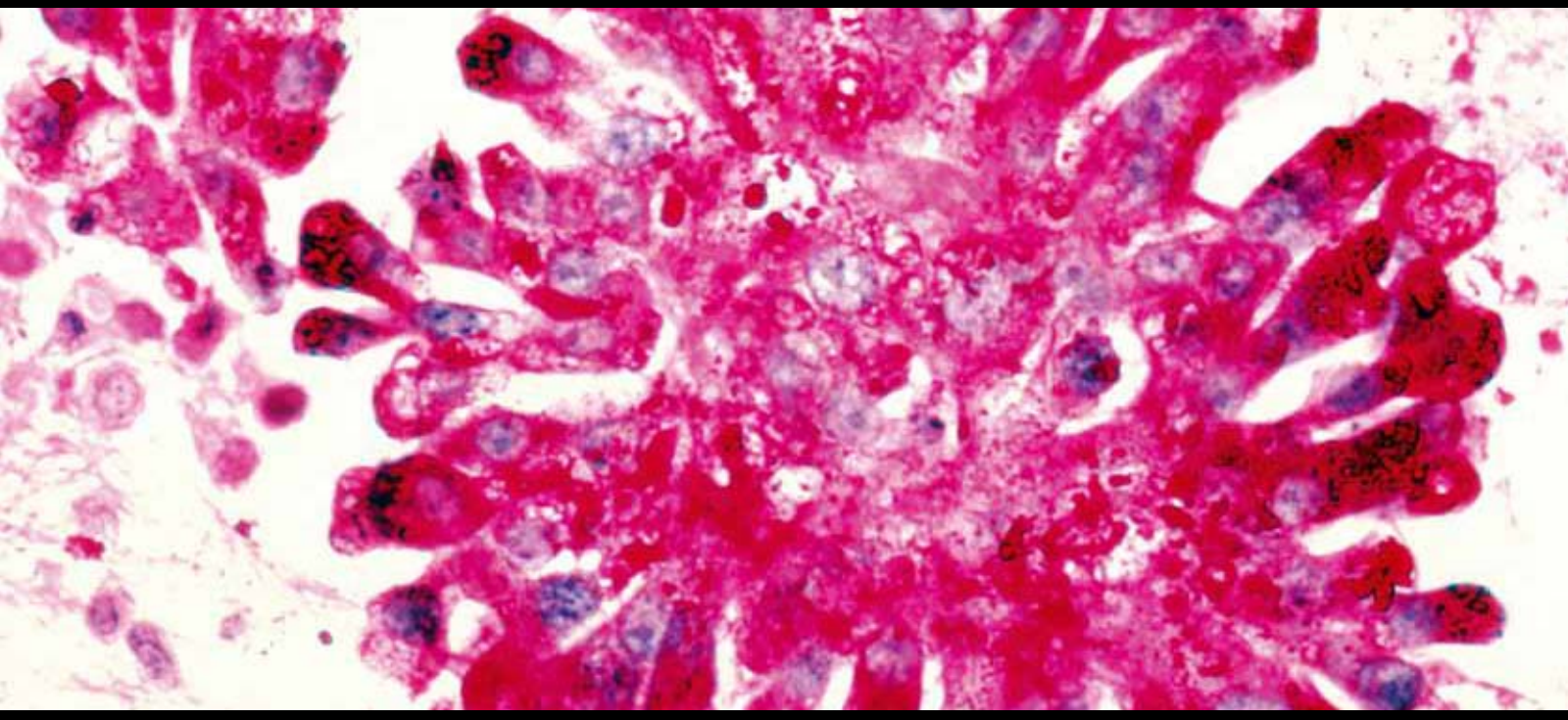
The Molecular Basis of Sarcoma

Guest Editors: Stephen L. Lessnick, Heinrich Kovar, and Peter Houghton



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Editorial Board

Contents

Contents

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Editorial
The Molecular Basis of Sarcoma

Stephen L. Lessnick,[1] Heinrich Kovar,[2] and Peter Houghton[3]

[1] *Huntsman Cancer Institute, Center for Children's Cancer Research, 2000 Circle of Hope, Salt Lake City, UT 84112, USA*
[2] *Children's Cancer Research Institute, St. Anna Kinderkrebsforschung, 1090 Vienna, Austria*
[3] *Center for Childhood Cancer, Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205, USA*

Correspondence should be addressed to Stephen L. Lessnick, stephen.lessnick@hci.utah.edu

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The sarcoma field is at a crossroads. There have been incredible advances in the identification and characterization of key genetic events associated with sarcoma development. For example, many chromosomal translocation breakpoints have been cloned, and the fusion proteins associated with those breakpoints have been subjected to rigorous molecular analysis. Similarly, there has been an explosion of molecularly targeted agents available for the treatment of patients with cancer. In cases where these targeted agents inhibit a key abnormality in sarcoma, such as activated KIT in gastrointestinal stromal tumor, they have been quite successful. Unfortunately, examples of such success are still limited. Many investigators in the field have hoped that deeper understanding of the molecular basis of sarcoma may lead to new therapeutic opportunities for this varied set of diseases.

It is with this spirit that the current special issue is presented. Twenty-three papers are included, which cover a vast range in the field and include a mix of both original research and timely reviews. Through the process of presenting a broad array of molecular topics related to sarcoma development, it is our hope that investigators and other interested parties will recognize both common threads and unique issues across many different subtypes of sarcoma and that such recognition will stimulate new research directions that will lead to new cures for patients suffering from these diseases.

In the first paper of the special issue, "*Epigenetic regulation of apoptosis and cell cycle in osteosarcoma*," Kleinerman and K. Rao-Bindal critically discuss frequency and prognostic impact of epigenetic inactivation of p16/p14ARF, HIC1, and RASSF1A in this disease, as well as the possible role of histone H3 lysine 27 monomethylation in osteosarcoma apoptosis. This provides unique insights into the role of epigenetics as a molecular basis for osteosarcoma development.

High-dose methotrexate is a mainstay of modern osteosarcoma therapy. While osteosarcomas respond well to high-dose methotrexate, they do not respond well to conventional doses of the drug. The second article, "*Impairment of methotrexate transport is common in osteosarcoma tumor samples*" by R. Sowers et al., demonstrates that methotrexate transport is impaired in high-grade osteosarcoma. This suggests that high doses of the agent are required to overcome this transport impairment, and implies that antifolate agents that are not dependent on the common transport pathway might be more effective for this disease.

In the third article, "*The molecular pathogenesis of osteosarcoma: a review*," M. L. Broadhead et al., provide an extensive and timely discussion of the current knowledge on the etiology of the disease and more frontline translational studies aiming at targeted osteosarcoma therapy.

In the next review, "*Osteosarcomagenesis: modeling cancer initiation in the mouse*," K. B. Jones traces the historical pathways for developing nonclinical models of osteosarcoma to the more contemporary genetically engineered mouse models and how they recapitulate the human disease. The work presents early approaches to creating non-clinical models that involved random mutagenesis, bone seeking radionuclides, external beam radiation, viral insertional mutagenesis and how these models informed human osteosarcoma. The review also details current approaches using gene targeting to engineer mouse models, and their value and limitations.

Although osteosarcoma displays features of poorly differentiated osteoprogenitors, the exact histogenesis is still unknown. In the fifth article, "*Defective osteogenic differentiation in the development of osteosarcoma*," W. R. Wagner

et al., assess the problem of osteosarcoma pathogenesis from a developmental angle. In their review they discuss osteosarcoma as a disease of impaired differentiation, and summarize the rationale and first data on the use of differentiation inducing agents as a potential therapeutic strategy in this disease.

Understanding normal development and how these processes go awry in cancer has important implications for understanding tumorigenesis, and for considering new therapeutic approaches. With this in mind, the sixth article, "*The role of RUNX2 in osteosarcoma oncogenesis*," by J. W. Martin et al., reviews the RUNX2 transcription factor and its potential role in osteosarcoma. RUNX2 is a DNA-binding transcription factor that is involved in normal bone development. RUNX2 is often overexpressed in osteosarcoma, and so its normal functions may also be important for the development of the tumor.

Continuing on with the osteosarcoma theme, the seventh article, "*Using epidemiology and genomics to understand osteosarcoma etiology*," by S. A. Savage and L. Mirabello, provides a comprehensive review of the epidemiology of osteosarcoma, and also reviews the known genomic mutations and variants that are associated with osteosarcoma development. This review is particularly timely given expanding interest in large-scale studies of pediatric cancer epidemiology and genomics.

Transcription factor fusions involving the EWS gene or one of its relatives FUS or TAF15 in sarcomas or rare leukemias are considered dominant oncogenes. However, since EWS family proteins are ubiquitously expressed housekeeping proteins, allelic rearrangements may result in haploinsufficiency or even a dominant negative effect on the remaining expressed allele. Nuclear localization is considered an essential prerequisite for the normal function of EWS family proteins. In the eighth paper, "*Tyrosine phosphorylation in the C-terminal nuclear localization and retention signal (C-NLS) of the EWS protein*," R. P. Leemann-Zakaryan et al., describe the role of C-terminal phosphorylation in physiological control of EWS nuclear localization, which is lost upon rearrangement with a transcription factor moiety in sarcoma.

In the ninth article, "*Dr. Jekyll and Mr. Hyde, the two faces of theFUS/EWS/TAF15 protein family*," H. Kovar reviews the interesting dual personality of the FUS/EWS/TAF15 proteins. On the one hand, these proteins have a variety of wild-type functions that are important to normal cellular behavior that are slowly being worked out. On the other hand, they are evil fusion partners in oncogenesis. It seems likely that the oncogenic functions (both transcriptional and nontranscriptional) are related to the normal functions of these proteins.

In the next article, "*Copy number alterations (CNAs) and methylation in Ewing's sarcoma*," M. S. Jahromi et al., provide a comprehensive literature review of available data reporting copy number alterations (CNA's), alterations in mitochondrial DNA, and gene silencing by methylation for Ewing's sarcoma. The potential implication of trisomy 8 and 12, gains on chromosome 2q, and deletion or methylation silencing of the CDKN2A (p16-INK4a) locus, or genes involved in the extrinsic death pathway for patient outcome are reviewed. This work provides an overview of intriguing data, limited by sample size, that points to the pathogenesis of Ewing's sarcoma, and the potential to impact treatment outcome through large-scale studies that are now possible using archival tissue.

In the eleventh article, "*Targeting angiogenesis in childhood sarcomas*," H. K. Bid and P. J. Houghton provide a timely and comprehensive evaluation of the literature regarding angiogenesis and vasculogenesis in sarcomas (particularly, pediatric sarcomas). In addition to reviewing the data regarding the biologic basis for these processes, the authors also review a series of therapeutic strategies based on inhibiting angiogenesis and vasculogenesis, in sarcoma.

In the twelfth article, "*Immune-based therapies for sarcoma*," S. M. Pollack et al., point out that there are great unmet needs in the systemic therapy of sarcomas and that nonchemotherapeutic strategies might be exploited for this role. The authors provide an important review of immunotherapy in sarcoma and discuss a variety of therapeutic trials and concepts in the field, including nonspecific immunomodulation and targeted immunotherapy approaches. This review highlights the opportunities, and remaining challenges, that exist in allowing immunotherapy to become a part of the armamentarium to treat sarcomas in the future.

IGF signaling is an important component of the machinery driving cellular growth in embryonal tissues and in many tumors. It was therefore expected that anti-IGF therapy should be a promising therapeutic option in the treatment of cancer, particularly of sarcomas in which IGF signaling is constitutively activated. In the thirteenth article, "*Targeting the insulin-like growth factor pathway in rhabdomyosarcomas: rationale and future perspectives*," A. S. Martins et al., discuss the role of IGF signaling in rhabdomyosarcoma and summarize first clinical experience and so far unexplored options of combination chemotherapy.

The article by L. E. S. Crose and C. M. Linardic, "*Receptor tyrosine kinases as therapeutic targets in rhabdomyosarcoma*," reviews the current knowledge regarding expression of tyrosine kinase receptors in rhabdomyosarcoma. The review presents up-to-date information on expression in clinical samples of specific receptor kinases including members of the epidermal, hepatocyte, fibroblast, platelet and insulin-like growth factors and their potential role in tumorigenesis as shown in genetically engineered models. The review details current ongoing clinical studies of agents that target these receptors and discusses the future development of these agents in the context of contemporary therapeutic approaches to rhabdomyosarcoma.

Among the various cellular stresses which tumor cells have to evade in order to survive and proliferate is a markedly increased production of reactive oxygen species (ROS). In the fifteenth paper, "*The role of mirk kinase in sarcomas*", E. Friedman discusses how the activity of the serine/threonine kinase Mirk/dyrkB may prevent apoptosis of osteosarcoma and rhabdomyosarcoma cells by increasing the expression of antioxidant scavenger proteins. Dr. Friedman reviews the literature on the role of Mirk as a potential prognostic

biomarker in osteosarcoma, and on the therapeutic promise of Mirk inhibition in combination of chemotherapy with conventional anticancer drugs.

The sixteenth paper, "*miRNA profiling: how to bypass the current difficulties in the diagnosis and treatment of sarcomas*," A. Gougelet et al., demonstrate that real-time quantitative PCR approaches focused on microRNA (miRNA) signatures provide a new prognostic and diagnostic approach in two important sarcoma types: osteosarcoma and rhabdomyosarcoma. In the case of osteosarcoma, miRNA profiles changed in unique and predictive ways following exposure to chemotherapeutic agents, which suggests that such profiles might be used in a prognostic fashion. In the case of rhabdomyosarcoma, miRNA profiles were diagnostic of each of the subtypes of rhabdomyosarcoma. Thus, miRNA profiling may have important use in the diagnostic and prognostic analysis of at least some types of sarcoma.

The next paper, "*Delineation of chondroid lipoma; an immunohistochemical and molecular biological analysis*," by de Vreeze et al., presents a study from the Dutch Pathology Registry of chondroid lipomas, extremely rare benign tumors. Chondroid lipoma may exhibit histologic features resembling myoepithelioma, myxoid liposarcoma, extraskeletal myxoid chondrosarcoma, hibernoma, and other lipomatous or chondroid neoplasms, resulting in difficulties in accurate diagnosis with an appropriate treatment. The aim of this study was to delineate chondroid lipoma from several morphologic mimics by the means of immunohistochemistry. Although these tumors show high expression of CCND1, the authors rule out the CCND1 and FUS genes as candidates involved in the t(11;16)(q13;p13) previously reported as a recurrent translocation in this rare benign lipomatous tumor.

While chondrocytes are mesenchymal in nature, chondrosarcomas exhibit features of epithelial cells as well. The eighteenth article, "*Human chondrosarcoma cells acquire an epithelial-like gene expression pattern via an epigenetic switch: evidence for mesenchymal-epithelial transition during sarcomagenesis*," by M. P. Fitzgerald et al., provides evidence for epigenetic activation of a set of epithelial markers in chondrosarcomas, and downregulation of snail, as compared to chondrocytes. These data suggest that chondrosarcomas undergo a mesenchymal to epithelial transition via an epigenetic pathway.

In the next article, "*Spinal chondrosarcoma: a review,*" P. Katonis and colleagues present a comprehensive review of spinal chondrosarcoma, a rare variant comprising 10 percent of chondrosarcoma patients. The review covers histologic classification and molecular characteristics associated with disease progression, subtype classification, and risk factors. Radiologic features associated with diagnosis and staging and current approaches to therapy and prognosis are discussed.

Dermatofibrosarcoma protuberans (DFSP) is a rare cutaneous-origin sarcoma associated with constitutive activation of the receptor tyrosine kinase (RTK) PDGFR by chromosomal rearrangement with the collagen gene COL1A1. In the twentieth paper, "*Advances in molecular characterization and targeted therapy in dermatofibrosarcoma protuberans (DFSP)*," P. Rutkowski et al., discuss their clinical results obtained with the broad spectrum RTK inhibitor Imatinib in the treatment of inoperable and/or metastatic and/or recurrent cases of DFSP.

Clinically, distinguishing benign uterine leiomyoma from malignant uterine leiomyosarcoma (LMS) remains a challenge. The next article, "*Molecular approach to uterine leiomyosarcoma: LMP2-deficient mice as an animal model of spontaneous uterine leiomyosarcoma*," by T. Hayashi et al., reviews the molecular pathogenesis of LMS, with specific reference to the role of LMP2, a gene encoding a component of the immunoproteasome, in development of uterine leiomyosarcoma. They report that uterine LMS occurred in female LMP2-deficient mice at age of 6 months and the incidence at 14 months of age was about 40%. They identify LMP2, a single IFN-γ-responsive gene product, as obligatory for tumor surveillance and demonstrate a tissue-specific role for LMP2 in protection from spontaneous neoplasms of the uterus. The potential for use of LMP2 expression as a diagnostic marker to distinguish leiomyoma from LMS is proposed.

Recently, great advances have been made in understanding the molecular basis of different types of liposarcoma. In the twenty-second article, "*Liposarcoma: molecular genetics and therapeutics*," S. Young et al., review the molecular basis for liposarcoma, with a focus on recent molecular genetic data from techniques such as fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH). In some cases, these molecular changes (such as amplification of MDM2 and CDK4) suggest rational therapeutic approaches for these diseases, which are also reviewed.

Sarcomas tend to metastasize to lungs and bones, and prevention of metastasis is considered the holy grail of cancer treatment. In the final article of the issue, "*The role of chemokine receptor CXCR4 in the biologic behavior of human soft tissue sarcoma*", R. H. Kim et al., review the growing body of evidence that chemokine receptors, specifically CXCR4, play an important role in homing of sarcoma cells to lung and bones. Data are discussed that imply CXCR4 inhibitors as promising add-ons to classical chemotherapy to prevent deadly metastases in sarcoma patients.

Clearly there are great challenges, but also great opportunities, to link the molecular basis of sarcoma (and all of its relevant associated phenotypes) to new diagnostic, prognostic, and therapeutic approaches for this complex group of malignancies. We hope that the articles in this special issue provide a strong stimulus for such a linkage and will help to spur ongoing advances that will ultimately transform the care of patients with sarcoma.

Stephen L. Lessnick
Heinrich Kovar
Peter Houghton

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Research Article

Epigenetic Regulation of Apoptosis and Cell Cycle in Osteosarcoma

Krithi Rao-Bindal[1] and Eugenie S. Kleinerman[1,2]

[1] *Department of Pediatrics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA*
[2] *Division of Pediatrics, Unit 87, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*

Correspondence should be addressed to Eugenie S. Kleinerman, ekleiner@mdanderson.org

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The role of genetic mutations in the development of osteosarcoma, such as alterations in p53 and Rb, is well understood. However, the significance of epigenetic mechanisms in the progression of osteosarcoma remains unclear and is increasingly being investigated. Recent evidence suggests that epigenetic alterations such as methylation and histone modifications of genes involved in cell cycle regulation and apoptosis may contribute to the pathogenesis of this tumor. Importantly, understanding the molecular mechanisms of regulation of these pathways may give insight into novel therapeutic strategies for patients with osteosarcoma. This paper serves to summarize the described epigenetic mechanisms in the tumorigenesis of osteosarcoma, specifically those pertaining to apoptosis and cell cycle regulation.

1. Introduction

Osteosarcoma is the most common primary malignant tumor of the bone in children and adolescents. As a result of recent advances in chemotherapy, long-term survival rates for osteosarcoma patients with no detectable metastases at diagnosis have improved dramatically. However, for patients that present with metastasis or have disease recurrence, the long-term survival rate is less than 20% [1–3]. Therefore, there is an ongoing need to understand the biology of osteosarcoma progression and metastasis in order to identify new therapeutic approaches.

Numerous studies have investigated the pathogenesis of osteosarcoma. This tumor has generally been associated with alterations in genes involved in cell cycle regulation and apoptosis. Most notably, the p53 and retinoblastoma protein (Rb) pathways have been shown to play a role in the progression of osteosarcoma [4–7]. However, much of the focus has been on understanding point mutations or deletions, disregarding any potential role of epigenetic mechanisms in the inactivation of these and other important pathways.

Epigenetics involves changes in the activation of genes without altering the basic structure of DNA. This includes but is not limited to CpG island methylation within gene promoter regions and acetylation, deacetylation, and/or methylation of histone proteins [8, 9]. Epigenetic regulation has been considered a mechanism for the inactivation of tumor suppressor pathways in several types of cancer. These changes can impact gene expression, but how this may contribute to the process of tumorigenesis requires further investigation.

Recent advances in the study of the role of epigenetics in the progression of osteosarcoma have increased the understanding of the pathogenesis of this disease, an area which is complex and not well defined. Altering gene expression and the signaling pathways that control the cell cycle and apoptosis can contribute to the tumorigenic process and cell transformation from a normal to a malignant phenotype. This paper serves to review what is currently known about the effects of aberrant methylation and other epigenetic mechanisms on the regulation of cell cycle and apoptosis in osteosarcoma.

2. Rb Pathway

Retinoblastoma protein (Rb) is a tumor suppressor protein that is inactivated in several types of cancer [10]. It has been shown to play a role in cell cycle control by inhibiting entry into the S-phase, thus creating a G1 checkpoint [11, 12]. About 70% of human primary osteosarcoma tumors have molecular aberrations in the Rb gene. The most common alterations include genetic deletions, mutations, and structural rearrangements [1, 13–15]. While inactivation by hypermethylation of the Rb gene has been shown to contribute to pathogenesis of other tumor types such as retinoblastoma [16], analysis of patient samples has suggested that this inactivation may not play an essential role in the progression of osteosarcoma. In one study, only 6 of 76 patients displayed heterozygous Rb methylation and 6 out of 41 patients displayed Rb promoter methylation. It is important to note, however, that loss of the Rb gene was only detectable in 37.2% of these patients, which is considerably lower than previously reported data [17]. Therefore, further analysis of Rb methylation in osteosarcoma is warranted. It has been shown that Rb-dependent G1 arrest involves $p16^{INK4A}$ inhibition of cyclin D/cdk4 and cyclin D/cdk6 complexes, which normally initiate the phosphorylation of Rb [18]. Therefore, alterations in Rb, cyclin D, cdk4/6, or $p16^{INK4A}$ may result in a loss of the G1 checkpoint, leading to the accumulation of genetic damage which may contribute to tumor development (Figure 1). Until recently, epigenetic modifications of the $p16^{INK4A}$ gene, a tumor suppressor that is often altered in osteosarcoma cell lines [19], were not investigated. In a study with p16-negative osteosarcoma samples, 8/15 had total or partial CpG methylation of the $p16^{INK4A}$ promoter and 6/15 were pRb-negative [20]. Overall, these data suggest that in addition to other mechanisms of Rb pathway inhibition, promoter methylation of either Rb or $p16^{INK4A}$ may play a role in the disruption of cell cycle control, promoting the development of osteosarcoma.

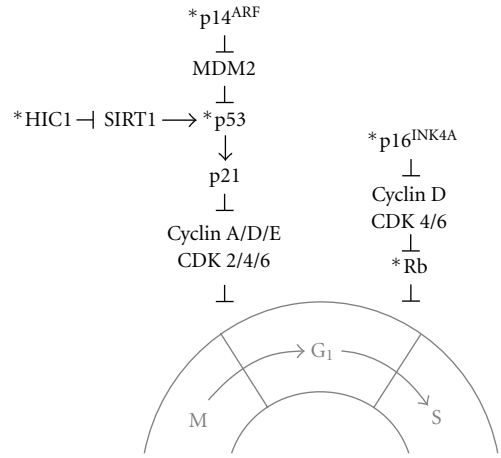

Figure 1: Schematic model of epigenetic events that regulate cell cycle progression in osteosarcoma. The cell cycle regulators Rb, p53, $p16^{INK4A}$, $p14^{ARF}$, and HIC1 have been found to be hypermethylated at the gene promoter in osteosarcoma (*). These alterations may contribute to dysregulation of cell cycle control (loss of the G1 checkpoint) and may promote tumor development.

3. p53 Pathway

The p53 gene (TP53) is known as the most commonly mutated gene in human cancers [1, 21–23]. P53 plays an important role in the regulation of apoptosis, cell cycle arrest, and DNA repair. When DNA damage occurs, p53 upregulates WAF/CIP resulting in increased p21 protein. P21 can then bind to and inhibit G1-S/CDK and S/CDK complexes to arrest cell division. If damage is irreparable, activated p53 can directly regulate the expression of apoptotic genes, resulting in the initiation of apoptosis [23]. The frequency of p53 alterations in osteosarcoma ranges from ~30% point mutations to ~80% allelic loss, suggesting that p53 status plays an important role in the tumorigenesis of osteosarcoma [1]. However, few groups have investigated the role of epigenetic regulation of p53 pathways in osteosarcoma. Recently a novel protein, hypermethylated in cancer (HIC1), was identified to modulate p53-dependent apoptosis (Figure 1). Inactivation of HIC1 results in the upregulation of SIRT1 deacetylase which then deacetylates and inactivates p53. This results in the circumvention of apoptosis and then cells are able to survive DNA damage, a process that may promote tumor development [24]. In order to investigate the mechanism for loss of function of HIC1 and p53 in osteosarcoma, Chen et al. analyzed the regulation of HIC1 in tumors of $HIC1^{+/-}$ $p53^{+/-}$ mice. Eight of 13 osteosarcomas demonstrated HIC1 1b promoter hypermethylation and 2/13 had hypermethylation of the HIC1 1a promoter. Both alterations were associated with loss of HIC1 expression, whereas tumors with abundant HIC1 expression had no apparent hypermethylation of the HIC1 promoter. In addition, 2/4 osteosarcomas in $p53^{+/-}$ mice had abundant HIC1 1b hypermethylation. This suggests that loss of HIC1 function resulting from promoter hypermethylation, along with inactivation of p53, is associated with the development of osteosarcoma. To further examine whether HIC1 promoter hypermethylation with p53 inactivation was important in the development of human osteosarcomas, Chen et al. analyzed 44 osteosarcoma patient samples. It was demonstrated that 8/21 (38%) tumors with p53 mutations and 2/23 (9%) without p53 mutations were characterized by HIC1 promoter hypermethylation [25]. In addition, it has been found that 17% of pediatric osteosarcomas display hypermethylation of the HIC1 promoter [26]. This further validates that in addition to mutation or deletion of the p53 gene, regulation of the p53 pathway by HIC1 promoter hypermethylation resulting in p53 inactivation also plays an important role in the development of osteosarcoma.

4. $p14^{ARF}$/CDKN2A

The $p14^{ARF}$ protein, which is encoded by the CDKN2A gene, is critical in the regulation of cell cycle control (Figure 1). $P14^{ARF}$ regulates p53 function by inhibiting MDM2, allowing p53 to upregulate p21 expression. P21 can then bind to and inactivate cyclin/CDK complexes, resulting in G1 arrest [27].

In an examination of tissue samples from 59 osteosarcoma patients, it was demonstrated that epigenetic alterations in $p14^{ARF}$ correlated with poor prognosis. Using methylation-specific polymerase chain reaction (PCR), it was shown that 15/32 (47%) osteosarcomas had aberrant methylation of the $p14^{ARF}$ gene (CDKN2A) promoter. As anticipated, these 15 osteosarcomas with methylated $p14^{ARF}$ showed negative or weak expression of the $p14^{ARF}$ protein. This confirms that methylation of the $p14^{ARF}$ gene promoter is associated with loss of protein expression. Methylation did not correlate with age, gender, location of tumor, tumor volume, stage, histologic subtype, or chemotherapeutic response. Interestingly, the patients with $p14^{ARF}$ methylation had a lower median survival than the patients without $p14^{ARF}$ methylation, which was statistically significant according to Kaplan-Meier's survival analysis. This suggests that aberrant $p14^{ARF}$ promoter methylation correlates with poor survival in patients with osteosarcoma. In addition, 9/14 patients with $p14^{ARF}$ promoter methylation developed metastases. Associated deaths correlated with the incidence of metastasis but were not significant due to the size of the cohort [28]. Overall, these data suggest that $p14^{ARF}$ promoter methylation may result in the loss of p53-dependent G1 arrest, which may promote tumor development. However, further study into the significance of $p14^{ARF}$ promoter methylation in primary osteosarcoma and metastasis is warranted and may provide further insight into the importance of $p14^{ARF}$ methylation in osteosarcoma progression.

5. RASSF1A

Ras association domain family 1A (RASSF1A) is a newly identified tumor suppressor gene that is involved in pathways regulating cell cycle arrest and apoptosis. Although unclear, RASSF1A has been identified to be involved in death receptor-dependent apoptosis [29]. RASSF1A has been demonstrated to be silenced in cancers of the ovary, nasopharynx, kidney, stomach, prostate, urinary bladder, thyroid, and neuroblastoma [30–37]. The method of RASSF1A silencing in all of these tumor types was determined to be a result of aberrant methylation of the RASSF1A promoter. Recently, RASSF1 was also shown to be a tumor suppressor in osteosarcoma. Lim et al. investigated the expression of RASSF1A in primary osteosarcomas and cell lines and demonstrated a lack of RASSF1A expression in 4/10 primary and 5/6 cell lines. Upon treatment of these RASSF1A-negative cell lines with the demethylating agent 5-aza-2′-deoxycytidine, RASFF1A expression was upregulated. This suggests that RASSF1A promoter methylation may be a possible mechanism for the transcriptional silencing of RASSF1A. In contrast with these results, Lim et al. found that several primary osteosarcomas and one cell line (SAOS-2) did not display methylation of the RASSF1A promoter [38]. Therefore, further studies incorporating a greater number of osteosarcoma samples and cell lines are warranted. Additionally, Hou et al. performed a thorough analysis of promoter hypermethylation of a wide of array of genes in osteosarcoma and normal tissues using Q-MSP analysis. RASSF1A promoter hypermethylation was present in 14.29% (mean) of tumor tissues and in 1.29% (mean) of normal tissues, demonstrating that promoter methylation is a method of RASSF1A silencing in osteosarcoma [39]. This validates that loss of RASSF1A by promoter methylation may play a role in the development of osteosarcoma by dysregulation of cell cycle control and apoptosis.

6. Monomethyl Histone H3 Lysine 27

Several groups have demonstrated the various histone modifications that occur during apoptosis, termed the "apoptotic histone mark" [40]. Histone tails are known to be posttranslationally modified by acetylation, methylation, phosphorylation and ubiquitination of lysine, and/or arginine residues [41, 42]. One such modification, methylation of lysine 27 in histone H3, was associated with gene repression and has been implicated in tumorigenesis [43–45]. In osteosarcoma cells, induction of lysine 27 methylation in histone H3 has been associated with caspase-dependent apoptosis and cell cycle arrest, suggesting that this epigenetic mark may play a possible role in these processes [46]. Conversely, osteosarcoma cells undergoing apoptosis displayed elevated levels of monomethylated histone H3 lysine 27 [47]. These studies are the first to provide evidence that epigenetic modification of histone H3 by lysine 27 methylation may be linked with apoptosis in osteosarcoma. Although a correlative study, this finding suggests a possible role for histone modifications in the induction of apoptosis in osteosarcoma.

7. Discussion

Osteosarcoma is a relatively rare disease, accounting for about 5% of all pediatric cancers. However, more than 30% of patients with osteosarcoma die of pulmonary metastasis within 5 years after diagnosis [48]. While adjuvant chemotherapy has improved overall survival rates compared to surgery alone, the fatality rates have remained unchanged for more than 20 years. Therefore, there is an ongoing need for new therapeutic strategies and a more thorough understanding of the genetic and molecular mechanisms that participate in the development of osteosarcoma and in the metastatic process, particularly to the lung. Multiple pathways have been implicated in the pathogenesis of osteosarcoma. Specifically, pathways involving cell cycle and apoptosis have been found to play a role in tumorigenesis. While there is abundant evidence that pathways involving p53, Rb, and many key mediators of cell cycle regulation and apoptosis contribute to osteosarcoma, how these critical genes are altered is not clearly understood. Recent advances in the study of epigenetics have shown that these pathways may be regulated by methylation, histone modifications, and other epigenetic mechanisms. This paper highlights the importance of these epigenetic events in the development of osteosarcoma, specifically pertaining to cell cycle regulation and apoptosis. Overall, a growing body of evidence suggests that use of therapeutic agents that target epigenetic mechanisms may be beneficial for patients with osteosarcoma.

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Research Article

Impairment of Methotrexate Transport Is Common in Osteosarcoma Tumor Samples

Rebecca Sowers,[1] Bethanne D. Wenzel,[2,3] Condon Richardson,[4] Paul A. Meyers,[2] John H. Healey,[5] Adam S. Levy,[1] and Richard Gorlick[1]

[1] *Division of Hematology/Oncology, Department of Pediatrics, The Children's Hospital at Montefiore, The Albert Einstein College of Medicine of Yeshiva University, 3415 Bainbridge Avenue, Rosenthal Rm 300, Bronx, NY 10467, USA*
[2] *Department of Pediatrics, Memorial Sloan-Kettering Cancer Center New York, NY 10065, USA*
[3] *c/o Veterans Administration New York Harbor Healthcare System, Narrows Institute for Biomedical Research Brooklyn, NY 11209, USA*
[4] *Charlotte Kimelman Cancer Institute, 9048 Sugar Estate, St. Thomas USVI 00802, British Virgin Islands*
[5] *Division of Orthopaedic Surgery, Department of Surgery, Memorial Sloan-Kettering Cancer Center New York, NY 10065, USA*

Correspondence should be addressed to Richard Gorlick, rgorlick@montefiore.org

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Osteosarcoma does not respond well to conventional dose methotrexate but does respond to high-dose methotrexate. Previous work has indicated that this resistance may be due to impaired transport of methotrexate across the cell membrane. In this study, the PT430 competitive displacement assay was adapted to evaluate methotrexate transport in 69 high-grade osteosarcoma tumor samples. All samples studied were shown to have relatively impaired methotrexate transport by PT430 assay. Ninety-nine percent of the samples had less than 20% PT430 displacement by methotrexate. Eighty-eight percent exhibited displacement by methotrexate at less than 50% of the displacement by trimetrexate. The high frequency of impaired transport suggests the presence of decreased functionality of the reduced folate carrier protein. The overwhelming presence of impaired transport may explain why methotrexate needs to be given in high doses to be effective in osteosarcoma therapy and suggests that reduced folate carrier-independent antifolates should be explored.

1. Introduction

Although osteosarcoma is uncommon among the general population, it is the most common primary malignant bone tumor in children and adolescents [1]. It is believed that osteosarcoma is not responsive to conventional dose methotrexate in contrast to high-dose methotrexate [2, 3]. Most current multi-agent treatment regimens include the administration of high-dose methotrexate with the combination regimens having a five-year disease-free survival rate of 60% or greater [1]. This requirement for methotrexate in high doses for effectiveness may be explained by an intrinsic resistance of osteosarcoma to transport the drug across the cell membrane.

Methotrexate is a structural analog of folic acid and acts by binding and inhibiting dihydrofolate reductase (DHFR), a key enzyme required for intracellular folate metabolism [4]. Intracellular methotrexate undergoes polyglutamylation whereby the polyglutamylated methotrexate is preferentially retained in the cell and ultimately results in DHFR inhibition [5–7]. Resistance to methotrexate in model systems has been attributed to several causes including loss of or decreased reduced folate carrier (RFC) function [8], increased DHFR expression potentially as a result of gene amplification [9], and diminished intracellular retention of methotrexate secondary to decreased polyglutamylation [7]. Additionally, changes in downstream efflux pathways could affect the intracellular concentration of methotrexate [10].

Methotrexate can be transported by at least three routes: the folate receptors, the reduced folate carrier, and the proton coupled folate transporter [10]. The proton coupled folate transporter has optimal transport when in an acidic environment [10]. The folate receptors have a higher affinity for folic acid as compared with the reduced folates while the RFC has a higher affinity for reduced folates and methotrexate as compared with folic acid [3]. The RFC has an exponentially greater cycling rate than folate receptors. The role of folate receptors in antifolate transport may be relevant only when RFC function is quite low unless the antifolate in question has a particularly high affinity for the folate receptor or if the folate receptor is highly expressed [10].

Trimetrexate does not require the RFC for cell entry; however, limited clinical studies have been performed using trimetrexate for the treatment of pediatric solid tumors. Some studies have suggested that methotrexate transport defective cells are more sensitive to trimetrexate [11] and could potentially overcome methotrexate transport resistance [7].

Previous work has demonstrated that over 50% of osteosarcoma samples have at least one sequence alteration in the RFC [12]. Another study has shown decreased RFC mRNA expression occurs in 65% of osteosarcoma samples obtained at biopsy and in 50% of metastatic or recurrent samples [13]. The same study concluded that 10% of osteosarcoma samples have increased DHFR mRNA expression at time of biopsy and 62% of metastatic or recurrent samples have increased DHFR. PT430, a fluorescent lysine analog of methotrexate, competes with both methotrexate and trimetrexate for DHFR binding. Where PT430 is displaced by trimetrexate and not by methotrexate, the difference in displacement can be attributed to defective transport of methotrexate into the cell [14]. In this report the PT430 competitive displacement assay has been adapted to assess methotrexate transport in osteosarcoma.

2. Materials and Methods

2.1. Sample Collection. Osteosarcoma samples were collected at Memorial Sloan-Kettering Cancer Center between November 1997 and June 2001 after obtaining written informed consent in accordance with a biology study approved by the Memorial Hospital Institutional Review Board. Additional samples were collected as part of the Children's Oncology Group P9851 Osteosarcoma Biology Study also after obtaining written informed consent. All samples were confirmed to have a pathologic diagnosis of osteosarcoma.

2.2. Establishment of Short-Term Cell Cultures. Approximately 25 mg of fresh tumor were finely minced using a sterile scalpel. The minced tissue was incubated for at least two hours in 5 mLs of disaggregation media composed of MEM-alpha media, 20% FCS (HyClone, Logan, UT), 0.6% collagenase Type 2 (Worthington Biochemical, Lakewood, NJ), and 0.002% DNAseI (Promega, Madison, WI). After incubation, the slurry was passed through a 70 μm cell strainer. The filtered solution was centrifuged at 200 $\times$g and the resulting cell pellet was resuspended in 20 mls of cell culture media (MEM-alpha media + 20% FCS + 1% pen-strep) and subsequently plated in a Corning T75 flask. Cells were passaged after reaching 80% confluence.

2.3. PT430 Competitive Displacement Assay. The PT430 competitive displacement assay was performed as previously described [8, 14] with the following modifications. The assay was performed with the osteosarcoma cells placed in suspension as described originally with the cells trypsinized to place them in suspension. The trypsinized cells were centrifuged at 200 xg and the resulting cell pellet was resuspended in 5 mLs of media. Cells were kept in suspension through the completion of the assay. Cells were incubated in 20 μM PT430 for four hours at 37° instead of the referenced two hours. Methotrexate and trimetrexate were added to final concentrations of 1 μM each. PT430 was synthesized and kindly provided by Dr. Joel Wright and Dr. Andre Rosowsky of the Dana-Farber Cancer Institute. Methotrexate and trimetrexate displacement were calculated relative to the peak uptake correcting all samples for autofluorescence as has been described previously.

2.4. GD2 Antibody Labeling. GD2 antibody labeling was performed to verify that the cell population consisted of osteosarcoma cells and not fibroblasts. 1×10^7/ml cells were incubated with 100 μg 3F8 mouse monoclonal antibody for 1 hour at 4 degrees C on a rotating platform. The 3F8 antibody is specific for the GD2 cell surface protein which is present on osteosarcoma cells but is absent on fibroblasts. Following incubation with the primary antibody, cells were washed twice with PBS and then incubated with 50 μg of mouse IgG-FITC secondary antibody (Research Diagnostics Inc., Flanders, NJ). Secondary antibody incubation was for 15 minutes at 4 degrees C on a rotating platform. After incubation, cells were washed twice with PBS. The remaining pellet was resuspended in PBS and analyzed by flow cytometry. (The 3F8 antibody was kindly provided by Dr. N. K. Cheung, Memorial Sloan-Kettering Cancer Center).

3. Results

Tumor samples were obtained from 69 patients with confirmed diagnosis of osteosarcoma. Fifty of the 69 samples were obtained at time of biopsy, 11 samples were obtained at time of definitive surgery, and the remaining eight were obtained at the time of relapse or at the site of metastatic disease. GD2 antibody labeling of a subset of patient samples demonstrated that more than eighty-five percent of cells were osteosarcoma tumor cells (data not shown).

PT430 displacement by methotrexate and trimetrexate in the 69 osteosarcoma patient samples is summarized in Table 1 and Figure 1. Sixty eight of 69 samples (99%) had less than 20% displacement by methotrexate with 17 samples evidencing a complete lack of PT430 displacement

Table 1: Displacement of methotrexate and trimexate by PT430 grouped by sample type.

		All samples	Biopsy	Definitive surgery	Metastatic disease or relapse
% PT430 Displacement by methotrexate	*n*	69	50	11	8
	Range	0%–50.97%	0%–19.28%	0%–50.97%	1.13%–14.71%
	Median	3.10%	2.29%	5.81%	7.62%
	Average	4.88%	3.60%	8.89%	7.36%
% PT430 Displacement by trimetrexate	Range	3.27%–86.69%	3.27%–59.01%	6.84%–86.69%	12.48%–83.13%
	Median	21.05%	18.24%	30.50%	27.23%
	Average	24.66%	20.63%	32.81%	38.69%
MTX : TMTX Differential displacement ratio[a]	Range	0.00–3.60	0.00–3.60	0.00–0.95	0.04–0.64
	Median	0.15	0.12	0.22	0.13
	Average	0.25	0.24	0.27	0.25

[a]The MTX : TMTX differential displacement ratio represents the percentage of PT430 displaced by methotrexate in relation to the percentage of PT430 displaced by trimetrexate. For example, a sample with methotrexate displacement of 25% and trimetrexate displacement of 50% would have an MTX : TMTX differential displacement ratio of 0.50.

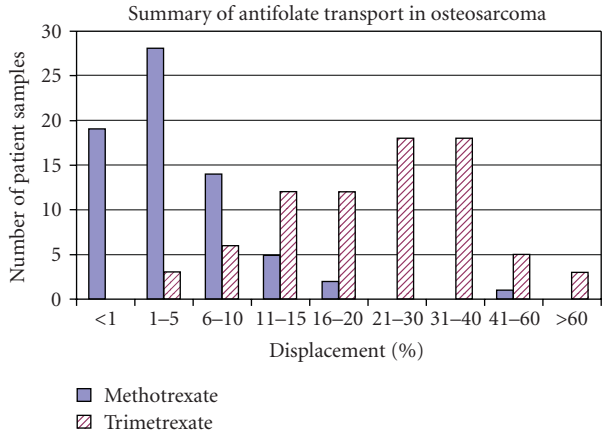

Figure 1: Summary of percentage PT430 displacement by methotrexate and trimetrexate in 69 osteosarcoma patient samples. Methotrexate displacement of PT430 was less than 20% in 99% of the samples.

by methotrexate. In comparison, trimetrexate displacement of PT430 was measurable in all 69 patient samples. Displacement by methotrexate among the 69 samples ranged from 0% to 51% (mean = 4.9%); displacement by trimetrexate ranged from 3.3% to 86.7% (mean = 24.7%).

In 60 of the 69 samples, displacement by methotrexate (MTX) was less than half the displacement by trimetrexate (TMTX) suggesting an RFC-mediated transport defect. This is represented by an MTX : TMTX differential displacement ratio where the percentage displacement by methotrexate is compared to the percentage displacement by trimetrexate (Table 1). In two of the 69 samples, PT430 displacement by methotrexate was greater than displacement by trimetrexate which may be related to P-glycoprotein expression. Nine samples had elevated peak PT430 accumulation signifying possible DHFR overexpression [8, 15].

All 50 biopsy samples exhibited less than 20% methotrexate displacement of PT430 (range = 0% to 19.3%; mean = 3.6%) with 16 samples displaying 0% displacement by methotrexate. Trimetrexate displacement of PT430 ranged from 3.3% to 59% (mean = 20.6%). Forty five of the 50 biopsy samples (90%) exhibited methotrexate displacement of PT430 at less than half of the trimetrexate displacement. The average MTX : TMTX differential displacement ratio of the biopsy samples was 0.24 indicating that, on average, methotrexate displacement of PT430 was only 24% of the PT430 amount that was displaced by trimetrexate.

Of the 11 definitive surgery samples, 10 samples displayed less than 20% methotrexate displacement of PT430 (range = 0% to 51%; mean = 8.9%). One sample displayed 0% methotrexate displacement of PT430 and one sample displayed 51% methotrexate displacement. Trimetrexate displacement of PT430 ranged from 6.8% to 86.7% with an average of 32.8%. Nine of the 11 definitive surgery samples (82%) displayed methotrexate displacement of PT430 at less than half of the corresponding trimetrexate displacement with an average MTX : TMTX differential displacement ratio of 0.27.

All eight samples representative of metastatic disease or relapse had methotrexate displacement of less than 20% (range = 1.1% to 14.7%; mean = 7.4%). Six of the eight samples (75%) showed methotrexate displacement at less than half of trimetrexate displacement. Trimetrexate displacement for the eight samples ranged from 12.5% to 93.1%; mean = 38.7%. The average MTX:TMTX differential displacement ratio was 0.25.

4. Discussion

Methotrexate treatment is a valuable component of current treatment protocols for osteosarcoma [16–19]. Unfortunately, high-dose methotrexate therapy is associated with considerable costs and morbidity, although significant neurotoxicity and renal toxicity are rarely observed. The improved efficacy of high-dose methotrexate as compared to conventional dose methotrexate suggests that osteosarcoma may have intrinsic methotrexate resistance which can be

overcome by achieving a high extracellular drug concentration [4, 10]. One possibility for intrinsic methotrexate resistance would be a transport defect. A clearer understanding of the relative methotrexate-resistance of osteosarcoma may direct better treatment strategies.

This study sought to determine whether the impaired cell entry of methotrexate is involved in the observed intrinsic resistance to methotrexate in osteosarcoma. When PT430 is displaced by trimetrexate and not by methotrexate, the difference in displacement can be attributed to defective transport of methotrexate into the cell [14]. Currently, there is no standard for what is considered functionally defective methotrexate transport in osteosarcoma. For the PT430 assays described in this study, methotrexate was added to a final concentration of 1 μM. Of note, this is a clinically relevant methotrexate level, and typical plasma concentrations following high-dose methotrexate for osteosarcoma patients are $<10\,\mu$M at 24 hours and $<1\,\mu$M at 48 hours. In acute lymphocytic leukemia, defective transport has been defined as less than 40% displacement of PT430 by methotrexate [20]. A recent study investigating impairments in antifolate transport in retinoblastoma tumors suggested a reduced folate carrier protein defect was indicated in those samples whose PT430 displacement by methotrexate was less than half the displacement by trimetrexate [12].

In this study, 99% of the osteosarcoma patient samples exhibited less than 20% displacement of PT430 by methotrexate, and 88% had methotrexate displacement at less than half of the corresponding trimetrexate displacement of PT430 ($n = 69$). This is strong evidence that osteosarcoma harbors some level of intrinsic resistance to methotrexate due to impaired transport. Only nine of the 69 samples (13%) exhibited elevated peak PT430 levels suggestive of DHFR overexpression. These results suggest that methotrexate resistance is a result of impaired transport via the reduced folate carrier rather than DHFR overexpression. Given the evident intrinsic methotrexate resistance in osteosarcoma, evaluation of antifolate agents that do not rely on transport via the RFC is warranted for recurrent or refractory disease. Potentially, the degree of intrinsic methotrexate resistance could be determined at diagnosis and could help define individualized therapy for osteosarcoma.

Forty five of the 50 biopsy samples (90%) had an MTX : TMTX differential displacement ratio of <0.50; that is, displacement of PT430 by methotrexate was less than half of the displacement by trimetrexate. The human lymphoblast cell line CCRF-CEM is known to be sensitive to methotrexate and exhibited an MTX : TMTX differential displacement ratio of 0.99 (data not shown). Nine of 11 patient samples (82%) taken at time of definitive surgery exhibited methotrexate displacement at less than half of the corresponding trimetrexate displacement. Similarly, six of eight samples (75%) taken either at time of relapse or from metastatic disease had methotrexate displacement at less than half of trimetrexate displacement. In summary, functional methotrexate defects were observed in osteosarcoma regardless of sample type: biopsy, definitive resection, or relapse.

Since methotrexate enters the cell primarily through the reduced folate carrier, it could be plausibly concluded that the prevalence of impaired methotrexate transport in osteosarcoma is due to altered function of the reduced folate carrier. It has been previously reported that over 50% of osteosarcoma samples have decreased reduced folate carrier expression at time of diagnosis as determined by semiquantitative PCR [13]. The decreased reduced folate carrier expression was associated with inferior response to preoperative chemotherapy, but functional methotrexate transport was not assessed in these samples.

Sequence alterations of the reduced folate carrier have been shown to occur in osteosarcoma. The functional significance of several reduced folate carrier sequence variants was evaluated by transfecting several different altered human reduce folate carrier fusion proteins into a reduced folate carrier-null hamster cell line [21] or null HeLa cell line [22]. Four of the altered hamster lines resulted in less effective transport of methotrexate as compared to wild type. Likewise, four of the altered HeLa lines resulted in either reduced or abolished methotrexate transport in comparison to wild type. It is expected that these sequence variants in human osteosarcoma cell lines would yield a similar result. Other reported alterations to the reduced folate carrier include multiple sequence variants in exons 2 and 3 [9], promoter methylation [23], and high-frequency splice variants [24].

Although therapeutically informative, functional transport assays are not likely to be prognostic given the high frequency of transport defects. Changes in reduced folate carrier expression level as opposed to sequence alterations may be prognostic as the relevance may be the mechanism of functional inactivation. It remains unclear why even at diagnosis, prior to methotrexate exposure, the methotrexate-reduced folate carrier transport pathway is functionally inactivated. Further characterization of the reduced folate carrier alterations may provide more insight into basic folate transport mechanisms and steps in osteosarcoma tumor pathogenesis.

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Review Article

The Molecular Pathogenesis of Osteosarcoma: A Review

Matthew L. Broadhead,[1] Jonathan C. M. Clark,[1] Damian E. Myers,[1] Crispin R. Dass,[2] and Peter F. M. Choong[1,3]

[1] *Department of Orthopaedics, Department of Surgery, University of Melbourne, St. Vincent's Hospital, SVHM, L3, Daly Wing, 35 Victoria Parade, Fitzroy VIC 3065, Australia*
[2] *School of Biomedical and Health Sciences, Victoria University, St. Albans, VIC 3021, Australia*
[3] *Sarcoma Service, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia*

Correspondence should be addressed to Peter F. M. Choong, sarcoma@bigpond.net.au

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Osteosarcoma is the most common primary malignancy of bone. It arises in bone during periods of rapid growth and primarily affects adolescents and young adults. The 5-year survival rate for osteosarcoma is 60%–70%, with no significant improvements in prognosis since the advent of multiagent chemotherapy. Diagnosis, staging, and surgical management of osteosarcoma remain focused on our anatomical understanding of the disease. As our knowledge of the molecular pathogenesis of osteosarcoma expands, potential therapeutic targets are being identified. A comprehensive understanding of these mechanisms is essential if we are to improve the prognosis of patients with osteosarcoma through tumour-targeted therapies. This paper will outline the pathogenic mechanisms of osteosarcoma oncogenesis and progression and will discuss some of the more frontline translational studies performed to date in search of novel, safer, and more targeted drugs for disease management.

1. Introduction

Osteosarcoma is a relatively uncommon cancer although it is the most common primary malignancy to arise from bone. While incidence is low, osteosarcoma predominately affects adolescents and young adults, and if untreated it is fatal. Despite modern treatment protocols that combine chemotherapy, surgery, and sometimes radiotherapy, the 5-year survival rate for patients diagnosed with osteosarcoma remains at 60%–70% [1]. Current treatments for osteosarcoma are associated with significant morbidity, and a period of rehabilitation may be required following surgery for osteosarcoma. Hence, there is a real need to optimise current treatment strategies and to develop novel approaches for treating osteosarcoma.

Traditionally, our understanding of osteosarcoma has been largely anatomical. Osteosarcoma arises most commonly in the metaphyseal region of long bones, within the medullary cavity, and penetrates the cortex of the bone to involve the surrounding soft tissues. A pseudocapsule forms around the penetrating tumour [2]. Histologically, osteosarcoma is characterised as a highly cellular tumour composed of pleomorphic spindle-shaped cells capable of producing an osteoid matrix. Current standards for staging and surgical resection rely on this anatomical knowledge [3]. However, recent developments in molecular biology have provided insight into the molecular pathogenesis of osteosarcoma. Through the identification of tumour pathways and specific mediators of osteosarcoma progression, novel approaches for targeting osteosarcoma are being developed. This paper will review our current understanding of the molecular pathogenesis of osteosarcoma.

2. Pathogenesis

2.1. Bone Growth and Tumorigenesis. Osteosarcoma has a predilection for developing in rapidly growing bone. A number of studies have established a correlation between the rapid bone growth experienced during puberty and osteosarcoma development [4, 5]. Fifty-six percent of all osteosarcomas present around the knee [2]. The epiphyseal growth plates of the distal femur and proximal tibia are responsible for a great deal of the increase in height that occurs during puberty. Additionally, the peak age of

osteosarcoma development is slightly earlier for females, an observation that may be explained by the relatively earlier growth spurt experienced by girls [6]. There is a male:female ratio of 1.5 : 1 for osteosarcoma, and patients affected by the disease are taller compared to the normal population of the same age group [7]. Patients affected by Paget's disease, a disorder characterised by both excessive bone formation and breakdown, also have a higher incidence of osteosarcoma [2].

2.2. Environmental Factors. Physical, chemical, and biological agents have been suggested as carcinogens for osteosarcoma. Among these, the role of ultraviolet and ionising radiation is the best established. The initial pathogenic link between radiation exposure and osteosarcoma was noted in female radium dial workers who applied radium to watch faces to make them luminescent [8]. However, radiation exposure is implicated in only 2% of cases of osteosarcoma [9] and is not thought to play a major role in paediatric disease. An interval of 10–20 years between exposure and osteosarcoma formation has been observed [10]. When radiotherapy is used in children as a treatment agent for a solid tumour, 5.4% develop a secondary neoplasm, and 25% of these are sarcomas [11].

The chemical agents linked to osteosarcoma formation include methylcholanthrene and chromium salts [12], beryllium oxide [13], zinc beryllium silicate [14], asbestos, and aniline dyes [15]. Previously, a viral origin had been suggested for osteosarcoma. This stemmed from the detection of simian virus 40 (SV40) in osteosarcoma cells. However, the presence of SV40 in these cells was later concluded to be the result of presence of SV40 viral units as contamination in the polio-virus vaccine that these patients had received [16, 17]. Studies evaluating the role of SV40 in the pathogenesis of mesothelioma have suggested that detection of SV40 in human cancers may in fact be due to laboratory contamination by plasmids containing SV40 sequences [18, 19].

2.3. Chromosomal Abnormalities. A number of chromosomal and genetic syndromes have been linked to osteosarcoma. Osteosarcoma has been reported in patients with Bloom syndrome, Rothmund-Thompson syndrome, Werner syndrome, Li-Fraumeni syndrome, and hereditary retinoblastoma [15]. Bloom, Rothmund-Thompson, and Werner [20] syndromes are characterised by genetic defects in the RecQ helicase family. DNA-helicases are responsible for separation of double-stranded DNA prior to replication [21, 22]. Mutations in these genes confer a higher risk of multiple malignancies.

A recent study of pretherapeutic biopsy specimens has identified amplifications of chromosomes 6p21, 8q24, and 12q14, as well as loss of heterozygosity of 10q21.1, as being among the most common genomic alterations in osteosarcoma. Furthermore, it was concluded that patients carrying these alleles had a poorer prognosis [23]. Numerical chromosomal abnormalities associated with osteosarcoma include loss of chromosomes 9, 10, 13, and 17 as well as gain of chromosome 1 [24].

2.4. Tumour Suppressor Gene Dysfunction. When human cells are exposed to environmental insults, such as those discussed above, somatic DNA may be damaged. Such DNA damage may not necessarily give rise to a malignant cell line, as there are a number of tumour-suppressor mechanisms in place. These mechanisms may either repair the DNA damage or induce apoptosis of these cells. The p53 and retinoblastoma (Rb) genes are well-known tumour-suppressor genes. However, tumour suppressor genes may themselves become mutated, resulting in the loss of their protective function. As a result, additional somatic mutations may accumulate, giving rise to a cell line that replicates without restraint. Mutations in both the p53 and Rb genes have been proven to be involved in osteosarcoma pathogenesis [6].

The p53 gene is mutated in 50% of all cancers and 22% of osteosarcomas [24]. DNA damage results in phosphorylation of p53, which is constitutively inhibited by Mdm2. Phosphorylation allows p53 dissociation from Mdm2. p53 exerts its tumour-suppressor effects via the activation of proapoptotic Bax and p21. The latter binds and inactivates G_1/S-Cdk and S-Cdk complexes, causing arrest of the cell cycle in G_1 [25].

Recently, p53 mutations have been shown to result in impaired DNA repair mechanisms and disrupted antiangiogenesis activity [26]. For osteosarcoma, the prototypical condition of p53 mutation is Li-Fraumeni syndrome. This syndrome is characterised by an autosomal dominant mutation of p53 leading to the development of multiple cancers including osteosarcoma [27]. Li-Fraumeni syndrome and germ-line mutations of p53 in osteosarcomas are rare, however [28], and in many osteosarcoma cell lines, a mutation in the first intron of the p53 gene occurs [29] though other point mutations have also been reported [30].

While p53 has been implicated in the oncogenesis of osteosarcoma, it is unclear whether p53 mutation or loss may affect tumour behaviour. Using the p53-null SaOS-2 osteosarcoma cell line, Ganjavi et al. [31] showed that adenoviral-mediated gene transfer of wild-type p53 resulted in reduced cell viability and increased sensitivity to chemotherapeutic agents. A recent study published by Hu et al. [32] showed that p53 expression was higher in low Rosen grade osteosarcomas (Rosen grade 1: <50% necrosis; grade 2: 50%–90% necrosis; grade 3: >90% necrosis; grade 4: 100% necrosis; grade 1 + 2 = low-grade; grade 3 + 4 = high grade). p53 expression correlated with reduced metastatic disease and improved survival for these patients. p53 mutation has also been shown to be more common in high-grade conventional osteosarcomas versus low grade central osteosarcomas [33]. However, other studies differ such as that of Lonardo et al. [34], which found no relationship between p53 and histological grade. Univariate analysis performed by Park et al. [35] showed no correlation between survival and the p53 protein, while coexpression of p53 and P-glycoprotein was associated with a poorer prognosis.

In addition to p53, the Rb tumour suppressor has also been implicated in the tumorigenesis of osteosarcoma. The Rb gene is critical to cell-cycle control, and inherited mutation of the Rb gene causes retinoblastoma syndrome, a condition that predisposes a patient to multiple malignancies including osteosarcoma. The Rb protein regulates the cell

cycle by binding the transcription factor E2F. E2F is held inactive by Rb until the CDK4/cyclin D complex phosphorylates Rb. Mutations of Rb allow for the continuous cycling of cells [25].

Both germ-line and somatic mutations of Rb confer an increased risk of osteosarcoma. Loss of the Rb gene may even explain the familial risk of osteosarcoma [36]. However, it has yet to be determined whether Rb gene loss or suppression gives rise to more aggressive tumours with poorer prognosis. Loss of heterozygosity for Rb has been reported to confer both an improved and poorer prognosis for patients [37–40]. In terms of response to chemotherapeutic treatment, Iida et al. [41] showed that the SaOS-2 osteosarcoma cell line, lacking active Rb, was less sensitive to the growth-suppressing effect of methotrexate compared to cell lines with wild-type Rb gene. Further studies are warranted to investigate the role of Rb on chemosensitivity of osteosarcoma cells.

2.5. Transcription Factors. Transcription is the process of forming single-stranded messenger RNA (mRNA) sequences from double-stranded DNA. Transcription factors facilitate binding of promoter sequences for specific genes to initiate the process. While transcription is usually tightly regulated, deregulation may occur in osteosarcoma, as with other cancers. Excess production of transcription factors, or the production of a new overactive transcription factor, may result from gene rearrangement.

The activator protein 1 complex (AP-1) is a regulator of transcription that controls cell proliferation, differentiation, and bone metabolism. AP-1 is comprised of Fos and Jun proteins, products of the c-fos and c-jun proto-oncogenes, respectively. Fos and Jun are found to be significantly upregulated in high-grade osteosarcomas compared with benign osteoblastic lesions and low-grade osteosarcomas [42, 43] and are associated with the propensity to develop metastases [44]. Fos and Jun double-transgenic mice are found to develop osteosarcomas with a higher frequency than c-Fos only transgenic mice [45]. Most recently, Leaner et al. [46] showed that inhibition of AP-1-mediated transcription caused reduced migration, invasion, and metastasis in a murine model of osteosarcoma. Another approach has been to target the Jun component of AP-1. The DNA enzyme Dz13 cleaves human c-Jun mRNA and is capable of inhibiting osteosarcoma growth and progression in a clinically relevant murine model when delivered by nanoparticle vector [47].

Myc is a transcription factor that acts in the nucleus to stimulate cell growth and division. Myc amplification has been implicated in osteosarcoma pathogenesis and resistance to chemotherapeutics. Overexpression of Myc in bone marrow stromal cells leads to osteosarcoma development and loss of adipogenesis [48]. Myc is amplified in U2OS osteosarcoma cell-line variants with the highest resistance to doxorubicin, and gain of Myc was found in SaOS-2 methotrexate-resistant variants [49]. Additionally, Myc has been examined as a therapeutic target for osteosarcoma. Downregulation of Myc enhanced the therapeutic activity of methotrexate against osteosarcoma cells [50]. Adenovirus-mediated transfection with the antisense Myc fragment led to cell-cycle arrest and enhanced apoptosis in the MG-63 osteosarcoma cell line [51]. Using a conditional transgenic mouse model, Arvanitis et al. [52] showed that Myc inactivation caused proliferative arrest and promoted differentiation in osteosarcoma. Additionally, using positron emission tomography (PET), these tumours exhibited reduced metabolic activity as demonstrated by reduced uptake of [^{18}F]-fluorodeoxyglucose ([^{18}F]-FDG).

2.6. Growth Factors. Osteosarcoma cells produce a range of growth factors that exert autocrine and paracrine effects. Dysregulated expression of growth factors such as transforming growth factor (TGF), insulin-like growth factor (IGF), and connective tissue growth factor (CTGF) leads to the accelerated proliferation of cells. Growth factor receptors may be overexpressed and constitutively activated. Signal transduction associated with these receptors may also be overactivated.

Transforming growth factor beta (TGF-β) proteins are a large family of dimeric proteins secreted by cells. Like many other growth factors, they influence a wide variety of cell process such as differentiation, proliferation, apoptosis, and matrix production. Bone morphogenic proteins (BMPs) make up a large component of the TGF-β family. High-grade osteosarcomas are found to express TGF-β1 in significantly higher amounts than low-grade osteosarcomas [53]. Navid et al. [54] examined the autocrine role of TGF-β on two osteosarcoma cell lines, demonstrating a 30%–50% reduction in growth when osteosarcoma cells were cultured in the presence of TGF-β-blocking antibody. Smad activation was implicated downstream of TGF-β with an inability to phosphorylate the Rb protein. Most recently, Hu et al. [55, 56] have shown an association between increased susceptibility and metastasis of osteosarcoma with TGFR1 variants, TGFBR1*6A, and Int7G24A.

IGF (insulin-like growth factor)-I and IGF-II are growth factors that are often overexpressed by osteosarcomas. These ligands bind corresponding receptors such as IGF-1R, leading to activation of the PI3K and MAPK transduction pathways. This, then, supports cell proliferation and inhibition of apoptosis [57]. The growth-stimulating effect of IGF has been targeted for osteosarcoma. Lentivirus-mediated shRNA targeting IGF-R1 enhanced the chemosensitivity of osteosarcoma cells to docetaxel and cisplatin [58]. The use of monoclonal antibodies targeting IGF-R1 was also effective in enhancing antitumour response [59, 60].

Connective tissue growth factor (CTGF) is related to a number of proteins in the CCN family (CTGF/Cyr61/Cef10/NOVH). This protein family appears to act via integrin signalling pathways [61] and, like TGF-β, has a diverse range of functions including adhesion, migration, proliferation, survival, angiogenesis, and differentiation. Nishida et al. [62] showed that CTGF is a potent stimulator for the proliferation of SaOS-2 cells, leading to increased expression of type I collagen, alkaline phosphatase, osteopontin, and osteocalcin, markers for bone cell differentiation and maturation. A related protein, CCN3, was found to be overexpressed in osteosarcoma and associated with a worse prognosis [63].

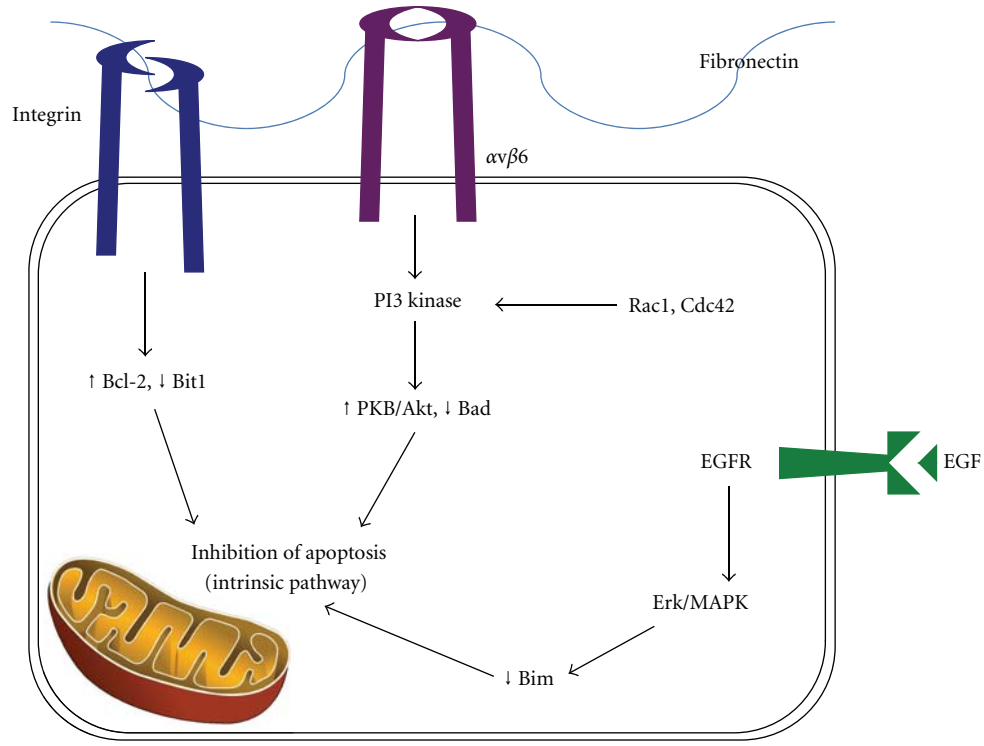

Figure 1: Pathways disrupting anoikis.

Parathyroid hormone (PTH), parathyroid hormone-related peptide (PTHrP), and the receptor (PTHR1) have been implicated in the progression and metastasis of osteosarcoma. PTHrP was discovered as the humoral factor associated with tumour metastasis and hypercalcaemia [64]. The role of PTHrP and PTHR1 in osteoclast signalling will be discussed later. In terms of direct effects on osteosarcoma cells, when HOS osteosarcoma cells were overexpressed with PTHR1, increased proliferation, motility, and invasion through Matrigel were observed [65]. Gagiannis et al. [66] recently showed that PTHrP confers chemoresistance in osteosarcoma by blocking signalling via p53, death-receptor and mitochondrial pathways of apoptosis. PTHrP downregulated expression of proapoptotic Bax and PUMA and upregulated antiapoptotic Bcl-2 and Bcl-xl. Berdiaki et al. [67], using MG-63 and SaOS-2 osteosarcoma cell lines, showed that PTH peptides enhanced osteosarcoma cell migration through the regulation of hyaluron metabolism. However, a previous study showed that overexpression of PTHrP in a murine osteoblastic osteosarcoma cell line reduced cell proliferation by 80% [68]. Further studies are required to determine the prognostic significance of PTH/PTHrP/PTHR1 signalling in osteosarcoma.

2.7. Osteosarcoma Cell Proliferation, Apoptosis, and Anchorage-Independent Growth. Cancer cells are relatively resistant to apoptosis, and this ability to avoid elimination contributes to the ability of osteosarcoma cells to proliferate without restriction. Apoptosis consists of initiation and execution phases. During initiation, enzymes responsible for the cleavage of vital cellular proteins, known as caspases, are activated. Execution refers to the actual process of hydrolysis performed by activated caspases. Both extrinsic and intrinsic pathways regulate the initiation phase. The extrinsic pathway is a death receptor-initiated pathway, while the intrinsic pathway relies on increased mitochondrial permeability. Both proapoptotic and antiapoptotic factors interact with these pathways, and these have been discussed in a previous review [69].

Anoikis is a form of apoptosis that is induced when cells are no longer attached to a basement membrane or matrix. This is of particular interest in osteosarcoma given the propensity of osteosarcoma cells to detach from matrix components and to metastasise. Osteosarcoma cells are resistant to anoikis and proliferate despite deranged cell-cell and cell-matrix attachments. This resistance to anoikis is termed anchorage-independent growth (AIG).

The pathways causing anoikis disruption and leading to anchorage-independent growth are complex. They involve interactions between integrin signalling, Rho GTPases, PI3 kinase, and PKB/Akt activation, along with many key components of the intrinsic and extrinsic apoptosis pathways (Figure 1). For example, when normal cells adhere to surrounding matrix via integrin-fibronectin binding, the Bcl-2 inhibitor Bit1 is suppressed allowing Bcl-2 to prevent apoptosis via the intrinsic pathway [70]. Another pathway involves the exchange of integrin subunits resulting in the production of abnormal integrins, such as $\alpha v\beta 6$, which can upregulate PI3 kinase function [71]. PI3 kinase can then activate PKB/Akt which inhibits the proapoptotic factor Bad, leading

to cancer cell survival [72]. Rho GTPases such as Rac1 and Cdc42 can also upregulate PI3 kinase with similar consequences [73]. Increased epidermal growth factor-receptor (EGF/EGFR) binding with subsequent extracellular signal-regulated kinase (Erk)/microtubule-associated protein kinase (MAPK) signalling leading to inhibition of Bim has also been described [74]. This suppresses cell death, as Bim would normally act to increase mitochondrial outer membrane permeability allowing release of cytochrome *c* and then the activation of executioner caspases.

2.8. Tumour Angiogenesis. Tumour angiogenesis is essential for sustained osteosarcoma growth and metastasis. Without a supporting vasculature, osteosarcoma cells would be unable to obtain the nutrients and oxygen necessary for proliferation. Metastasis to the lungs and bone, the most common sites for osteosarcoma spread, also relies on the formation and maintenance of blood vessels. Radiation therapies, while compromising tumour cells, also destroy the vascular component of tumours and block the supply of nutrients. So, radio- and chemotherapies act by these dual actions. This aspect is discussed below.

A balance between pro-angiogenic and antiangiogenic factors regulates angiogenesis, and this balance is tipped towards the favour of neovascularisation by tissue hypoxia, acidosis, oncogene activation, and loss of tumour suppressor gene function. A hypoxic and acidotic microenvironment exists around proliferating osteosarcoma cells, and these conditions stimulate deubiquitination of von Hippel Lindau protein. Von Hippel Lindau protein releases hypoxia-inducible factor-1α (HIF-1α), allows HIF-1α to bind to the promoter region of the vascular endothelial growth factor (VEGF) gene [75], and upregulats it. TGF-α, and fibroblast growth factor (FGF) may also upregulate VEGF [76].

VEGF is the best-characterised pro-angiogenic factor, and it stimulates the processes of endothelial cell proliferation, migration, and blood vessel maturation. A number of different VEGF molecules exist (VEGF-A through to VEGF-E), and these proteins bind to VEGF receptors (VEGFR1-3) [77]. VEGF-A has the broadest angiogenic effect. Upon VEGF-A binding to VEGFR2, a number of divergent signalling pathways are initiated [77]. Nitric oxide (NO) is released by endothelial cells, leading to vasodilation and increased vascular permeability [78]. Endothelial cell proliferation and cycling are stimulated via phospholipase Cγ (PLCγ), protein kinase C (PKC), and the c-Raf-MEK-MAPK cascades [77]. Rearrangement of the actin cytoskeleton, necessary for endothelial cell migration occurs via phosphorylation of T cell-specific adapter (TSAd) and interaction with Src, another protein kinase [79]. The net result of all these changes is the formation of an immature, irregular, and leaky vascular network.

The immature and inefficient nature of the vessels so produced facilitates feedback loops for further vessel formation. Upregulation of HIF-1α and VEGF [80] again occurs as the leaky vasculature is unable to meet the metabolic demands of the proliferating osteosarcoma cells. Additionally, VEGF upregulates matrix metalloproteinase (MMP) and plasmin activity [81]. These proteases break down extracellular matrix, which releases any VEGF combined with heparin proteoglycan in the matrix. VEGF also induces antiapoptotic factors Bcl-2, and survivin, ensuring ongoing endothelial proliferation [82]. In addition to VEGF, the proliferating tumour cells release a number of other pro-angiogenic factors. These include FGF, platelet-derived growth factor (PDGF), angiopoietin1 (Ang1), and ephrin-B2 [83, 84].

While it is known that osteosarcoma is a relatively vascular tumour, the prognostic significance of this is yet to be determined. There have been studies suggesting both a correlation [85, 86] and lack of association [87] between VEGF expression and osteosarcoma microvascular density and metastases at diagnosis. This may relate to a greater tumour dependence on functionally mature vessels. One study that demonstrated a survival advantage associated with increased osteosarcoma microvascular density [88] attributed this advantage to improved tissue penetration by chemotherapeutic agents.

As previously mentioned, angiogenesis is regulated by the balance between pro-angiogenic and antiangiogenic factors. Antiangiogenic proteins such as thrombospondin 1, TGF-β [89], troponin I, pigment epithelial-derived factor (PEDF) [90], and reversion-inducing cysteine rich protein with Kazal motifs (RECK) [91] are downregulated in osteosarcoma. These antiangiogenic molecules are particularly important for embryogenesis and physiological processes such as wound healing and menstruation; however, they also play a protective mechanism against osteosarcoma progression. For example, troponin I and PEDF are expressed predominately within the avascular zones of the cartilaginous growth plate [92, 93] and are likely to contribute to growth plate resistance to osteosarcoma invasion from a typical metaphyseal location. In addition to inhibiting angiogenesis, PEDF exerts direct effects on osteosarcoma cells. Ek et al. [94, 95] have demonstrated apoptosis induction in osteosarcoma cell lines treated with PEDF. Also, in a murine model of orthotopic osteosarcoma, tumour volume was reduced by PEDF, which was associated with reduced microvascular density. There was decreased tumour metastases and reduced size of metastatic tumours in lung.

2.9. Cell Adhesion and Migration. Osteosarcoma is a highly metastatic tumour, and pulmonary metatases are the most common cause of death. The metastatic sequence involves the detachment of osteosarcoma cells from the primary tumour, adhesion to the extracellular matrix, local migration and invasion through stromal tissue, intravasation, and extravasation. The ability of osteosarcoma cells to metastasise by such a pathway relies on complex cell-cell and cell-matrix interactions.

The extracellular matrix is composed of various protein fibrils and growth factors. The proteins include fibronectin, collagens, proteoglycans, and laminins. Osteosarcoma cells may also produce matrix proteins. The extracellular matrix provides a developing tumour with a supporting scaffold and facilitates blood vessel formation. Osteosarcoma cells adhere to matrix components via cell-surface receptors. These receptors are more than just a physical point of attachment; they also provide a link between matrix proteins

and the cytoskeleton. The principle receptor proteins are the integrins, which bind to the matrix protein fibronectin. There are 24 different integrin heterodimer molecules consisting of different α and β subunits [96].

The integrins also play a role in cell signaling, particularly in pathways critical to cell migration. Integrin-binding proteins such as talin become associated with the cytoplasmic domain and act, via adaptor proteins such as vinculin, paxillin, and α-actin, for the upregulation of protein kinases [97]. The key enzymes involved here are focal adhesion kinase (FAK), protein kinase C (PKC), PI3 kinase, Src, and the RhoA GTPases.

The relative activities of these enzymes underlie conformational changes in cell architecture. For example, there is a shifting balance between two of the RhoA GTPases: Rac1 and RhoA. High Rac1 expression suppresses RhoA and induces the formation of membrane ruffles. These membrane changes facilitate cell spreading and migration [98]. Conversely, high RhoA with low Rac1 leads to membrane retraction. These two processes are coordinated such that in cell migration, the leading edge of the cell is demonstrating actin polymerisation and lamellipoedia, while the trailing edge is undergoing actin disassembly. Inhibition of RhoA pathways has been shown to reduce osteosarcoma cell migration and invasion [99].

In general, cells migrate towards ligand-dense matrix and towards more rigid matrix [100], indicating a constant intracellular response to extracellular adhesion and tension. Tumour stroma is more rigid than normal connective tissue matrix, and this generates integrin clustering, activation of intracellular signalling pathways, decreases cell-to-cell contacts, and stimulates tumour growth [101].

The ezrin protein also has a role in cell-cell interactions, signal transduction, linkage between actin filaments, and cell membrane receptors such as CD44, which binds hyaluronan in the extracellular matrix. When ezrin is overexpressed, it is associated with an increase in metastasis [102]. Increased ezrin expression in paediatric osteosarcoma patients is associated with reduced disease-free intervals, and downregulation of ezrin expression in a mouse model of human osteosarcoma has been shown to reduce pulmonary metastasis [103].

2.10. Tumor Invasion. Invasion of the surrounding tissues by osteosarcoma also involves degradation of the extracellular matrix. Matrix metalloproteinases (MMPs) are principally involved in the breakdown of the extracellular matrix, although roles in tumour angiogenesis have also been established.

MMPs are a family of zinc-dependent endopeptidases that are involved in a range of physiological processes including inflammation, wound healing, embryogenesis, and fracture healing. In normal tissues, MMPs are regulated by natural inhibitors such as tissue inhibitors of MMPs (TIMPs), RECK, and α2 macroglobulin [104]. In the setting of osteosarcoma, MMPs break down extracellular collagens, facilitating both tumour and endothelial cell invasion. MMPs may be designated as gelatinases, collagenasesm, or stromeolysins. Gelatinases break down denatured collagens and type IV collagen. Collagenases break down type I, type II, and type III collagen, and stromeolysins break down proteoglycan (found in articular cartilage), type III, type IV (in basement membranes), and type V collagen, as well as casein and fibronectin [105].

In addition to clearing a pathway for invading osteosarcoma cells, the role of MMPs in angiogenesis has already been mentioned. Remodelling of vessel walls by MMPs gives rise to a thin and leaky vascular network that allows passage of tumour cells into the bloodstream [106]. Furthermore, MMP-9 releases VEGF stored within the extracellular matrix [107], and VEGF is able to upregulate MMP-2 [108]. The specific importance of the gelatinases MMP-2 and MMP-9 to tumour progression has been delineated in an *in vivo* study, where combined MMP-2/MMP-9 deficiency in mice significantly impaired tumour angiogenesis and invasion [109].

The urokinase plasminogen activator (uPA) system is the other key regulator of osteosarcoma invasion, which interacts with MMPs. The ligand uPA binds to its receptor uPAR to become active. Once activated, uPA cleaves plasminogen to plasmin. Plasmin breaks down the extracellular matrix but also activates pro-MMPs. A cascade of activation is hence established [110, 111]. The role of the uPA-uPAR system is well established in osteosarcoma pathogenesis. An inverse relationship between uPA levels and survival time has been demonstrated [112]. Downregulation of uPAR in an *in vivo* osteosarcoma model resulted in reduced primary tumour growth and fewer metastases [113].

2.11. Osteoclast Function. Osteosarcoma invasion of bone relies on interactions between the bone matrix, osteosarcoma cells, osteoblasts, and osteoclasts (Figure 2). Osteoclasts are the principle bone-resorbing cells, and the substantial osteolysis exhibited by some osteosarcomas is the direct result of increased osteoclastic activity. During the initial stages of osteosarcoma invasion, growth factors such as TGF-β are released from the degraded bone matrix and act on osteosarcoma cells, stimulating the release of PTHrP, interleukin-6 (IL-6) and interleukin-11 (IL-11) [114, 115]. These cytokines then stimulate osteoclasts, facilitating further invasion and release of proresorptive cytokines.

Osteoblasts are, in fact, mediators in this process of bone resorption. Osteosarcoma cells release endothelin-1 (ET-1), VEGF, and PDGF in response to the hypoxic and acidotic conditions. These factors have predominantly osteoblast-stimulatory functions [116, 117]. PTHrP and IL-11 also act on osteoblasts, stimulating increased expression of receptor activator of nuclear factor κB ligand (RANKL). RANKL is a key mediator of osteoclast differentiation and activity, and osteosarcoma cells have been noted to produce RANKL independently [118].

RANKL activates osteoclasts through binding to RANK on the osteoclast surface. RANK expression is under control of cytokines IL-1, IL-6, IL-8, tumour necrosis factor-α (TNF-α), PTHrP, and TGF-α [119]. Receptor-ligand binding initiates a cascade of events through binding of TRAF-6, leading to activation of both NFκB and MAPK pathways, with a resulting increase in nuclear factor of activated T-cells (NFATc1) activity. RANK/RANKL also activates the

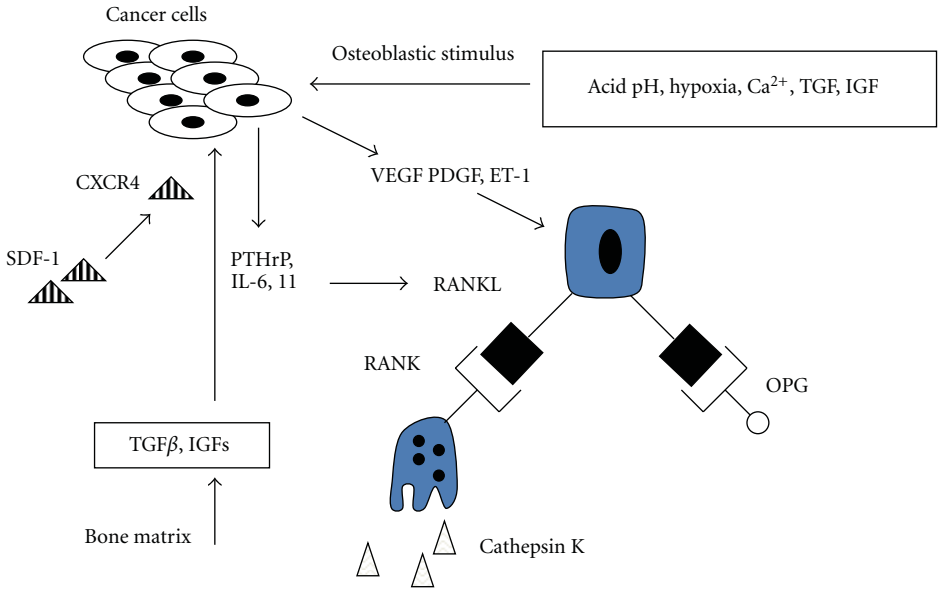

Figure 2: Vicious cycle of osteolysis by osteoclasts.

c-Fos component of AP-1, resulting in additional NFATc1 upregulation. NFATc1 is thus a common end-point for effecting transcription of genes involved in osteoclast activity and maturation [120].

Activated osteoclasts release proteases to resorb the nonmineralised components of bone. Cathepsin K (Cat K) is a cysteine protease selectively produced by osteoclasts for breakdown of collagen I, osteopontin, and osteonectin [121]. Cat K is also produced by some cancer cells to aid invasion [122]. This protease is essential for osteoclast function in normal bone remodelling and also in pathological states of osteolysis. For patients with high-grade metastatic osteosarcoma, low Cat K levels at the time of diagnosis confers a better prognosis [123].

c-Src is a nonreceptor tyrosine kinase present within osteoclasts [124] and is involved in pathways regulating cell growth, survival, and migration [125]. Osteoclast survival occurs through c-Src mediating phosphatidylinositol 3-kinase and TRAF-6 interaction, with resulting Akt/mTOR (mammalian target of rapamycin) pathway activation and then inhibition of caspase-3 [126]. Podosome assembly, vesicle transport, secretion of proteases, and organisation of microtubules are all regulated by c-Src pathway activity [127]. For osteosarcoma, inhibition of c-Src induces apoptosis and inhibits invasion *in vitro*. Primary tumour volume in a murine model of osteosarcoma was also reduced by c-Src inhibition [128].

Osteoclast pathways of differentiation, maturation, and activation have potential as therapeutic targets. Inhibition of bone resorption at the tumour-bone interface may lead to reduced local invasion by osteosarcoma. The central role that RANKL plays in osteoclast function makes it a particularly attractive target. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and strongly suppresses osteoclast differentiation both *in vitro* and *in vivo* [129]. OPG gene therapy has been applied to a murine model of osteosarcoma and successfully suppressed osteolytic activity. There were a reduced number of osteoclasts associated with tumours, leading to reduced local osteosarcoma progression and improved survival [130].

3. Summary and Future Directions

Osteosarcoma is a relatively uncommon malignancy, with an overall incidence of 5 cases per million persons per year. However, among childhood malignancies, osteosarcoma is the eighth most common. Only leukaemias, lymphomas, and neurological malignancies are more common. Osteosarcoma accounts for 8.9% of cancer-related deaths in children and carries an overall 5-year survival rate of 60%–70% [131]. However, being a disease that affects patients in the prime of their lives, incidence and survival rates do not accurately reflect the true burden of this disease. The burden to patients and the community is particularly high as our current treatments combine chemotherapy, often disabling surgery, and prolonged periods of rehabilitation. The disability-adjusted life year (DALY) was put forward by the World Health Organisation (WHO) as a measure of overall burden of disease. It is the number of years lost due to disability, poor health, or premature death. For sarcomas, an average of 17 life years per patient is lost, compared to 6.5 for bowel, lung, and breast cancers. For this reason, the treatment of osteosarcoma is a major public health issue.

Ottaviani and Jaffe [131] recently published a review of the epidemiology of osteosarcoma. Death rates for osteosarcoma are declining by a small 1.3% per year. Indeed, there has been no significant improvement in prognosis for patients with osteosarcoma since the advent of multiagent chemotherapy. Prior to chemotherapy, the overall survival rate for osteosarcoma was a dismal 20% [132]. However,

the challenges we now face are paradoxically the result of our application of modern chemotherapeutics. Resistance to chemotherapy and the recurrence of disease, commonly in the form of pulmonary metastases despite successful surgical resection, are the two greatest challenges we face in regards to the development of therapies for osteosarcoma.

Our understanding of the molecular basis of osteosarcoma has advanced considerably over recent decades. The processes involved in osteosarcoma oncogenesis have been outlined above, and it is our hope that a molecular understanding of the disease will lead to targeted treatment of osteosarcoma. As is evident from the discussion above, there are potentially multiple targets, and we must identify and develop those with the most promise. Therapeutic approaches may not target osteosarcoma cells themselves but may seek to intervene in the complex biology between osteosarcomas cells, osteocytes, osteoblasts, osteoclasts, and even endothelial cells. Indeed, some of the more promising therapeutic agents developed exploit multiple tumorigenic pathways. For example, the potent antiangiogenic pigment epithelium derived factor (PEDF) inhibits the supporting vasculature of the developing tumour whilst also inhibiting proliferation, invasion, and metastasis of osteosarcoma cells [94, 133–135]. Similarly, reversion-inducing cysteine rich protein with Kazal motifs (RECK) has been shown to reduce microvascular density, tumour invasion, and metastasis independently [136].

In this paper we have sought to outline the molecular pathogenesis of osteosarcoma with some reference to potential therapeutic targets currently under investigation. The genetic basis of osteosarcoma has been presented and discussed, along with the role of key transcription factors and growth factors. The processes of osteosarcoma cell proliferation, apoptosis, adhesion, invasion, and metastasis represent potential biological targets for treating osteosarcoma. Osteoclast and endothelial cells may also be targeted. However, the study of pathogenic mechanisms is in itself not enough. Translational studies are critical if an effective treatment for osteosarcoma is to arise from this understanding of osteosarcoma biology. The past decade has revealed a great deal about osteosarcoma pathogenesis, and only with further translational studies will, we see which of the many potential targets and combination of therapies prove to be the most effective in treatment of this debilitating tumour.

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Review Article

Osteosarcomagenesis: Modeling Cancer Initiation in the Mouse

Kevin B. Jones

Department of Orthopaedic Surgery and Center for Children's Cancer Research, Huntsman Cancer Institute, The University of Utah, Salt Lake City, UT 84112, USA

Correspondence should be addressed to Kevin B. Jones, kevin.jones@hci.utah.edu

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Osteosarcoma remains a deadly malignancy afflicting adolescents and young adults. The lack of a precursor and the panoply of genetic aberrations present in identified osteosarcomas makes study of its initiation difficult. A number of candidate hypotheses have been tested in the mouse, a species with a higher background incidence of osteosarcoma. Chemical carcinogens, external beam radiation, and bone-seeking heavy metal radioisotopes have all proven to be osteosarcomagenic in wild-type mice. A number of oncogenes, introduced via integrating viruses or aberrantly activated from heritable genetic loci, participate in and can individually drive osteosarcomagenesis. Germline and conditional gene ablations in the form of some but not all aneuploidy-inducing genes, conventional tumor suppressors, and factors that function normally in mesenchymal differentiation have also proven osteosarcomagenic, especially in combinations that silence the *Rb1* and *p53* pathways. This paper reviews the rich history of mouse models of osteosarcomagenesis, what they have taught us about the human disease, and what future mouse experiments yet promise to teach.

1. Introduction

Osteosarcoma is the most common primary bone malignancy and a leading cause of cancer death in adolescents and young adults [1]. Phenotypically, osteosarcoma adheres to a narrowly defined pattern of disease.

Most osteosarcomas arise in the 2nd and 3rd decades of life in the metaphyses of long bones, especially near the major growth centers of the distal femur, proximal tibia, and proximal humerus [1, 2]. When osteosarcoma rarely develops in a patient over 40, it is usually secondary to prior radiation exposure or Paget's metabolic disease of bone. The vast majority of osteosarcomas (~95 percent) present as high-grade neoplasms, with microscopic metastatic disease at presentation the expectation in every case [3]. Intermediate and low-grade variants of osteosarcoma are extremely scarce [4]; benign bone-forming neoplasms are also much more rare than conventional osteosarcoma itself. There is no identifiable precursor to osteosarcoma.

Despite this narrow clinical phenotype, the genotype of osteosarcoma aligns best with high-grade carcinomas, by its many cytogenetic aberrations and multiple mutations. It is difficult to discern which of these many derangements are causative of, as opposed to resultant from oncogenic transformation. Naturally, when the final state of these cells fails to readily highlight the pathway of transformation that engendered them, and no precursor lesion is known, scientists turn to model systems to investigate cancer initiation hypotheses.

Mouse models of human diseases have proven useful in both mechanistic biological understanding of pathogenesis and preclinical evaluation of medical interventions. For the field of cancer research, most mouse models have been xenografts of human cells into immunocompromised mice. Recent efforts have extended to genetic mouse models of disease, given the ability to manipulate the murine genome with predictable facility. The modeling of osteosarcoma specifically in mice predates the wide availability of xenografts and even the technological innovations that permitted gene targeting in the mouse.

Although much remains unknown regarding osteosarcomagenesis, rodent models of osteosarcoma initiation have taught us much and promise to have much more yet to teach.

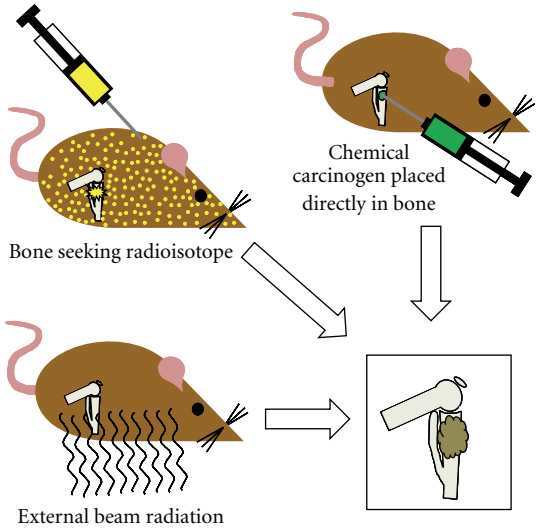

FIGURE 1: Osteosarcomagenesis can be induced in genetically wild-type mice through a variety of means. Most heavy metal radioisotopes will naturally home to ossifying bone matrix in the metabolically active metaphyses of long bones, emitting their radiation locally after embedding. External beam radiation in varied forms has also proven successfully osteosarcomagenic. Although a few chemical carcinogens administered systemically by oral or intravenous application in mice have proven sufficient, most osteosarcomagenic chemical compounds have been orthotopically implanted in the tibia or femur.

2. Random Mutagenesis and Induced Chromosomal Instability

The background rate of osteosarcomagenesis in rodents is higher than the background rate of most carcinomas and much higher than the natural incidence of osteosarcoma in humans, but is still low. Control mice in most induction studies have under five percent natural lifetime incidence of osteosarcoma. A mouse strain with a higher basal incidence of osteosarcoma was reported in 1938 but may have carried germline mutations in some important tumor suppressors yet unrecognized [5].

2.1. Chemical Carcinogens. The first report of chemically induced murine osteosarcomagenesis was also in 1938. Drs. Brunschwig and Bissell surgically placed crystals of 1,2-benzpyrene mixed in cholesterol into the tibial medullary cavities of a few mice. One of these formed a radiographically and histologically verified osteosarcoma 8.5 months later [6]. The same authors had previously produced nonosteogenic sarcomas by the application of 3-methylcholanthrene [7]. Many reports of chemical carcinogen-induced osteosarcomas in mice and rats have followed, but a theme that began with these first two attempts has persisted: bulky-adduct-forming heterocyclic compounds have generally proven more efficient in the induction of osteosarcomagenesis than methylating agents, which tend to produce fibrosarcomas [8–10].

The mechanisms by which these different classes of agents generate genetic instability are chromosomal instability from bulky-adduct-forming agents and microsatellite instability derived from a mismatch repair defect in methylating agents [11]. This observation says nothing definitive about human osteosarcomagenesis but suggests that chromosomal instability is an important element of the genetic distress and instability involved in pathogenesis.

Most chemically induced mouse osteosarcomas have arisen from orthotopically implanted chemical carcinogens (Figure 1), arguing that environmental exposures to chemical carcinogens may not play a large role in sporadic human osteosarcoma incidence. One exception to this is the oral administration of 1-(2-hydroxy-ethyl)-1-nitrosurea to rats, which generated osteogenic or chondrogenic sarcomas in 58 percent. Overall, chemical implant-related osteosarcomagenesis has taught us primarily about the effects of varied methods of DNA damage, rather than necessarily recapitulating the actual etiology of the human disease. Although two reports have considered an increased incidence of osteosarcoma in pediatric and adult populations exposed to pesticides, the links are yet to be verified in second populations [12, 13].

2.2. Radiation. Since the observation of frequent osteosarcomas arising among radium dial painters in 1931 [14], there have been many attempts to induce osteosarcomas by application of radiation to the rodent skeleton. Some of this work in rabbits actually predated Brunschwig's chemical induction of a mouse osteosarcoma [15, 16]. Both external beam and internal exposure to filtered and unfiltered rays from radium, thorium, and roentgen radiation have proven sufficient to induce osteosarcomas in rodents at high enough doses [17–20]. Beyond fitting the theme of DNA damage readily inducing osteosarcoma, this has an obvious correlate with human osteosarcomagenesis, as exposure to environmental or therapeutically/diagnostically applied external beam radiation is known to increase risk for osteosarcoma [21]. Osteosarcoma is one of the more common radiation-induced secondary cancers in humans.

Mice have otherwise been induced to initiate osteosarcomagenesis with radioactive heavy metals [22–30]. These ions tend naturally to home to the bone and incorporate in the hydroxy-apatite crystals that mineralize the ossifying matrix, each ion usually replacing a calcium ion in the structure. Most of the successfully osteosarcomagenic radioisotopes have been alpha emitters. One can certainly speculate as to the particular variety of genomic damage caused by alpha particles, but this observation may have had as much to do with simple anatomical localization of the damage to osteoprogenitors near the matrix mineralization front, as alpha particles do not traverse multiple tissue planes efficiently, due to their large size. Certainly, beta-emitters that home to bone have also proven osteosarcomagenic.

2.3. Viral Insertional Mutagenesis. Genomic distress can also be generated by infection with integrating retroviruses. Many of the early mouse models of leukemia and lymphoma were

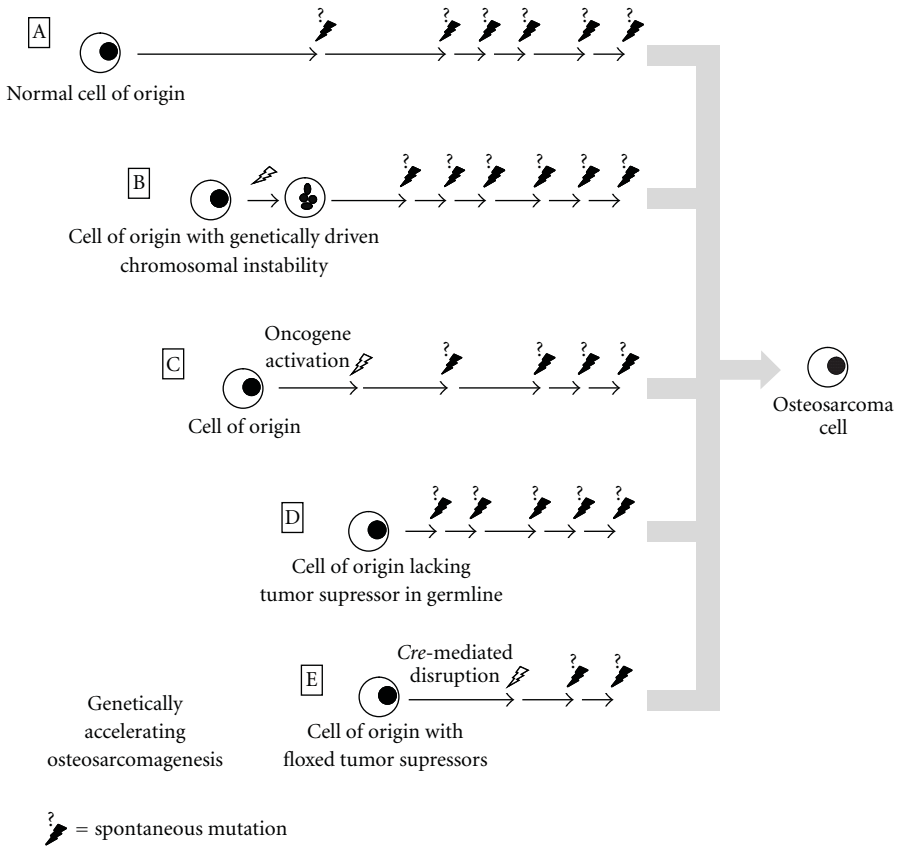

Figure 2: Genetically wild-type cells can naturally accrue sufficient mutations to initiate osteosarcomagenesis in mice as evidenced by the background incidence of osteosarcomas a little under 5 percent in most strains (A). Genetically induced aneuploidy alone, in most of its forms, is inefficient in osteosarcomagenesis (B). Expression of oncogenes as transgenes using native promoters, introduced via insertional viral vectors or unmasked by radiation, can lead to benign and malignant bone neoplasia in mice (C). Inherited germline deletion of tumor suppressors in heterozygosity or homozygosity or generation of mouse chimeras of cells with and without such deletions to avoid serious developmental defects have proven the efficiency of many gene inactivations in osteosarcomagenesis (D). Another means by which severe developmental phenotypes may be eschewed or potential cells of origin tested is by the use of conditional oncogene activation or conditional tumor suppressor ablation, specified either temporally or, by tissue, spatially (E); combinations of tumor suppressor deletions, possible in conditional ablation methods, have been very efficient at driving osteosarcomagenesis.

driven by the mutations caused by integrating viral DNA [31]. Cloning of these sites was the first major source of information regarding tumor suppressors and oncogenes in general. There are no reports of osteosarcomas arising in mice exposed to such insertional mutagenesis specifically, but the contribution of such insertional mutations to the osteosarcomagenesis induced by oncogene-expressing integrating viruses has not fully been explored.

2.4. Gene Targeting. Bombarding the genome with a chemical, radiation, or an integrating virus will necessarily have pleiotropic effects. There have been other attempts to distress the genome in a more orderly fashion, by genetic manipulation of mice (Figure 2). For instance, aneuploidy can be readily induced via hypomorphic or ablated alleles of certain cell cycle checkpoint and mitotic spindle assembly proteins. Such aneuploidy induced from *UbcH10* disruption [32], *Bub3* and/or *Rae1* disruption [33], or *RanBP2* disruption [34] generates no statistically significant increase in the number of sarcomas arising. In contrast, homozygosity for hypomorphic *Bub1* does increase sarcoma incidence to nearly 15 percent (from a 5-percent background) over the 2-year lifespan typical of these mice [35]. The authors did not identify how many, if any, of these sarcomas were osteosarcomas. In another study the same group added *Bub1*-induced aneuploidy to a background of heterozygosity for a panel of tumor suppressors and identified induced loss of heterozygosity of these tumor suppressors as one mechanism of action for *Bub1* oncogenesis [36].

Insertional mutagenesis can also be driven by transposons in a controlled genetic fashion in murine somatic cells. Similar to oncogenic mutations from insertional retroviruses, most of the tumors that form in *Sleeping Beauty* transposon mice, when it is activated throughout the body, are leukemias and lymphomas [37]. Soft-tissue and a few bone sarcomas have been generated but only with cooperative mutations in *p19Arf* [38].

3. Oncogenes

The other mouse models of osteosarcoma that predated the age of gene targeting were derived from insertional viruses that expressed transforming oncogenes. FBJ murine osteosarcoma virus and RFB osteoma viruses were identified from spontaneous murine tumors and the FBR osteosarcoma virus from noncellular extracts from a radiation-induced murine osteosarcoma [39]. All of these viruses were eventually noted to cause high expression of c-*fos*, which became almost the definitional osteosarcoma oncogene [40, 41]. When transgenic mouse technology was possible in the late 1980s, mice transgenic for high c-*fos* expression similarly formed osteosarcomas consistently [42]. Then, c-*Jun*, another powerful oncogene, was found to potentiate c-*fos*-driven osteosarcomagenesis when coexpressed [43]. In screening a number of spontaneous and radiation-induced murine osteosarcomas, a variety of oncogenes were found to be expressed, including c-*abl*, c-*bas*, c-*fos*, K-*ras*, and c-*myc*. No particular oncogene appeared to predominate [44]. So important were these oncogenes felt to be initially that a theory became prominent in the literature during the 1980s and 1990s that radiation-induced osteosarcomagenesis may have as much to do with unsilencing of oncogenes as it does with DNA and chromosomal damage [45, 46].

Perhaps involved with steps other than initiation, other nonviral oncogenes have been found to have potential importance in mouse osteosarcomagenesis. Comparative genomic hybridization on osteosarcomas arising in *p53* heterozygous mice identified a recurrent amplification of mouse chromosomal region 9A1 [47]. This corresponds to an amplification of chromosome 11 common in human osteosarcomas. The antiapoptotic genes *Birc1* and *Birc2*, as well as *matrix metalloproteinase 13* were all found to be upregulated by this amplification. Knockdown of the expression of each impeded tumor growth following engraftments into immunocompromised mice. Further, *ezrin* has been highlighted as a major contributor to the metastatic phenotype in a particular *in vivo*-passaged murine osteosarcoma cell line [48].

No discussion of oncogenes and their role would be complete without mention of the Simian vacuolating virus 40 (SV40), a polyoma virus that readily causes osteosarcoma in hamsters. Expression cassettes from the virus have proven effective in producing osteosarcomas in mice as well [49–52]. As the oncogenes produced function more as a means of inactivating specific tumor suppressors, we will discuss them in greater detail below, acknowledging that, while SV40 T antigens function as tumor suppressors, they are themselves overexpressed and pro-oncogenic, similar to other oncogenes.

4. Tumor Suppressor Silencing

4.1. Retinoblastoma and p53 Pathways. The first clues that murine osteosarcomagenesis can be driven by *Rb1* and *p53* pathway disruptions came in the high incidence of osteosarcomas in mice with transgenic expression of the SV40 large T antigen [49–52], which is known to bind and silence members of the *Rb1* and *p53* cell cycle checkpoint pathways, effectively disrupting them. Conditional genetic ablation of *Rb1* and *p53* specifically using the *Cre*-lox system has also recently proven sufficient to drive short-latency osteosarcomagenesis [53, 54]. Both research teams, who published essentially identical mouse models based on this technique, concluded that *p53* silencing is necessary and *Rb1* silencing cooperative—but insufficient alone—to drive osteosarcomagenesis.

Germline heterozygosity for *p53* ablation engenders a variety of cancers, but approximately 25 percent of mice heterozygous for *p53* knockout will develop osteosarcomas [55]. While the variety of tumors common in patients with Li-Fraumeni syndrome is generally replicated in heterozygous *p53*-deficient mice, the relative rates of each are quite different. Lymphomas are much more common in murine than in human Li Fraumeni, and carcinomas much less common [56]. Homozygous knockout of *p53* has a lower incidence of osteosarcoma (4 percent), most likely due to the high incidence of early mortality from lymphoma formation [55]. Further studies have reverse-translated specific disease-causing missense mutations from human cancer patients into the mouse and have shown that some can behave more like dominant negative than silent alleles. Heterozygosity for a particular such missense mutation, replacing the arginine at amino acid residue 172 with a histidine, generated a 50% incidence of osteosarcoma with shorter latency than typical for heterozygous *p53* ablation [57]. Other experiments have conditionally ablated *p53* alone in osteoprogenitor cells. Homozygosity for this tissue-specific *p53* silencing efficiently produces osteosarcomas with near-complete penetrance [58].

Germline homozygous deletion of *Rb1* is not compatible with life. Heterozygous Germline *Rb1* disruption does not generate osteosarcoma with any detectably increased incidence, in stark contrast to humans heterozygous for germline *RB* ablation, who have a 500-fold increased incidence of osteosarcoma [59]. *Rb1* heterozygous mice also do not develop retinoblastomas, whereas most humans with the homologous genotype will have bilateral retinoblastomas either congenitally or during infancy. This is possibly due to the reduced stochastic likelihood of losing heterozygosity in retinal cells given that they have undergone fewer cell cycles prior to terminal differentiation in a mouse than in a human. However, mouse chimeras bearing cells with *Rb1* homozygous deletion also do not form retinoblastomas or osteosarcomas. The explanation for this lack of tumorigenesis came in the discovery of a redundancy in the mouse (but not in humans) among the pocket proteins, *Rb1*, *p107*, and *p130* [60]. It was first discovered, with further chimera experiments, that *p107* ablation with *Rb1* ablation efficiently produces retinoblastomas and early mortality in mice [61]. Similar chimera experiments with homozygous *p107* deletion and heterozygous *Rb1* deletion were performed to permit appreciation of the broader tumor spectrum, typical of humans heterozygous for *Rb1* disruption. Eight of the 53 chimeric mice observed developed osteosarcomas, most of which had lost heterozygosity for *Rb1* in the tumor cells [62]. These data, although overlooked in recent articles

arguing from mouse data for the predominance of the *p53* pathway as critical and *Rb1* as only cooperative, suggest that a slight variation in pocket protein biology between mouse and human may have masked the importance of the retinoblastoma pathway overall in mouse osteosarcomagenesis.

The *INK4a/ARF* locus in the mouse and human has implications for both the *p53* and *Rb1* pathways. Although disruption of at least the *INK4a* portion is common in human osteosarcomas that otherwise lack *RB* silencing [63], it has received little attention in the mouse. Beginning from cultured murine mesenchymal progenitors, *INK4a/ARF* disruption in tandem with c-*myc* oncogene expression has proven sufficient to generate cells that will form osteosarcomas when injected orthotopically into syngeneic mice [64]. *In vivo* osteosarcomagenesis experiments have yet to be performed with respect to *INK4a/ARF*.

4.2. Osteoprogenitor Differentiation Program Factors. Speculation has reigned over the consideration that *Runx2*, the definitional preosteoblast transcription factor, functions as a tumor suppressor gene in osteosarcoma. Other *Runx* family genes have been implicated in cancers [65]. The semidifferentiated state of osteoid producing osteosarcoma cells certainly raises questions about the *Runx2*-mediated juncture in osteoblast differentiation. While Runx2-driven osteoblast differentiation is clearly truncated in osteosarcoma, expression tends to be higher than in osteoblasts and correlates with a poor prognosis [66, 67]. Experiments in mice and using *ex vivo* murine cells in culture have identified interactions between *Runx2* and both *p53* and *Rb1* defining the early stages of the osteoblast differentiation program [58, 68]. One of the ways that *Rb1* loss is felt to prime for osteosarcomagenesis is by disrupting the feed-forward cycle between *Runx2* and $p27^{kip1}$ [69]. Disruption of this feed-forward loop results in osteoprogenitors failing to terminally differentiate and exit cell cycle [70]. This explains the dysfunctional presence of *Runx2* in human osteosarcomas.

Prkar1α was recently proposed as another tumor suppressor gene involved in osteosarcomagenesis in the mouse [71]. Disruption of this gene reduced the latency to osteosarcomagenesis in mice transgenic for SV40 large T and small t antigens expressed by the osteocalcin promoter. *Prkar1α* is involved with osteoblast-osteoclast *RANK/RANKL* interactions and osteoclast differentiation and activation programs. A potential challenge to the role of *Prkar1α* disruption more generally in osteosarcomagenesis is that the differentiated osteoblasts in which this model initiate T-antigen-mediated genomic distress are also more likely to be embedded within tight anatomic constraints, perhaps overemphasizing their need to recruit bone destroying cells to permit geographical expansion and invasiveness. *Prkar1α* may be a critical tumor suppressor for this particular model and perhaps a subset of osteosarcomas, but not critical overall. A correlation between human osteosarcoma expression of *Prkar1α* and response to chemotherapy was also discussed in the same paper but rendered somewhat equivocal results [71].

4.3. Other General Tumor Suppressors. One recent finding is that *Wnt-inhibitory factor-1* (*Wif1*) functions as a tumor suppressor in osteosarcomas. It was identified among genes frequently silenced by promoter hypermethylation in a panel of human osteosarcoma samples [72]. Mice homozygous for targeted deletion of *Wif1* were noted to have a slight tendency toward osteosarcomagenesis (2 of 13 mice); when exposed to beta-emitting calcium 45 radioisotope, the typical latency to osteosarcomagenesis was shortened by two months compared to wild-type controls [72]. *Wif1* knockout essentially enhances *Wnt* signaling, offering the possibility of a therapeutic target in *Wnt* signaling itself.

Hypermethylated in cancer 1 (*Hic1*), by its name, is another epigenetically modified locus that has also been reported to have a tumor suppressor function in osteosarcoma. Mice homozygous for both *p53* and *Hic1* deletion have a similar life expectancy overall, compared to *p53* deletion alone, but much more frequently develop osteosarcomas [73]. *Hic1* and *p53* are located in close proximity on mouse chromosome 11 and human chromosome 17. Heterozygous deletion of *trans* alleles of *p53* and *Hic1* results in increased incidence of metastatic osteosarcomas over either allele alone [73]. Interestingly, in human tumors, *Hic1* is only hypermethylated in tumors with *p53* mutations, suggesting that it is a dependent, necessarily secondary tumor suppressor.

Wwox is a tumor suppressor gene located in one of the most fragile loci in the mammalian genome. While heterozygotes can be induced to generate a variety of tumors, mice homozygous for Germline deletion of *Wwox* form bone surface lesions suggestive of chondroblastic osteosarcomas and then die at a very young age with no additional tumorigenesis noted [74]. *Wwox* expression has been assessed in human tumors as well, where it appeared to have reduced or absent expression in a majority of osteosarcomas and increased in response to chemotherapy [75].

Mice bearing deletion of the *Xpa* gene and heterozygosity for *p53* developed more frequent osteosarcomas than controls when exposed to diethylstilbestrol, but *Xpa* knockout alone was not different than controls, suggesting that *p53* played a more critical role in these experiments [76]. Although *Xpa* disruption has only received minimal attention in human osteosarcomas, one investigation of polymorphisms in it and other nucleotide excision repair genes found shorter event-free survival from osteosarcoma in cisplatin-treated patients bearing a polymorphism in *ERCC2*. Similarly, cooperative disruption of *p53* and *Brca2* generated osteosarcomas in mice, but the increased incidence over baseline was primarily attributed to the former tumor suppressor [77]. A single case of osteosarcoma in a patient with a germline *Brca2* mutation has been reported, but the association has not been investigated further [78].

4.4. Surprises. After congenital bilateral retinoblastoma and Li Fraumeni syndromes (from *RB* and *p53* heterozygous inactivation in the germline, resp.), the next highest incidence of osteosarcoma in humans occurs in a heritable syndrome called Rothmund-Thomson syndrome, which results from homozygosity for *RECQL4 helicase* ablation. A model of *Recql4 helicase* disruption has been generated in

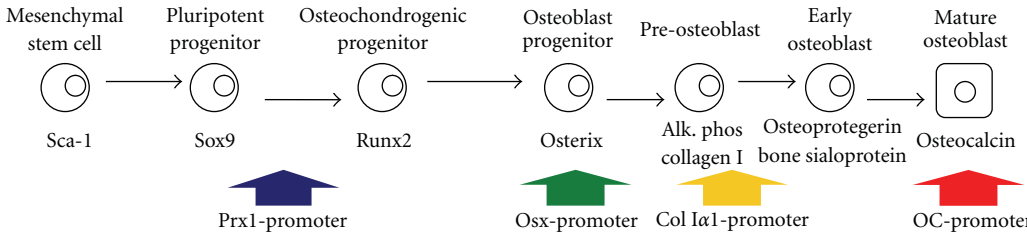

Figure 3: Osteosarcomagenesis has been tested from a range of cells of origin along the differentiation pathway of osteoblasts. *Cre*-recombinase expression driven from the *Prx1*, *osterix*, and *collagen 1a1* promoters has generated osteosarcomas in mice when used for the conditional inactivation of *p53* with or without *Rb1*. The osteocalcin promoter, defining a late stage of osteoblast differentiation usually not expressed in osteosarcoma cells directly, has proven sufficient for osteosarcomagenesis, when used to drive SV40 large and small T antigens.

the mouse by gene targeting, but osteosarcomas have not been identified, despite recapitulation of other features of the autosomal recessive heritable disorder [79, 80]. Because *RECQL4 helicase* has not been found to be mutated or deleted in sporadic human osteosarcomas, it is felt that its disruption represents more of a route to generate genomic instability generally than the silencing of a specific tumor suppressor important to osteosarcomagenesis.

One of the first gene-targeted mouse models noted to develop frequent osteosarcomas is the *merlin* or *Nf2* knockout [81]. There is no human correlate to this. *NF2* deletion has not been detected in human osteosarcomas [82]. Surprisingly, many attempts at acoustic schwannoma (the tumor that predictably arises in *NF2* heterozygous humans) formation in these mice have been thwarted by their strong propensity toward osteosarcomagenesis. The only direct link that has fit with other knowledge of osteosarcoma so far is that the *Nf2* and *p53* genes are in relative proximity in the genome and the osteosarcomas that form often lose one or both alleles of *p53* as well. However, overexpression of *ezrin* is important to human osteosarcomagenesis, especially development of the metastatic phenotype [48]. *Nf2*, or *merlin*, is named as the moesin_ezrin_radixin_like protein. A possible relationship between *merlin* disruption in the mouse and *ezrin* overexpression in humans has yet to be deciphered for osteosarcomagenesis.

5. Tissue of Origin

The anatomic placement of most successfully osteosarcomagenic chemicals in the metabolically active metaphyses of long bones and the natural homing of alpha-emitting heavy metals to the same both make some philosophical comment on the osteosarcoma tissue of origin. That said, a wide variety of cell lineages are typically active even within this constrained anatomy. Osteosarcomas are pathologically defined as malignant appearing cells producing bone, but this can be a small minority of the overall tissue volume of a given tumor. There can be osteoblastic, chondroblastic, fibroblastic, and telangiectatic tissue types in any single osteosarcoma. This fact has long raised the question of what cell within the bone is the cell in which the program of oncogenesis begins: marrow stromal cells, mesenchymal progenitor cells, stem cells, differentiated osteoblasts, or hematopoietic progenitors.

The tools for conditional gene disruption and oncogene activation have greatly enhanced our capacity to test for specific cell types of origin (Figure 3). Of course, all that can really be tested with any of these experiments is osteosarcomagenic sufficiency or lack thereof of the induced derangement in the given cell expressing a specific promoter. A variety of promoters have proven sufficient to drive osteosarcomagenesis enacting the specific conditional derangements planned. SV40 transgenic promoters included the alpha-amylase promoter [49, 52], the heat shock protein 70 promoter from Drosophila [50], and the myelin basic protein promoter [51], each of which managed to generate osteosarcomas in mice, confirming that each was expressed in at least some sufficient cells of origin.

With *Cre*-lox conditional ablation of *p53* as the given derangement, different mesenchymal and preosteoblast promoters have proven sufficient for osteosarcomagenesis. The first tried was a *collagen 1α1* promoter fragment, which also generated a number of lymphomas, suggesting leakiness into earlier mesenchyme as the possible source of sufficient originating cells [58]. Others have used the *Osterix* promoter to drive *Cre*-mediated conditional ablation of *p53* with or without tandem *Rb1* conditional ablation [53, 54]. This should be expressed in committed but not yet terminally differentiated osteoprogenitors. These mice formed some adipocytic tumors in addition to the high incidence of osteosarcoma, also suggesting some contribution of either dedifferentiation after tumor suppressor silencing or promoter leakiness into earlier multipotent mesenchyme. Finally, *Prx1-Cre* was used to drive tumor suppressor silencing in lateral plate mesoderm cells and what certainly remains pluripotent mesenchymal progenitors [83]. These mice developed a number of soft-tissue sarcomas as well as osteosarcomas, highlighting the originating cells' pluripotency.

The genetic mouse model initiating osteosarcomagenesis in the most differentiated cells uses the *osteocalcin* promoter to express large and small T antigens from SV40 [71]. Clearly, a sufficiently strong sledge hammer to the genome can produce osteosarcomas even from what should be cells that have exited cell cycle. It may be that a lesser hit in the same cells would prove insufficient to drive osteosarcomagenesis. All we know is that we have not pushed against the utter extreme yet for osteosarcomagenesis from more differentiated cells. Further, we are no closer to knowing which of these sufficient

osteoprogenitors is most frequently the actual cell of origin in humans.

6. Host Factors

Although this has received the least attention so far from investigators in the field, mice also have been manipulated to identify the effects of the background strain or presence of specific germline expressions or disruptions on other methods of inducing osteosarcomagenesis. Not long after the report of the first targeted disruption of *p53* in the mouse, a paper compared the cancer spectrum and incidence between the 129/Sv strain and a mixed C57BL/6+129/Sv strain, finding no significant difference for osteosarcoma specifically [84]. The rates of plutonium-induced osteosarcomagenesis were not significantly different between C3H/He, C57BL/6, and $B6C3F_1$ strains in another study [85].

Surgical hypophysectomy in mice prior to implantation of grafted tumors suggested the role of *Igf1* in osteosarcomagenesis [86]. Later host mice engineered genetically to have *Igf1* gene ablation demonstrated similar results [87, 88]. Certainly, these were models intended to ask specific questions about pathway contributions rather than identify the typical etiologies behind human osteosarcoma, but they highlight an area of research that is already taking off with respect to other cancers and host-tumor interactions. With the technology for deep sequencing of polymorphisms and their variance between strains of mice, we may eventually be able to understand much about the more subtle host genetic contributions to risk of osteosarcoma.

One notable and unusual study of host factors in osteosarcomagenesis came in the toxicity screening of teriparatide or PTH(1-34). Fischer 344 rats developed high rates of osteosarcoma from prolonged administration of the parathyroid hormone biological analog, even at lower doses [89]. No human correlate to this has been encountered, with only two osteosarcoma cases identified out of over 430,000 human individuals treated with the drug [90]. Treatment of human and murine cells as well as mice *in vivo* with teriparatide-generated DNA double-stranded breaks and chromosomal abnormalities [91]. While this may contribute mechanistically to osteosarcomagenesis in the rat, the mechanism by which PTH(1-34) generates DNA and chromosomal damage is not yet elucidated. Interestingly, treatment of Fischer 344 rats with the full-length PTH(1-84), which includes the C-terminal domain, did not induce osteosarcomas as efficiently at lower doses [92].

7. Conclusions

With the persistent elusiveness of a precursor lesion for osteosarcoma, even in very predictable genetic mouse models of the disease, we continue to learn most of what we know by candidate gene or candidate insult approaches, testing always for sufficiency in osteosarcomagenesis, but never necessity. We know that appropriately located chemical carcinogens or applied radiation rays or alpha-emitting radioisotopes have all proven sufficient to drive osteosarcomagenesis by driving mutagenesis and chromosomal instability, but genetically induced aneuploidy alone is not usually sufficient. We have learned that, when appropriately accounting for a mouse-specific *Rb1* redundancy from *p107*, disruption of either the *Rb1* pathway or *p53* alone in the mouse generally is sufficient to drive osteosarcomagenesis. Their combination is alarmingly efficient, consistent with the observation from human osteosarcomas that both pathways are usually disrupted by some means in tumors. We have learned that these disruptions can initiate osteosarcomagenesis in undifferentiated mesenchymal progenitors or even committed cell types.

Other pathways, oncogenes, and tumor suppressors have been identified, some of which are clearly dependent on other disruptions or the application of other genetic insults to drive osteosarcomagenesis. No doubt, improved oncogenomic techniques will inspire yet new mouse models and the testing of the sufficiency of newly identified tumor suppressor disruptions and oncogene activations in the mouse. The new fields of host and niche biology as the environments in which cancers initiate and develop can also be expected to bring new knowledge to osteosarcomagenesis as well as further innovative experimentation in the mouse.

There remain challenges to the use of the mouse as a model organism for human osteosarcomagenesis. First, rodents more readily form osteosarcomas than do humans. Second, there are a number of specific pathways that are difficult to translate between the two. Other challenges, such as the lack of true lamellar bone in the mouse, highlight critical discrepancies in healthy bone biology between the species. Many scientists argue that genetically engineered or transplanted syngeneic osteosarcomas in rats, which have lamellar bone structure, or spontaneous osteosarcomas in canines may provide better preclinical models for drug testing and so forth. Nonetheless, the mouse as preclinical model may be less enlightening than the mouse as testing platform for the induction of osteosarcomas. The breadth and depth of genomic understanding of the mouse and facility with which it can be experimentally manipulated will not soon be replicated in any other mammalian species. While care must be taken in interpreting results in the mouse, attending to interspecies variations, that same attention can broaden our understanding of this complex disease even further. For example, specific biological eccentricities such as the pocket protein redundancy in mice have not prevented illumination of the human disease; they just require more careful attention to all the available literature. In similar fashion, perhaps further interrogation of *Nf2* in murine osteosarcomagenesis may yet highlight pathways that are important, but unrecognized in the human diseasem.

Although mice and humans are decidedly different in many ways, most of the knowledge gained from mouse modeling has been validated in human clinical samples and cell lines. Clearly, the opportunity to prospectively test hypotheses in mouse-modeled osteosarcomagenesis is unique. There is doubtless much that remains to be learned from the mouse with regard to osteosarcoma initiation, progression, and metastasis.

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Review Article

Defective Osteogenic Differentiation in the Development of Osteosarcoma

Eric R. Wagner,[1] Gaurav Luther,[1] Gaohui Zhu,[1,2] Qing Luo,[1,2] Qiong Shi,[1,3] Stephanie H. Kim,[1] Jian-Li Gao,[1,3] Enyi Huang,[1,4] Yanhong Gao,[1,5] Ke Yang,[1,6] Linyuan Wang,[1] Chad Teven,[1] Xiaoji Luo,[1,3] Xing Liu,[1,3] Mi Li,[1,3] Ning Hu,[1,3] Yuxi Su,[1,2] Yang Bi,[1,2] Bai-Cheng He,[1,3] Ni Tang,[1,3] Jinyong Luo,[1,3] Liang Chen,[1,3] Guowei Zuo,[1,3] Richard Rames,[1] Rex C. Haydon,[1] Hue H. Luu,[1] and Tong-Chuan He[1,2,3]

[1] *Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC3079, Chicago, IL 60637, USA*
[2] *Stem Cell Biology and Therapy Laboratory, The Children's Hospital of Chongqing Medical University, Chongqing 400014, China*
[3] *Key Laboratory of Diagnostic Medicine, Chinese Ministry of Education and Affiliated Hospitals, Chongqing Medical University, Chongqing 400016, China*
[4] *School of Bioengineering, Chongqing University, Chongqing 400044, China*
[5] *Department of Geriatrics, Xinhua Hospital, Shanghai Jiaotong University, Shanghai 200092, China*
[6] *Department of Cell Biology, Third Military Medical University, Chongqing 400030, China*

Correspondence should be addressed to Hue H. Luu, hluu@surgery.bsd.uchicago.edu and Tong-Chuan He, tche@surgery.bsd.uchicago.edu

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Osteosarcoma (OS) is associated with poor prognosis due to its high incidence of metastasis and chemoresistance. It often arises in areas of rapid bone growth in long bones during the adolescent growth spurt. Although certain genetic conditions and alterations increase the risk of developing OS, the molecular pathogenesis is poorly understood. Recently, defects in differentiation have been linked to cancers, as they are associated with high cell proliferation. Treatments overcoming these defects enable terminal differentiation and subsequent tumor inhibition. OS development may be associated with defects in osteogenic differentiation. While early regulators of osteogenesis are unable to bypass these defects, late osteogenic regulators, including Runx2 and Osterix, are able to overcome some of the defects and inhibit tumor propagation through promoting osteogenic differentiation. Further understanding of the relationship between defects in osteogenic differentiation and tumor development holds tremendous potential in treating OS.

1. Introduction

Osteosarcoma is the most common primary malignant bone tumor. Most patients with osteosarcoma complain of symptoms for several months and initially present with a pathologic fracture [1, 2]. Although OS can occur in any bone, it frequently involves the metaphysis of long bones where high bone turnover occurs during longitudinal growth spurts [2]. Radiographic imaging, combined with biopsy, is required for definitive diagnosis [2]. However, a problem lies in the detection of the pulmonary metastases, as only around 15%–20% of patients will have radiographically detectable pulmonary metastases, while approximately 80% of the patients will either develop or already have radiographically undetectable micrometastases [1–4]. These pulmonary lesions are responsible for the high mortality associated with OS [1, 2]. Treatment of OS includes surgical resection of both primary and pulmonary lesions combined with radiotherapy [2]. However, due to the high suspicion for micrometastases, nearly all patients will also receive preoperative and postoperative chemotherapy with agents such as cisplatin, doxorubicin, methotrexate, and isofosfamide [1, 2, 5–7].

These agents expose patients to longterm toxicities, including hearing loss, cardiomyopathy, sterility, and hypomagnesemia [2, 8–13]. Even with this aggressive management, OS patients still have a poor prognosis. Patients who present without detectable metastases have a 70% longterm disease-free survival; once a metastasis has been detected, the disease is likely to relapse [1, 2, 5–7]. Thus, there is a critical need to identify metastatic markers that can accurately predict the presence or absence of metastatic disease at the time of diagnosis and provide both prognostic value and potential targets for novel therapies in the future.

Although the etiology underlying OS is poorly understood, the tumors often develop in settings of high bone turnover, such as the adolescent growth spurt [2]. Furthermore, numerous genetic and cytogenetic abnormalities have been associated with OS, including mutations of tumor suppressors and oncogenes, as well as chromosomal amplifications, deletions, rearrangements, and translocations [1, 2, 14]. The most common alterations are associated with chromosomes 1, 9, 10, 13, and 17, or involve the p53 and Rb genes [1]. Given the numerous alterations associated with OS, it is no surprise that no singular consensus mechanism can account for OS tumorigenesis. Recent investigations have focused on the role of osteogenic differentiation in the pathogenesis of OS. This is supported by the similarities between OS tumors cells and primitive osteoblasts [15]. It is plausible that the genetic and epigenetic alterations associated with OS alter the signaling pathways associated with osteogenic differentiation, arresting the cells as undifferentiated precursors. By approaching OS as a disease caused by differentiation defects, we not only acquire a unique understanding of OS pathogenesis, but suggest avenues for developing novel therapies that can target OS differentiation.

2. Molecular Biology of Osteosarcoma

2.1. Loss of Tumor Suppressors. Both sporadic and inherited mutations to pathways associated with p53 and Rb tumor suppressor genes are associated with osteosarcoma. Rb is a key regulator in the G1/S transition. In its hypophosphorylated state, Rb acts as a tumor suppressor by binding to and inactivating E2F, resulting in cell cycle arrest [16]. Cyclin D1 and CDK4 phosphorylate and inactivate Rb during the G1/S transition, thereby allowing cell cycle progression to occur [16]. Approximately 70% of sporadic OS cases have shown genetic alterations in the Rb1 locus, and individuals heterozygous for a germline inactivation of Rb1 have a 1,000-times greater probability of OS [1, 17–20]. Moreover, inactivation of the Rb1 locus has been implicated as a poor prognostic factor in patients with OS [1, 2, 14].

OS development has also been associated with another tumor suppressor in the Rb signaling pathway, $p16^{INK4A}$ [21]. It functions through inactivation of CDK4, causing cell cycle arrest at the G1/S transition. Alterations in $p16^{INK4A}$ cause an inability to regulate CDK4 and the G1/S transition, leading to an uninhibited cell cycle progression that mimics the Rb mutation phenotype. The downregulation of $p16^{INK4A}$ also serves as a poor prognostic factor in pediatric patients with OS [14, 22].

The tumor suppressor gene p53 maps to 17p13, a region that is frequently abnormal in patients with OS [14, 23]. The p53 gene product acts as a transcription factor that regulates cell cycle progression through apoptotic and DNA repair mechanisms, and has been implicated in the pathogenesis of a variety of human cancers, including OS [24–27]. In OS patients, studies have frequently found point mutations, gene rearrangements, and allelic loss at the p53 locus [1]. Furthermore, patients with the Li-Fraumeni syndrome, a disorder characterized by a germline mutation at the p53 locus, have a significantly higher risk of developing OS [28–30].

2.2. Induction of Oncogenes. Activation of a variety of oncogenes has been implicated in OS tumorigenesis. The c-Myc oncogene encodes for a transcription factor that regulates both cell proliferation and growth [31, 32]. It is reported that up to 12% of OS tumors have amplification at the c-Myc locus while the expression of Myc appears to be correlated with a higher risk for relapse [1, 33–36]. Furthermore, overexpression of c-Myc in $Ink4a/Arf^{-/-}$ bone marrow stromal cells leads to a malignant transformation [37]. Another oncogene associated with OS is MDM2, an important negative regulator of p53. It encodes a protein that inactivates the N-terminal transactivation domain of p53 and marks it for degradation via polyubiquitination [1, 23–25, 27]. Located at the 12q13 locus, MDM2 has been found to be amplified in up to 10% of OS tumors [38–40]. Finally, CDK4, an oncogene associated with the regulation of cell cycle progression, has shown high levels of expression in up to 65% of low-grade OS [41]. CDK4 forms a complex with cyclin D1 and phosphorylates RB, thereby releasing the E2F transcription factor and promoting cell cycle progression [1]. Other important oncogenes that have been reported in association with OS include, but are not limited to, FOS, ERBB2 and CCND1 [1].

2.3. Syndromes Associated with OS. A variety of syndromes show a predisposition to the development of OS. In patients affected by Paget's disease of the bone, approximately 1% will develop OS [42]. Paget's disease of bone results when there is a disconnection between osteoclast and osteoblast activity, resulting in largely deformed bone. Furthermore, Paget's disease accounts for a substantial fraction of patients over 60 years old with OS [42]. Another syndrome that increases the risk of OS is Rothmund-Thomson syndrome, an autosomal recessive disorder that results from a mutation in an RECQ helicase, resulting in photosensitivity, cataracts, and skeletal dysplasias [43]. In one study, 32% of patients with Rothmund-Thomson developed OS, with a tendency to occur at a younger age [43]. Finally, patients with neurofibromatosis 2 (NF2) have decreased expression levels of merlin, an ERM-related protein that acts as a tumor suppressor [44, 45]. Merlin increases the stability of p53 by inhibiting MDM2-mediated degradation, and the loss of merlin in NF2 is thought to destabilize p53 [46].

NF2 heterozygous mice showed a propensity of highly metastatic tumors, including poorly differentiated OS [46].

2.4. Dysregulation of Signaling Pathways. Recently, many investigations have focused on aberrations in cell signaling pathways that have been linked to the development of many different human tumors, including OS. One example is the TGFβ signaling pathway, which involves three distinct proteins (TGFβ 1–3) that are involved in cellular differentiation, cell growth, and apoptosis [47–50]. In OS tumors, there is significantly higher expression of TGFβ1 and TGFβ3 compared to TGFβ2 [51]. Expression levels of TGFβ3 strongly correlate with OS tumor progression [51]. Alterations in other signaling pathways that are implicated, but whose roles are less delineated in OS, include Shh, FGFR2, MET/HGF, and BMPs [1, 52–54]. Later, we discuss the signaling pathways associated with the Wnt proteins and Runx2, and their relationship with defects in osteogenic differentiation and subsequent OS tumor development.

2.5. Mesenchymal Stem Cell Differentiation. Mesenchymal stem cells (MSCs) are bone marrow stromal cells that can differentiate into osteogenic, chondrogenic, adipogenic, neurogenic, or myogenic lineages [55–58]. Osteogenic differentiation is a complex, tightly regulated process that is critical for proper bone formation and is influenced by a variety of endogenous and environmental factors [1, 59]. As MSCs pass through each successive stage of differentiation, they are thought to lose their proliferative capacity. Markers of the osteoblastic differentiation cascade include connective tissue growth factor (CTGF) (early), alkaline phosphatase (ALP), Osterix, Runx2 (early/middle), osteopontin (OPN), osteocalcin (OCN), and collagen 1a1 (Col 1a1) (late) [1, 15, 47, 57, 59–64] (Figure 1).

Many signaling pathways and associated regulatory genes control the complex MSC differentiation cascade [65]. For example, myogenic differentiaion is controlled by factors such as the MyoD and Mef2 family of transcription factors [58, 66, 67]. Commitment of MSCs to the adipogenic lineage is a two-phase process of cell determination and differentiation that is regulated in part by PPARγ, as well as BMPs 4 and 7 [57–59, 68, 69]. Chondrogenic differentiation is regulated by multiple transcription factors and growth factors, such as Sox9, BMP2, BMP7, and FGF2, many of which represent early regulators of the osteogenic differentiation pathway [57, 58]. The factors controlling these pathways are integral in regulating the osteogenic cascade through interpathway cross-talk and feedback cycles. Some of the most important of these molecules include the BMPs, PPARγ, Runx2, and the Wnts (Figure 1).

BMPs belong to the TGFβ superfamily of growth factors, which are considered pivotal regulators of early MSC commitment. The osteogenic BMPs include 2, 4, 6, 7, and 9, with BMP 6 and 9 showing the most potent osteogenic activity both in vitro and in vivo [1, 47, 57–59, 70–74]. BMP 4 and 7 also exhibit adipogenic activity, but commitment to the adipogenic or osteoblastic lineage is mutually exclusive [57, 59, 74–83]. These osteogenic BMPs are able to induce undifferentiated MSCs to express many early osteoblast progenitor markers, such as the connective tissue growth factor (CTGF), inhibitor of DNA binding (Id), alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2) [57, 75, 76, 84–87].

PPARγ is considered the main regulator of adipogenesis. However, it plays a crucial cross-regulatory role in osteoblastogenesis, as PPARγ expression shifts MSC differentiation from the osteogenic to the adipogenic cascade [59, 88]. For example, PPARγ-deficient mice show a lack of adipogenesis with an increase in osteogenic activity [59, 89]. Furthermore, PPARγ seems to be involved in BMP-induced osteogenesis, as PPARγ knockout mice fail to differentiate in response to BMP stimulation [59, 74, 85]. These results suggest that in addition adipogenesis, PPARγ may act as a differentiation regulator in conjunction with the osteogenic BMPs to promote MSC differentiation along an osteogenic lineage.

Runx2 is considered one of the master regulators in MSC osteoblast differentiation [58, 90–92]. Runx2 knockout is fatal in mice, leading to a cartilaginous skeleton without any ossification and delayed chondrocyte maturation [93, 94]. Moreover, Runx2 interacts with numerous transcriptional activators and repressors, which are crucial in osteogenesis, such as Rb, PTH/PTHrP, MAPLK, and histone deacetylases [58, 92, 95–97]. In particular, it is thought to be a critical regulator in the BMP-mediated osteogenic differentiation pathway [98].

Wnts are a group of highly conserved, secreted proteins, and are one of the major osteogenic regulators [58, 99–102]. Wnt genes are expressed in developing limbs and the Wnt coreceptor LRP5 has been shown to regulate bone formation [58, 103–105]. Osteoblast maturation is dependent on Wnt proteins, as Wnt deficient cells fail to undergo terminal differentiation in the presence of the hedgehog signaling proteins [106]. Overexpression of a Wnt antagonist leads to the presence of lytic bone lesions, while activation of Wnt/β-Catenin signaling is frequently observed in osteosarcoma [107, 108]. It appears Wnt molecules control both osteoblastic differentiation and cell proliferation while shunting away from chondrogenic differentiation [109].

The effect of terminal differentiation on stem cells is crucial in understanding oncogenesis. When cells progress down a differentiation cascade, they lose their proliferative capabilities in exchange for a differentiating potential. As a result, they are less responsive to growth factors and increasingly susceptible to apoptosis and cytotoxic agents such as chemotherapy [59]. Thus, it is conceivable that tumorigenesis may result from disruptions that prevent terminal differentiation, thereby allowing tumor-initiating cells to retain their highly proliferative precursor cell phenotypes.

3. Association between Differentiation Defects and Cancer

Stem cells are undifferentiated precursor cells that have a pluripotent ability to give rise to many different types of tissues. They are defined by their capacity for self-renewal, proliferation, and differentiation into mature cells of

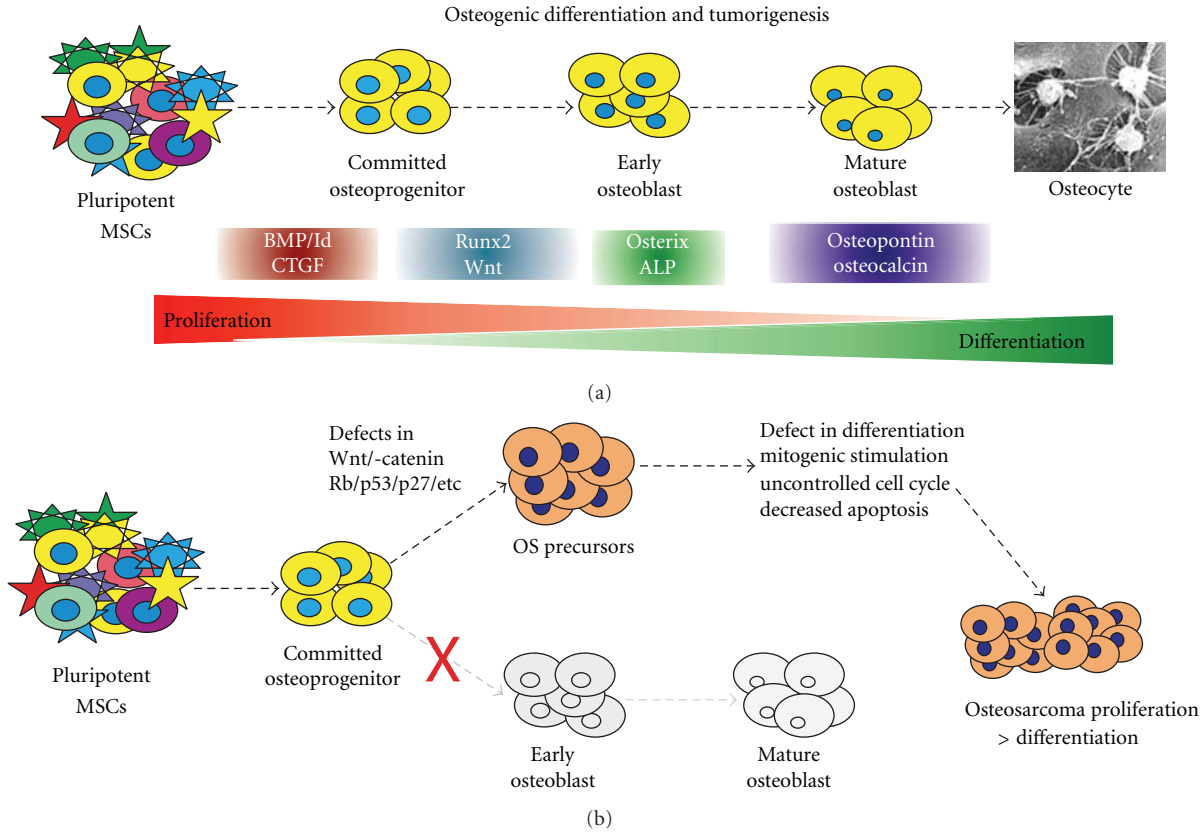

Figure 1: (a) Mesenchymal stem cells (MSCs) progress down the osteogenic differentiation cascade. MSCs are pluripotent bone marrow stromal cells that are able to differentiate into bone, muscle, tendon, and adipose tissue. Osteogenic differentiation of MSCs is a tightly regulated process by different signaling. Bone morphogenetic proteins (BMPs) and their downstream mediators, such as inhibitor of DNA binding (Id) proteins and connective tissue growth factor (CTGF), are early markers in the osteogenic differentiation cascade. Runx2 and Wnt proteins are important regulators of osteoblastic differentiation. Alkaline phosphatase and Osterix are early/middle markers, while osteocalcin and osteopontin are late markers of bone formation. (b) Defects in osteogenic differentiation lead to osteosarcoma (OS) development. If alterations in the MSC differentiation cascade block the progression to terminally differentiated osteoblasts or osteocytes, it is likely that tumorigenic precursors are formed. Such undifferentiated OS precursors would maintain the ability to proliferate and increase the risk for OS development. Although not well understood, some of the potential defects may include genetic and/or epigenetic changes in Wnt signaling, Rb, p53, and p27. These defects may lead to uncontrolled cell proliferation and disrupted differentiation. Thus, these alterations disrupt the delicate balance between proliferation and differentiation, leading to a tumorigenic phenotype.

a particular tissue. Recent studies have linked undifferentiated progenitor cells with tumorigenesis, and their similar ability to self-renew and proliferate [63]. A crucial aspect of stem cell biology is to regulate the balance between proliferation and terminal differentiation. A dysregulation of this balance in favor of proliferation appears to be associated with many different human tumors (Figure 1).

Both normal stem cells and cancer-initiating cells show a unique ability for self-renewal. Pathways that are normally associated with cancer are also crucial to stem cell proliferation, and vice versa. For example, the notch, Sonic hedgehog, and Wnt signaling pathways are associated with the regulation of the hematopoietic stem cell (HSC) pathway, development and oncogenesis [63, 106, 110–114]. Osteoblast maturation is dependent on Wnt proteins, as Wnt-deficient cells fail to undergo terminal differentiation in the presence of the hedgehog signaling proteins [106]. Overexpression of β-catenin in the Wnt pathway can expand the pool of transplantable HSCs from cultured HSCs by propagating stem cell division [62, 63]. Gli1, an intracellular mediator of the hedgehog family, regulates limb bud and osteogenic development [113, 114]. This pathway has also been linked to increased proliferation and tumorigenic transformation [114]. Furthermore, this link is demonstrated in the relationship between epidermal progenitor cells and epithelial cancers [115]. Tumorigenesis is thought to be a summation of multiple events over a period of time. If some of these alterations were blocked to arrest the progenitor cells in undifferentiated, highly proliferative state, it may explain the tumor cells' abilities of self-renewal and propagation [63, 116, 117].

Recently, the notion of "cancer stem cells" has taken shape, where a small subset of stem cells fail to undergo terminal differentiation and maintain their proliferative

capacities, enabling the tumor to continue to self-propagate and regenerate new cells [63, 118]. As reported by Reya et al., both cancer cells and stem cells maintain tremendous proliferative capacity and display similar phenotypic cellular markers [63]. Additionally, both tumors and stem cells consist of a heterogenous population of cells with different proliferative potentials at various stages of differentiation [63]. Thus, the cancer stem cells may be derived from normal undifferentiated progenitor cells, and are thought to drive tumorigenesis.

Multiple therapeutic interventions have targeted the defects in differentiation and are able to promote terminal differentiation of cancer cells and make them more susceptible to apoptosis. Furthermore, these therapies are able to target a specific tissue type, and therefore avoid the systemic toxicities of most chemotherapeutic agents. For example, in breast cancer the estrogen receptor (ER) blocks differentiation in part through induction of cellular proliferation [119]. Tamoxifen targets this receptor, enabling the cells to undergo differentiation and associated apoptosis [120]. PPARγ ligands and retinoids are able to treat liposarcoma through the induction of terminal differentiation [121–125]. In patients with prostate cancer, antiandrogens and retinoids can promote differentiation, and thus decrease tumorigenesis [126, 127]. Finally, clinical trials have suggested that ARA-C can induce complete remission in patients with AML by inducing the differentiation of myeloid leukemia cells [128]. While there are numerous examples of successful differentiation therapy, one particular example is seen in the treatment of Ewing's sarcoma, another primary bone tumor.

4. Ewing's Sarcoma: An Example of Differentiation Defects in a Bone Tumor

Ewing's sarcoma is the second most common malignant pediatric bone tumor [129]. A part of the molecular pathogenesis underlying Ewing's sarcoma is the overexpression of EWS/ETS or EWS/FLI-1 fusion oncogenes that prevent MSC differentiation along the adipogenic and osteogenic lineage [130]. The fusion protein carries out its functions by binding Runx2 and regulating the transcription of the hedgehog mediator Gli1 [130–133]. Silencing of this oncogene leads to the recovery of the MSCs differentiation capabilities [134]. Moreover, expression of this EWS/FLI-1 fusion protein in murine primary MSCs leads to the inhibition of MSC differentiation, and subsequent development of a EWS/FLI-1-dependent Ewing's sarcomas [129]. Collectively, these results suggest that inhibition of MSC differentiation may be crucial to the pathogenesis of Ewing's sarcoma, and that restoration of MSC differentiation potential may be an effective therapy in patients with Ewing's sarcoma.

5. Osteosarcoma as a Result of Differentiation Defects

OS cells share many similar features to undifferentiated osteoprogenitors, including a high proliferative capacity, resistance to apoptosis, and similar expression of many osteogenic markers, such as CTGF, Runx2, ALP, Osterix, and Osteocalcin [1, 15, 47, 57, 59–64]. Furthermore, the more aggressive OS phenotypes often resemble early progenitors, while less aggressive tumors seem to share more similarities with osteogenic MSCs that have progressed further along the differentiation cascade [55, 59].

Analysis of the expression of osteogenic markers in OS cells demonstrates an early osteogenic phenotype. Alkaline phosphatase, a well-documented early marker of osteogenesis, has a much lower expression in OS tumor cells when compared to hFOB1.19 cells, a committed osteoblastic line [64, 135]. Similarly, the late osteogenic markers osteopontin and osteocalcin are highly expressed in mature, differentiated osteoblasts, but are minimally expressed in both primary OS tumors and OS cell lines [47, 57, 136, 137]. CTGF, a multifunctional growth factor that is normally upregulated at the earliest stages of osteogenic differentiation, also shows elevated basal expression in human OS cells [76]. These results suggest that OS cells likely fail to undergo terminal differentiation, and that the degree of dedifferentiation may correlate with a worse prognosis.

By retaining a phenotype similar to undifferentiated osteoprogenitors, OS cells are able to maintain a capacity for uncontrolled proliferation. For example, it is well established that gradual telomere shortening is an effective mechanism of cell senescence when stem cells become terminally differentiated. However, more than 50% of OS cells utilize an alternative lengthening of telomere (ALT) pathway that prevents telomere shortening, allowing the tumor cells to evade senescence and resemble their stem cell progenitors [138]. As a result, OS cells demonstrate similar rates of proliferation, growth factors responsiveness, and capacity for self-renewal to osteoprogenitor stem cells [139]. Furthermore, the stage at which differentiation is interrupted likely correlates with the aggressiveness and metastatic potential of the various OS tumors.

The Runx2 and Wnt regulators of osteogenic differentiation are two examples of alterations in the differentiation cascade potentially underlying tumorigenesis (Figure 1). Runx2 is a member of the runt family of transcription factors that has been linked to a variety of human cancers such as leukemia and gastric cancer [98, 140, 141]. Runx2 is a master regulator of osteoblastic differentiation that is consistently altered in human OS [98]. Runx2 and its associated protein $p27^{KIP1}$, are important regulators of the G1 cell cycle checkpoint [98]. Runx2 also physically interacts with the hypophosphorylated form of Rb, a known coactivator of Runx2, to create a feed forward loop that promotes terminal cell cycle exit and the formation of a differentiated osteoblastic phenotype [98]. Additionally, Runx2 regulates BMP-induced osteogenesis, synergistically inducing many terminal differentiation markers [98]. Interestingly, Runx2 has a very low expression in OS cell lines. When considering the role of Runx2 in the cell cycle and terminal differentiation regulation associated with BMPs, Rb, and $p27^{KIP1}$, it is natural that any alterations would lead to uncontrolled proliferation and loss of differentiation. Accordingly, high-grade osteosarcomas show decreased expression of $p27^{KIP1}$, while lower-grade tumors have detectable $p27^{KIP1}$ levels.

Furthermore, dedifferentiated OS tumors have significantly lower levels of $p27^{KIP1}$ in comparison to well-differentiated OS. Since OS differentiation status bears prognostic significance, disruptions in the Runx2 pathway and loss of differentiation may be an important step in the development of highly aggressive, less differentiated OS tumors.

Wnt signaling pathway has been implicated in a variety of human diseases [62, 142, 143]. The canonical Wnt pathway involves binding of the Wnt glycoprotein to the transmembrane Frizzled receptor and LRP5/6 coreceptors [61, 144–146]. Ligand-receptor binding prevents downstream phosphorylation of β-catenin, allowing it to translocate to the nucleus and activate downstream genes that mediate cell proliferation and differentiation [61]. This canonical Wnt pathway plays a crucial role in osteoblast differentiation, as evidenced by the fact that Wnt3a expression leads to cell proliferation and suppression of osteogenic differentiation in adult MSCs [147]. Multiple aberrations in the Wnt signaling pathway have been associated with OS tumorigenesis [108, 148]. For example, elevated levels of β-Catenin, an important regulator of the Wnt pathway, are correlated with osteoprogenitor proliferation and OS metastasis [108, 148]. Furthermore, OS tumors overexpressing LRP5, a Wnt coreceptor, are associated with a poorer prognosis and decreased patient survival [149]. Therefore, it is reasonable to believe that deregulation of the Wnt signaling pathway may lead to OS tumorigenesis by preventing terminal osteogenic differentiation and promoting cell proliferation (Figure 1).

Given these results, it appears that a lack of terminal differentiation may not only be responsible for OS tumorigenesis, but may also predict its malignant potential. By preventing terminal differentiation, tumors can retain their proliferative phenotypes, responsiveness to growth factors, and overall aggressiveness. If osteosarcoma is a consequence of these differentiation defects, we can focus future research on identifying new therapies targeting cellular differentiation thereby avoiding some of the negative consequences associated with conventional chemotherapy.

6. Therapeutic Potential by Targeting Differentiation Defects in OS

Recent investigations have focused on the therapeutic potential to overcome differentiation defects associated with osteosarcoma, and therefore prevent tumorigenesis. Examples of such therapies have been detailed in previous studies and include agents such as nuclear receptor agonists, growth factors, and transcription factors [55, 59, 150–155] (Table 1). In addition to inducing terminal differentiation, these therapies can obviate the need for chemotherapy, thereby avoiding some of the toxicities and chemoresistance associated with current OS therapeutic regimens.

One example of potential OS differentiation agents are the nuclear receptor superfamily of proteins, including PPARγ, the retinoids, and estrogens. Various PPARγ agonists have shown the ability to prevent proliferation and induce osteoblastic differentiation in OS tumor cells [15, 153] (Table 1). When OS cells are exposed to these agents, they exhibit an increased susceptibility to apoptosis, decreased proliferative capacity, and an increase in the expression of differentiation markers such as alkaline phosphatase [59]. Similarly, treatment of OS cells with other members of the nuclear receptor superfamily, such as 9 cis-retinoic acid and all-trans retinoic acid, are able to induce differentiation and growth inhibition in human OS cell lines [150]. When these retinoic acid ligands are combined with troglitazone, a potent PPARγ agonist, there is a strong synergistic effect in inducing cellular apoptosis and differentiation [153]. Another nuclear receptor that has potential in OS therapies is the estrogen receptor. In previous studies, estrogen receptor antagonists, such as tamoxifen, Raloxifene, 17-beta estradiol, and SERMS, are able to inhibit proliferation and induce apoptosis in U2OS cell lines through varying mechanisms [156]. These studies also demonstrated that the decreased cell proliferation was associated with an increase in osteoblast differentiation markers [156].

Another nuclear receptor agonist that has the potential to serve as an OS differentiation inducer is 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (Table 1). 1,25(OH)2D3 can induce OS differentiation through a p21-dependent pathway [152]. The p21 is a downstream effector of p53 that regulates G1 cell cycle arrest [157]. However, since most OS cells contain absent or nonfunctional p53, this pathway is often interrupted [1]. Osteogenic differentiation of OS cells is associated with the expression of p21 [152]. 1,25(OH)2D3 has been shown to induce the expression of p21, and treatment of three different OS cell lines with exogenous 1,25(OH)2D3 induced cellular differentiation (as measured by ALP and OCN) and triggered apoptosis [151]. Taken together, these results suggest that 1,25(OH)2D3 may prevent OS tumorigenesis by inducing differentiation in a p21-dependent manner.

An interesting possibility for a differentiation agent is parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP), as they are both able to induce osteoblastic differentiation in MG63 OS cells [155] (Table 1). PTH/PTHrP ligands bind to the G protein family of transmembrane receptors, and the signal is transduced via a MAPK pathway that leads to the eventual phosphorylation of protein kinase A (PKA) and/or protein kinase C (PKC) [158]. Carpio et al. demonstrated that treatment of MG63 cell lines with PTHrP resulted in elevated levels of ALP and type 1 collagen, suggesting that these tumor cells underwent osteoblastic differentiation. Furthermore, transient transfection of the OS cells with inhibitors of this PTHrP pathway resulted in downregulation of both type 1 collagen and ALP, suggesting that the PTHrP-mediated cellular differentiation is likely a result of activation of the MAPK/PKA/PKC pathway [155]. Interestingly, PTH regulates the oncoprotein c-fos, which is a critical modulator of osteogenic differentiation and malignant transformation [159, 160]. Upregulating the expression of this oncoprotein leads to both malignant transformation and more aggressive tumors [159–161].

Interestingly, as potent osteogenic differentiation regulators BMPs are unable to promote OS cell terminal

Table 1: Summary of some currently used differentiation agents in human osteosarcoma cells. These differentiation agents are in general nonspecific differentiation-promoting agents, and are able to promote osteogenic differentiation in mesenchymal stem cells. These agents can inhibit the proliferation and induce apoptosis in OS cells.

Class	Target	Ligand	Possible mechanism	References
Nuclear receptor ligands	PPARy	Troglitazone Ciglitazone Pioglitazone	(i) Increased susceptibility to apoptosis (ii) Decreased proliferative capacity (iii) Increased differentiation (ALP Activity)	Haydon 2007, Logan 2004 [15, 146] Scotlandi 1996 [54] Deng 2008 [58]
	Retinoids	9 cis-retinoic acid All-trans retinoic acid	(i) Induced morphologic differentiation (ii) Inhibited anchorage-dependent growth (iii) Decreased proliferative capacity	Haydon 2002, Logan 2004 [15, 146] Luu 2004 [143]
	Estrogens	Tamoxifen Raloxifene 17-β Estradiol	(i) Increased apoptosis (ii) Decreased cell proliferation (iii) Increased osteoblastic differentiation markers	Hoang 2004 [149]
	Vitamin D	1,25-dihydroxyvitamin D3	(i) Decreased cell proliferation (increased p21 expression causing G1 arrest) (ii) Increased differentiation (ALP, OCN) (iii) Increased apoptosis	Cadigan 1997 [144] Wodarz 1998 [145]
Hormone (s)	Parathyroid hormone	Parathyroid Hormone-related peptide (PTHrP)	Increased differentiation via MAPK pathway (ALP, Type 1 Collagen)	Iwaya 2003 [148]
Growth factors	Bone morphogenetic proteins	BMP2 BMP4 BMP6 BMP9	(i) −Runx2: increased cell proliferation, no differentiation in OS cells (ii) +Runx2: decreased cell proliferation, increased OS cell differentiation	Reya 2001 [63]

differentiation (Table 1). BMPs play an essential role in the osteogenic differentiation of MSCs, and exposure of MSCs to the most osteogenic BMPs (2, 4, 6, and 9) result in the expression of osteoblast markers such as ALP, OCN, and OPN [47, 57, 58, 84, 136, 137, 162, 163]. When four different OS cell lines were exposed to these osteogenic BMPs, there was an increased expression of early target genes Id1, Id2, and Id3, but no change in ALP, OCN, and OPN levels [64]. Furthermore, BMP exposure not only prevented differentiation, but actually promoted tumor growth and proliferation [64]. These results suggest that these OS cells may contain defects in the differentiation pathway that are regulated by osteogenic BMPs. Therefore, exogenous administration of BMPs fails to bypass the defects, but instead promotes tumor cell proliferation. However, when the cells were treated with adenovirus expressing Runx2 (even in the presence of osteogenic BMPs), the tumor growth was significantly inhibited, and these cells underwent terminal differentiation and apoptosis [64]. Collectively, these results suggest that Runx2 is able to bypass the differentiation defects that are downstream in the cascade from the BMPs, and thus, able to inhibit tumor progression through the induction of osteogenic differentiation (Table 1).

7. Concluding Remarks and Future Directions

Osteosarcoma is a complex disease whose etiology is likely from multiple sources, including rapid bone proliferation, an accumulation of mutations, and possible defects in differentiation. Recent investigations have focused on the factors regulating the osteogenic differentiation cascade of mesenchymal stem cells. Alterations in other differentiation pathways have already been established as critical etiologies in the pathogenesis of other cancers, such as breast, prostate, and the hematologic system. We have had success in overcoming these differentiation defects in these cancers, leading to the inhibition of the tumor cells with uncontrolled proliferation. We have recently shown that osteosarcoma, at least in part, results from defects in the osteogenic differentiation cascade. OS tumor cells share many cellular and morphologic features with undifferentiated osteogenic progenitors. As a result, osteogenic factors such as BMPs, are not able to bypass these defects, leading to cellular proliferation and tumor growth. Late osteogenic regulators, such as Runx2 and the retinoids, are able to overcome these defects and stimulate progression through the differentiation cascade. Further understanding of the relationship between defects in differentiation and tumor development holds tremendous potential in developing novel therapies to treat OS.

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Review Article

The Role of RUNX2 in Osteosarcoma Oncogenesis

J. W. Martin,[1] M. Zielenska,[2] G. S. Stein,[3] A. J. van Wijnen,[3] and J. A. Squire[1]

[1] *Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada K7L 3N6*
[2] *Department of Pathology and Laboratory Medicine, Hospital for Sick Children, Toronto, ON, Canada M5G 1X8*
[3] *Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01655, USA*

Correspondence should be addressed to J. A. Squire, squirej@queensu.ca

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Osteosarcoma is an aggressive but ill-understood cancer of bone that predominantly affects adolescents. Its rarity and biological heterogeneity have limited studies of its molecular basis. In recent years, an important role has emerged for the RUNX2 "platform protein" in osteosarcoma oncogenesis. RUNX proteins are DNA-binding transcription factors that regulate the expression of multiple genes involved in cellular differentiation and cell-cycle progression. RUNX2 is genetically essential for developing bone and osteoblast maturation. Studies of osteosarcoma tumours have revealed that the RUNX2 DNA copy number together with RNA and protein levels are highly elevated in osteosarcoma tumors. The protein is also important for metastatic bone disease of prostate and breast cancers, while RUNX2 may have both tumor suppressive and oncogenic roles in bone morphogenesis. This paper provides a synopsis of the current understanding of the functions of RUNX2 and its potential role in osteosarcoma and suggests directions for future study.

1. Introduction

Osteosarcoma is an aggressive cancer of bone with unknown etiology and often poor clinical outcome. It is the most common primary malignant tumour of bone, representing about 35% of bone cancer cases [1], and it predominantly affects individuals in their second decade of life. Most often, tumours arise from osteoid-producing neoplastic cells in the metaphyses of the long bones, including the distal femur and proximal humerus [1], and less commonly in the axial skeleton and other nonlong bones [2]. Tumours frequently possess cells with extensive, complex genomic rearrangements, and few consistent changes have been observed across this heterogeneous disease.

No molecules for targeted therapy have been developed for osteosarcoma, and survival rates have not improved for several decades since the introduction of chemotherapy to treatment of the disease (reviewed in [3]). The current standard of care comprises limb-sparing surgery and combination neoadjuvant chemotherapy consisting of high dose methotrexate, doxorubicin, cisplatin, and ifosfamide [4]. Treatment of the bone tumours prior to the use of chemotherapy was solely surgical with a higher percentage of cases undergoing amputation and with an associated 5-year survival of about 15% [3, 5].

Ongoing studies continue to detect genes whose protein products may play a role in osteosarcoma oncogenesis and may have potential as therapeutic targets. The tumour suppressors p53 and pRB are inactivated at the DNA level in roughly 50%–70% of sporadic osteosarcomas [6], and germline inactivations of either of those proteins significantly increase risk for developing osteosarcoma [6, 7]. For example, Li-Fraumeni patients, who have p53 germ line mutations, have an increased incidence of osteosarcoma [8, 9]. A similar situation arises with RecQL helicase inactivations [6], which are also associated with chromosomal instability in osteosarcoma tumours [10]. This tumour is also characterised by a vastly heterogeneous array of complex genomic rearrangements, but their description is beyond the scope of this paper and can be retrieved in reports by our lab and others [11–21].

For the purpose of this paper, it will suffice to call attention to the chromosomal region 6p12-p21, which encompasses the *RUNX2* gene and experiences recurrent gain and amplification in osteosarcoma [11–17, 22]. In our lab, we have detected amplification-related overexpression

of the *RUNX2* gene in a subset of osteosarcoma tumours and identified a correlation between high *RUNX2* mRNA overexpression and poor tumour response to chemotherapy based on the percentage of tumour necrosis following treatment [23]. This prospective estimate of response is an indirect predictor of response that is routinely used as part of patient management. In a separate retrospective cohort of osteosarcoma patient specimens, we have also detected correlations between copy number gain of *RUNX2* and poor tumour necrosis in response to chemotherapy (measured by fluorescence *in situ* hybridisation) and between high RUNX2 protein levels and poor chemoresponse in the tumours [paper in preparation]. Furthermore, RUNX2 protein levels appear to be selectively deregulated in several osteosarcoma-derived cell culture models [24–27]. *RUNX2*/RUNX2 thus has potential as a predictive biomarker for osteosarcoma, but a better understanding of the gene and protein in the context of the disease is necessary before considering targeted treatments and diagnostic, prognostic, and predictive tests.

2. RUNX Family of Transcription Factor Genes

The three members of the mammalian *RUNX* family of tissue-specific transcription factor genes encode the DNA-binding α components of the core-binding factor (CBF) complex [28]. In the literature, the genes are also known by the family names *core-binding factor-α* (*CBFA*), *acute myeloid leukemia* (*AML*), and mouse *polyoma enhancer-binding protein 2α* (*PEBP2α*), depending on the context of their study [29]. The RUNX proteins, as part of the CBF complex, regulate differentiation, survival, and growth in a variety of tissues, but are specifically essential for definitive hematopoiesis (RUNX1), osteogenesis (RUNX2), as well as neurogenesis and gut development (RUNX3) (reviewed in [30]). *RUNX1/AML1/CBFA2/PEBP2αB* was discovered as a common chromosomal translocation target in chronic myelogenous and acute myeloid leukemias (reviewed in [31]), and its critical necessity for adult blood-cell production was discovered in *RUNX1*-null mice, which lacked definitive hematopoiesis [32, 33]. *RUNX3/AML2/CBFA3/PEBP2αC* expression is necessary for development of neuronal networks [34, 35] and the gastrointestinal tract [36], and its inactivation is strongly associated with gastric cancer [37]. *RUNX2/AML3/CBFA1/PEBP2αA* encodes an essential determinant of osteoblast differentiation [38, 39] that regulates the expression of many genes during bone development (reviewed in [40]).

3. RUNX2 Structure-Function Relationship

The *RUNX2* gene occupies approximately 220 kbp on chromosome 6 near the border between cytobands 6p21.1 [28, 41] and 6p12.3 (UCSC Genome Browser, March 2006 hg18 assembly), and the RUNX2 protein exists as two major isoforms [42] (Figure 1). Two distinct promoters for the *RUNX2* gene, P1 and P2, give rise to two biologically unique transcripts [43] (Figure 1(b)), and alternative splicing contributes to at least three variants of the protein based on the at least eight exons known to make up the gene [41, 44] (Figure 1(b)). The *RUNX2* gene is a unique member of the *RUNX* family in that it produces the largest protein product (521 amino acids) [45], which possesses two domains distinct from its homologues: a short stretch of glutamine-alanine (QA) repeats at the N-terminus and a C-terminal proline/serine/threonine (PST) rich tract, both regions of which are necessary for full transactivation activity [46]. However, the protein has high-sequence identity with the other RUNX proteins, sharing with them the DNA-binding Runt domain, the nuclear localisation signal (NLS), the nuclear matrix targeting signal (NMTS), and a C-terminal VWRPY sequence, which allows interaction with corepressors transducin-like enhancer of split (TLE)/Groucho [47, 48] (Figure 1(c)).

The Runt domain is common among the RUNX proteins [51], and was first characterised in the Runt and Lozenge proteins of *Drosophila*, in which they are essential for the regulation of many developmental processes, including segmentation, sex determination, and hematopoiesis (reviewed in [52]). This domain confers the ability for binding to DNA and for heterodimerisation with CBFβ [53] to form the CBF complex. The CBFβ protein, though necessary for RUNX activity, does not directly affect transcription regulation itself, but rather allosterically increases the DNA-binding capacity of its RUNX partner [54, 55].

RUNX2 binds specific *cis*-acting elements via the conserved Runt domain to enhance transcription of genes in many tissues during embryogenesis, particularly in T-lymphocytes throughout development of the thymus [56] and developing cartilage [57]. However, its most significant function is in the regulation of osteoblast differentiation during bone development [45].

4. Importance of RUNX2 in Normal Skeletal Development

The significance of RUNX2 in skeletal development was first suggested by studies of the autosomal dominant disease cleidocranial dysplasia (CCD). Initially, linkage studies of kindreds with CCD led to the discovery that a single locus within cytoband 6p21 was associated with the disease [58, 59]. Higher resolution cytogenetic and sequencing analyses subsequently identified several mechanisms for heterozygous inactivation of the *RUNX2* gene: in-frame polyalanine expansions within the QA domain, heterozygous deletions due to chromosomal inversion, nonsense mutations, missense mutations, and frameshift mutations due to insertion or microdeletion, all of which resulted in *RUNX2* haploinsufficiency [60, 61]. Mouse studies demonstrated conclusively that *RUNX2* was necessary for normal bone development. Mice heterozygous for mutant *RUNX2* recapitulate human CCD, and mice homozygous for mutant *RUNX2* were deficient in osteoblasts and vascularisation of marrow due to a lack of osteoblast and endothelial differentiation of periosteal mesenchymal stem cells (MSCs) [38, 39, 62, 63].

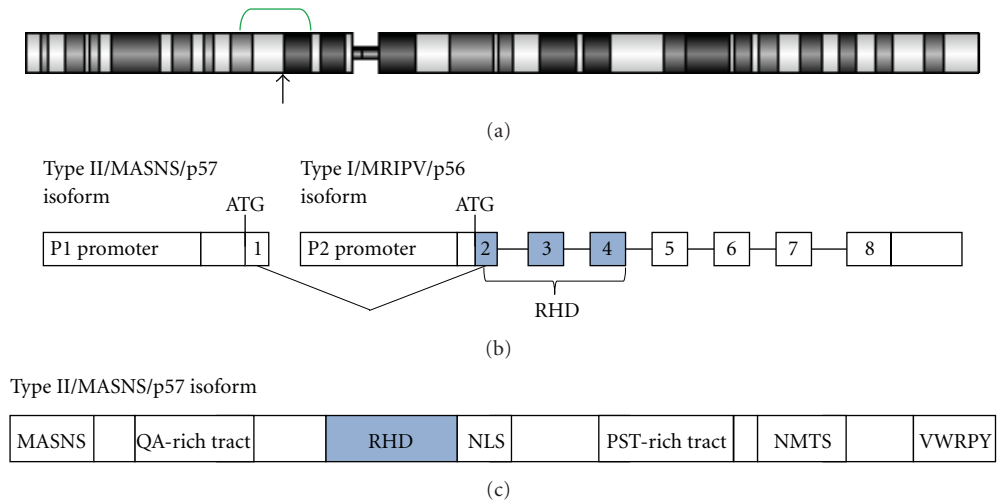

Figure 1: Chromosome 6 and *RUNX2*/RUNX2. (a) Chromosome 6 and location of *RUNX2*. The green bracket approximately spans the minimal common region of gain identified by array comparative genomic hybridisation (aCGH) studies of osteosarcomas, between cytobands 6p21.2 to 6p12.3 (spanning nucleotide positions 36,800,000 bp to 51,100,000 bp, resp.). All genomic information was obtained from UCSC Genome Browser (http://genome.ucsc.edu/), March 2006 (hg18) assembly. (b) Gene structure of *RUNX2*. Major isoforms MASNS and MRIPV are transcribed starting from promoters P1 and P2, respectively, and ATG indicates the start codon. The MRIPV isoform is encoded from exons 2–8, while the MASNS isoform is encoded from all eight exons. The Runt homology domain (RHD) is encoded from portions of exons 2, 3 and 4 (shaded). (c) Protein structure of RUNX2. The Type II/p57 isoform comprises 521 amino acids and begins with the bone-specific N-terminal MASNS polypeptide. It has a glutamine/alanine (QA) rich tract and a proline/serine/threonine (PST) rich tract that are both unique to RUNX2 in the RUNX family of proteins. The protein also possesses the RHD DNA-binding domain, the nuclear-localisation signal (NLS), the nuclear matrix targeting signal (NMTS), and the C-terminal VWRPY domain for TLE/Groucho corepressor interactions. Adapted from [44, 45, 49, 50].

In its capacity as a transcription factor necessary for osteoblast differentiation [64, 65] and full skeletal development [38, 39], RUNX2 acts as a "platform protein," in that it interacts with a variety of coactivator and corepressor proteins, including chromatin remodeling factors and epigenetic modifiers (reviewed in [45]). Transcriptional regulation of *RUNX2* is also complex and affected by a variety of signaling pathways (a summary of protein-protein interactions and transcriptional regulators of *RUNX2* is shown in Figure 2). The complexity of RUNX2 signaling is further compounded by its autorepression [49], by its presence in at least two isoforms, and by its emerging relevance in the development of nonosteogenic cells [66].

5. Upstream Signaling and Transcription Regulation of RUNX2

Discrete *RUNX2* transcriptional activity is necessary for all stages of osteogenesis, and expression of the MASNS/p57 (Type II) isoform from the osteoblast-specific P1 promoter leads to the osteoblast-specific isoform of the protein [67]. The MRIPV/p56 (Type I) isoform of RUNX2, expressed from the chondrocyte-specific P2 promoter [68], is required for chondrocyte hypertrophy and maturation, in a role subject to repression by the chondrocyte-specific transcription factor SOX9 [69, 70]. Upstream *RUNX2* promoter elements bind a variety of factors which form important branches of embryogenic pathways, including Hedgehog (Hh), canonical Wnt, mitogen-activated protein kinase (MAPK), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP)/transforming growth factor β (TGFβ) (Figure 2).

During endochondral ossification, one of the first events to begin differentiation of osteoprogenitor cells from MSCs is the transcriptional activation of *RUNX2* by Indian hedgehog (Ihh) [71, 72], which is itself upregulated by RUNX2 [73]. Other essential signals are the insulin-like growth factors (IGFs), which are implicated in early osteogenesis. IGF signaling activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, with AKT2 being required for both BMP2 signaling and for *RUNX2* transcriptional activation [74, 75]. The canonical Wnt protein T-cell factor 1 (TCF1), with betacatenin, also upregulates *RUNX2* expression in MSCs [76], but further studies have shown that Wnt signaling is most critical in the transition from RUNX2+Osterix1− osteoprogenitors to RUNX2+Osterix1+ cells [77], and in subsequent osteoblast maturation [72].

During progression of osteogenesis, numerous other factors regulate the expression of *RUNX2*. SP1, ETS1, and ELK1 all stimulate *RUNX2* expression, the former two predominating during osteoblast proliferation and early differentiation, and the latter protein maintaining basal *RUNX2* transcriptional activity in later stages of differentiation [78]. Transcriptional activation of *RUNX2* is also facilitated by the BMP2 signaling cascade via the homeodomain proteins DLX3 and DLX5 [79] and by MAPK/Ras/ERK signaling in response to mechanical stress [80, 81]. FGFs stimulate bone formation through the protein kinase C (PKC) pathway, with FGF2/FGFR2 activating expression of *RUNX2*, as well as transcriptional activity of the RUNX2 protein [82].

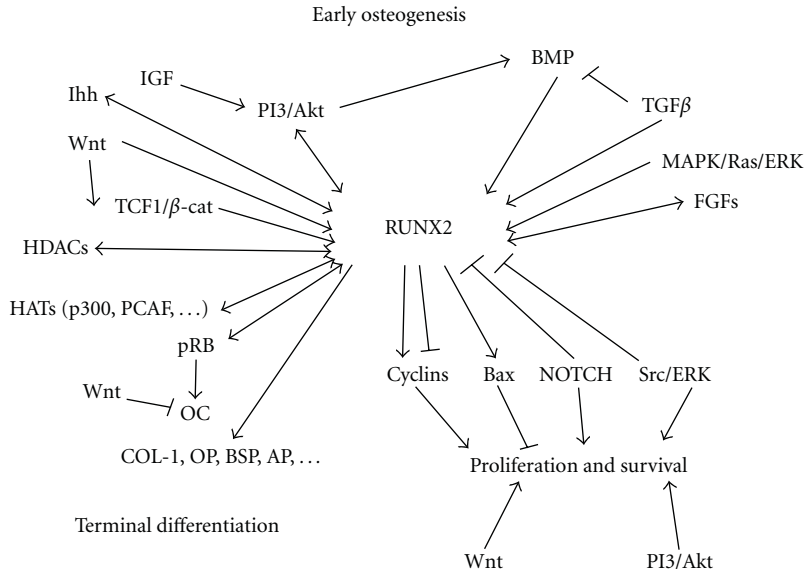

Figure 2: *RUNX2* transcription and RUNX2 activity are influenced by many signaling molecules during osteoblast development. Summarised here, a large number of complex protein-protein interactions characterise RUNX2 activity, and transcription of *RUNX2* and protein levels of the encoded product are influenced by a multitude of factors depending on the stage of osteoblast differentiation (see text for detailed descriptions). Arrows indicate protein-protein interactions and/or transcriptional upregulation whereas connections ending with a flat arrowhead indicate inhibitory effects.

On the other hand, expression of *RUNX2* is reduced by 1,25-$(OH)_2$-vitamin D3 (VD3) [83], peroxisome proliferation-activated receptor gamma 2 (PPARγ2) [84], and tumour necrosis factor alpha (TNFα) at the transcriptional and posttranscriptional levels [85]. NKX3.2/BAPX1 is upregulated by SOX9 in terminal chondrogenesis to reduce expression of *RUNX2* [86, 87]. Cyclic AMP signaling promotes proteasome-mediated degradation of RUNX2 [88], and RUNX2 activity is modulated by residue-specific phosphorylation [89], binding by inhibitory proteins such as coactivator activator (CoAA) [90], and acetylation of the protein [91].

6. RUNX2 Signaling in Osteogenesis Has Potential for Deregulation in Oncogenesis

RUNX2 regulates osteoblast lineage determination and expansion, osteoblast maturation, and terminal differentiation via a complex variety of pathways. Early osteoblast progenitor cells arise from pluripotent MSCs due to direct interactions of RUNX2 with broadly acting developmental pathways. Canonical Wnt factors and Hh family members are well known to inhibit adipogenic or chondrogenic differentiation of MSCs and to promote a preosteoblastic phenotype [92–94]. A number of relationships between RUNX2 and the canonical Wnt pathway have recently been shown to guide osteoblast commitment. In MSCs, RUNX2 forms a complex with lymphoid enhancer-binding factor 1 (LEF1), which is coactivated by betacatenin, to activate the *fibroblast growth factor 18* (*FGF18*) gene [95], whose product inhibits chondrogenesis and supports osteogenesis [96].

The canonical Wnt pathway in particular is important throughout osteoblast differentiation. Without Wnt signaling, RUNX2-mediated transcriptional activation of the *osterix* (*Osx1/SP7*) gene in osteoprogenitors cannot lead to further commitment to the osteoblast lineage [97]. Following lineage commitment, RUNX2 promotes differentiation, and a particularly important early step following commitment is the interaction between RUNX2 and SMAD proteins induced by BMP and TGFβ. In osteoprogenitors, BMP2 serves to induce *osterix* expression and promote osteoblast differentiation in a RUNX2-independent manner [98, 99], and in order for osteogenesis to approach completion, BMP/TGFβ signaling must be facilitated by the formation of the RUNX2-SMAD complex, which activates transcription of late osteoblast markers [100].

Proliferation and migration of committed osteoblasts precedes quiescence and terminal differentiation. Osteoblast proliferation and survival is promoted in large part by canonical Wnt signaling directly through LRP5 [101, 102] and indirectly via Src/ERK and PI3K/Akt [103]. Several studies have shown that RUNX2 attenuates osteoblast proliferation, and its protein levels are maximal during the G1 phase in which differentiation and growth occur. RUNX2 activity is maintained at high levels into the G0 phase if quiescence is induced, but is otherwise downregulated at the G1 to S transition and in the subsequent S, G2, and M phases [24, 89, 104]. Mitosis sees residual RUNX2 localised in active nucleolar organising regions to repress transcription of ribosomal RNA genes [105]. RUNX2 may support epigenetic regulation of protein-encoding genes during mitosis [106], a mechanism referred to as "bookmarking" [107]. *In vitro,* contact inhibition or serum deprivation

is associated with increased RUNX2 and cell-cycle exit, while RUNX2 deficiency induces increased growth potential [104]. Through activation by BMP/SMAD signaling, RUNX2 upregulates *BAX* expression to induce apoptosis in studies of the osteosarcoma cell line SAOS-2 [108].

Though its role in cell growth inhibition is well established, RUNX2 also promotes cell proliferation and survival. The maximal levels of RUNX2 during G1 may actually be necessary to stimulate continued cell division [24, 109]. RUNX2 represses transcription of *p21/CDKN1A/WAF1/CIP1*, which encodes a cyclin-dependent kinase inhibitor that arrests cells in G1 [110], and it activates *Gpr30* transcription and represses *Rgs2* transcription to increase cellular response to mitogenic signaling through cyclic AMP and G-protein-coupled receptor signaling pathways [109]. In converse to the finding that RUNX2 upregulates *BAX* expression in the SAOS-2 cell line [108], nitric oxide (NO) treatment of the MG-63 osteosarcoma cell line induces RUNX2-mediated *BCL2* expression, which promotes survival of the cells during oxidative stress [111]. NO signaling through cyclic guanosine 3′,5′-monophosphate (cGMP) may also cause site-specific phosphorylation of RUNX2 by protein kinase G (PKG), leading to upregulated transcription of the matrix metalloproteinase (MMP) gene *MMP13* [112]. MMP13 is one of several members of the MMP family with important roles in cartilage degradation during endochondral ossification and later bone remodeling (reviewed in [113]).

Additionally, during bone development and remodeling, RUNX2 and PI3K-Akt mutually upregulate each other to enhance chemotactic osteoblast migration [114], which occurs along gradients of platelet-derived growth factor (PDGF), TGFβ, and IGF [115–117]. Terminal osteoblast differentiation is accomplished through cell-cycle exit and complete expression of osteoblast phenotypic markers. RUNX2 induces higher levels of $p27^{KIP1}$/CDKN1B, which inhibits S-phase cyclin-dependent kinases to promote cell-cycle exit and causes dephosphorylation of pRB [118]. Active, hypophosphorylated pRB is necessary for cell-cycle exit at this stage [119] and, through cooperation with the transcription factor HES1 [120], the hypophosphorylated form of pRB is bound by RUNX2. The RUNX2-pRB complex then coactivates transcription of genes encoding late markers of osteoblast differentiation, including *osteocalcin* [121]. *Osteocalcin* is also activated by RUNX2 in complex with histone acetyltransferases (HATs) p300 and p300/cyclic AMP receptor element-binding protein binding protein-associated factor (PCAF) [122], as well as monocytic leukemia zinc finger protein (MOZ) and MOZ-related factor (MORF) [123]. Other late osteoblast markers include alkaline phosphatase (AP), osteopontin (OP), bone sialoprotein (BSP), and collagen type I (COL-1), all of which require RUNX2-SMAD signaling, induced by BMP/TGFβ, to be expressed [100] (Figure 2).

Depending on the phosphorylation level of RUNX2 and the stage of differentiation, it also interacts with several corepressor proteins. Histone deacetylases (HDACs) 6 and 3 interact with RUNX2 to repress *p21/CDKN1A/WAF1/CIP1* and *osteocalcin*, thus regulating osteoblast development during proliferation and terminal differentiation [110, 124]. The mSin3a, TLE/Groucho, and Yes-associated protein (YAP) corepressors form complexes with RUNX2 and other HDAC proteins to repress expression of osteoblast-specific genes, particularly *osteocalcin* [47, 125, 126], and HDAC4 induces transcriptional repression by binding RUNX2 to inhibit its intrinsic DNA-binding activity [127]. The transcriptional regulation and tissue-specific nature of RUNX2 activity thus depends a great deal on the proteins it forms multisubunit complexes with, and studies are ongoing to characterise the complex relationship between RUNX2 and the downstream factors that control osteoblast development.

7. Potential Significance of RUNX2 in Osteosarcoma

During development of normal bone, RUNX2 levels increase gradually after commitment of MSCs to the osteoblast lineage to maximal levels in early osteoblasts (Figure 3(a)). Several recent studies of osteosarcoma specimens have reported constitutively high protein levels of RUNX2. Although such studies of RUNX2 in clinical samples are rare, they are compelling in their findings. Andela et al. [128] published the earliest report we could find of RUNX2 immunoreactivity in osteosarcomas; the researchers tested 11 pathology specimens of the cancer and found RUNX2 immunopositivity in all of them. A comprehensive DNA-mRNA-protein analysis of patient samples by Lu et al. [12] found mRNA overexpression of *RUNX2* in 13 of 13 samples with genomic amplification in 8 of the 13.

Three more recently published studies were successful in linking *RUNX2* expression with measures of clinical course in patients with osteosarcoma. In a study of 22 osteosarcomas by our lab, mRNA overexpression of *RUNX2* was on average 3.3 times higher in tumours that had responded poorly (<90% necrosis) to neoadjuvant chemotherapy relative to tumours with good response (>90% necrosis). Compared to normal human osteoblasts, every tumour specimen had higher *RUNX2* mRNA expression [23]. Similarly, Won and colleagues observed low RUNX2 expression in 60% (29/48) of cores and high RUNX2 expression in 23% (11/48) of cores. In this study, high RUNX2 expression was significantly correlated with metastasis and predicted a trend towards lower survival [131]. Another study analysed the comparative immunoreactivity of RUNX2 in different types of patient samples, finding positive staining in 60% (12/20) of biopsy samples and 73% (8/11) of metastatic tumours. Interestingly, this same study found only 16% (4/25) of postchemotherapeutic resections were positive for RUNX2 staining [132]. Thus, the results of these recent studies are suggestive of predictive value of RUNX2.

The function of RUNX2 in osteosarcoma has not yet been identified, but given the complex functionality of RUNX2 in developing osteoblasts, deregulation of the protein could act during osteosarcoma pathogenesis. Significantly, cell cycle-dependent regulation of RUNX2 is absent in the cell line

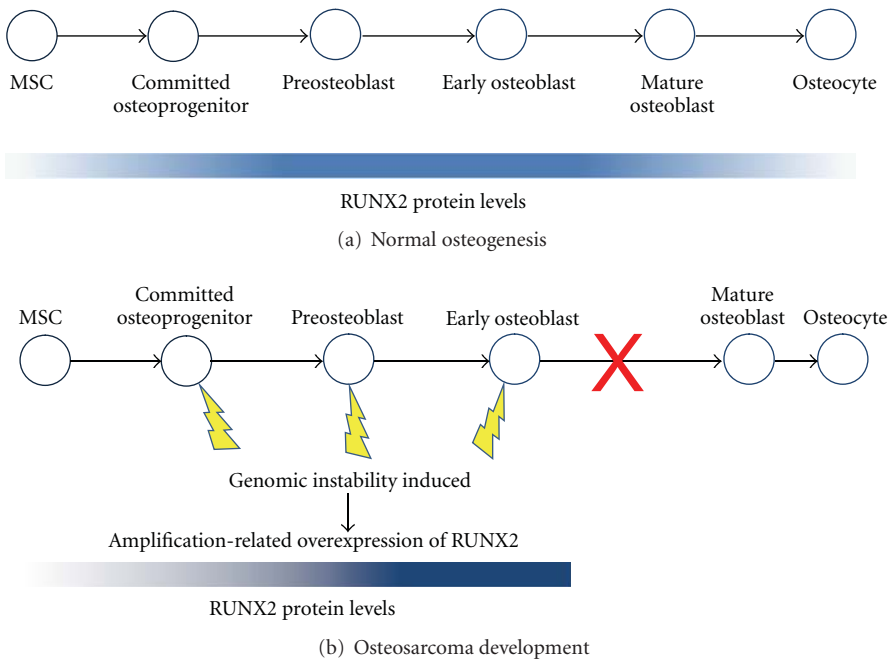

Figure 3: Osteoblast differentiation and RUNX2 protein levels. (a) In normal osteogenesis initiating in MSCs, overall RUNX2 protein levels are maximal in preosteoblasts and early mature osteoblasts, after gradually increasing during commitment. Overall RUNX2 levels are very low in mature osteoblasts and osteocytes [129]. RUNX2 activity and levels are modulated according to cell-cycle stage by posttranslational modification and transcriptional regulation of *RUNX2*, respectively. (b) In osteosarcoma development, genomic instability is induced (lightning bolts), for example by inactivation of pRB or p53, in cells committed to the osteoid lineage. Extensive rearrangements occur, with amplification of chromosome 6p12-p21 being a frequent early event in many cases. Amplification-related overexpression of *RUNX2* could result, leading to high levels of RUNX2 protein throughout the cell cycle and disrupted regulation of RUNX2 activity. Consequently, osteoblast differentiation is halted before or during maturation and characteristics of immature osteoblast-like cells are retained in the resulting osteosarcoma. Adapted from [130].

SAOS-2 and the protein is maintained at high levels throughout the cell cycle, particularly during the G1 to S transition when it is normally downregulated [24]. Previously published studies have shown that RUNX2 interacts specifically with hypophosphorylated pRB during initiation of cell-cycle withdrawal during terminal osteoblast differentiation [118, 121, 133]. Inactivation of pRB is very common to a small subset of tumours including osteosarcoma [134], and in particular, 50%–70% of osteosarcomas do not have functional pRB [6]. In the absence of pRB, RUNX2-pRB-induced cell-cycle exit would not be possible, and this could lead to uninhibited proliferation of osteoprogenitor cells, as well as increased genomic instability [135].

Apart from the pRB–RUNX2 connection, there is evidence indicating that normal RUNX2 function in bone is linked to the p53-MDM2 pathway [136]. The p53 pathway is perturbed in Li-Fraumeni patients, and there is increased osteosarcoma incidence in Li-Fraumeni families [8, 9]. Furthermore, bone-specific knockout of p53 is dominant over loss of pRB in the predisposition to osteosarcoma in mouse models [119, 137]. RUNX2-dependent osteoblastic differentiation is compromised when the p53-MDM2 pathway is genetically perturbed, and loss of p53 function increases the differentiation-related accumulation of RUNX2 [138]. In contrast to primary or immortalised osteoblasts, which normally have low RUNX2 levels, loss of p53 correlates with elevated RUNX2 protein levels in several growth factor-independent osteosarcoma cell lines [26, 27]. Hence, it is conceivable that loss of p53 function in osteosarcomas is permissive for or even contributes to the elevated protein levels that are observed in osteosarcoma patient samples with 6p12-6p21 gene amplifications [11–17, 22].

Cell cycle-dependent activity of RUNX2 is regulated by cyclin-dependent kinase- (CDK-) mediated phosphorylation [89], and the $p27^{KIP1}$/CDKN1B cyclin-dependent kinase inhibitor is also required for terminal differentiation and cell-cycle exit by interaction with RUNX2. Protein levels of $p27^{KIP1}$ are reduced in the undifferentiated subtype of osteosarcoma [118]. Our own aCGH analysis of 15 osteosarcoma patient samples detected loss of *CDKN1B* in nine of 15 samples (our unpublished data). RUNX2 signaling in the absence of the tumour suppressors pRB and $p27^{KIP1}$ would, therefore, be limited in its capacity to halt proliferation and induce osteoblast maturation. Similarly, reduced expression of the $p21^{CIP1}$/CDKN1A cyclin-dependent kinase inhibitor may occur as a result of elevated RUNX2 protein levels (which transcriptionally represses the $p21^{CIP1}$/CDKN1A gene) [110] and the concurrent loss of p53 (which is the major transactivator of $p21^{CIP1}$/CDKN1A)[139]. Reduced $p21^{CIP1}$ levels would prevent cell-growth arrest and DNA repair following DNA damage during chemotherapy and radiation of osteosarcomas in the clinic.

Clearly, the prodifferentiation and tumour suppressor function of RUNX2 has potential for deregulation, in that MSCs committed to the osteoblast lineage could be stalled in their differentiation before development of the mature osteoblast phenotype. Recently, it was found that Notch1 inhibits RUNX2 directly by binding it [140] and indirectly by upregulating cyclin D1-dependent kinase CDK4, which ubiquitinates RUNX2 [141]. An association has been found between upregulated Notch signaling and lung metastatic potential in osteosarcoma cell lines [142], but no functional studies have yet linked inactivation of RUNX2 directly to osteosarcoma metastasis.

Contrary to the tumour suppressor-like behaviour of RUNX2 that has been described by previously published studies of the protein [24, 104, 143], several recent studies have identified RUNX2 as potentially having a direct role in promoting neoplasia, particularly in prostate and breast cancers. To begin with, RUNX2 is highly integrated, often through reciprocal activation pathways, with PI3K/Akt, Wnt, BMP/TGFβ, MAPK/ERK, and Notch signaling, all of which can be activated in osteosarcomas and other tumours [144–147]. A comprehensive study by Akech et al. [148] demonstrated that overexpression of *RUNX2* in prostate cancer cells inoculated into bone led to activation of genes necessary for osteolytic disease, *PTH-related protein* (*PTHrP*) and *interleukin 8* (*IL8*). Both PTHrP and RUNX2 activate expression of receptor activator of nuclear factor-κB ligand (RANKL), which stimulates osteoclast formation and subsequent bone resorption [149, 150] whereas IL8 promotes osteolysis through osteoclast formation independent of RANKL [151]. Interestingly, osteosarcomas are frequently mixed osteolytic and osteoblastic tumours [1], and RANK/RANKL is overexpressed in subsets of the tumours [152]. Akech et al. [148] also detected that prostate cancer overexpression of *RUNX2* activated genes necessary for metastasis and invasion (*MMP2, MMP9, MMP13*), angiogenesis (*VEGF, osteopontin*), and survival (*survivin*). These findings are consistent with other studies of the metastasis-promoting role of RUNX2 in prostate cancer cell lines [153–155] and metastatic patient specimens [156]. The results support similar observations of the requirement for *RUNX2* expression in metastatic breast cancer-associated osteolytic disease [154, 157, 158].

RUNX2 appears to have dual roles as a tumour suppressor (described above) and as an oncoprotein, depending on its cellular levels and context, and its regulation. In T-cell lymphomas, overexpression of *RUNX2* and the *MYC* oncogene leads to cooperation between the encoded proteins that maintains survival and proliferation in the cancer cells [159]. In pituitary tumours, RUNX2 upregulates the anoikis suppressor galectin-3 (LGALS3) [160], which may also facilitate osteosarcoma metastasis [161]. The role of the protein in bone tumourigenesis is complicated, however, by incomplete knowledge of consequences of its deregulation in osteoblasts. High levels of RUNX2 inhibit apoptosis of osteoblasts in the presence of parathyroid hormone (PTH), which stimulates bone turnover [162]. Interaction between overexpressed RUNX2 and the protein product of proto-oncogene *FOS*, whose overexpression in mice led to development of the first osteosarcoma mouse model [163], upregulates transcription of the metastasis-associated gene *MMP13* via transcription factor AP-1 [164] and has potential for other roles in oncogenesis [165].

8. Conclusions and Future Directions

The dual roles of RUNX2 must be tightly regulated during osteoblast differentiation for normal bone development. Other studies have noted the resemblance of some osteosarcomas to committed osteoprogenitor cells that have undergone cell-cycle deregulation and have been blocked in their differentiation towards osteocytes [118, 130, 166–168]. Additionally, there is a range of differentiation status among osteosarcomas [1] that is reflected in the well-described osteosarcoma cell lines [26, 118, 169–173] and has been demonstrated in the development of mouse models of the disease [119, 137]. Disruption of RUNX2 signaling by high levels of the protein in osteoblast progenitor cells (Figure 3(b)) could significantly interrupt osteoblast differentiation and cell-cycle regulation.

It is possible that *RUNX2* overexpression resulting from gain and amplification of chromosome 6p12-p21 is a causative factor in osteosarcoma pathogenesis, because it is consistently overexpressed in patient specimens [12, 128, 131, 132], because of its oncogenic potential, and because of the potential for its tumour suppressor functions to be deregulated. Its overexpression at the protein level is likely driven by its genetic amplification at the DNA level [12, 174], our unpublished data] and facilitated by disrupted degradation [27, 132]. The instability of chromosome 6p12-p21 that leads to *RUNX2* gain and amplification has been demonstrated by many studies of patient samples, including biopsies [11–13, 15, 17], and thus it is probably an early event in osteosarcoma pathogenesis.

The complexity of osteosarcoma has continually posed a serious problem to understanding the etiology of the disease and identifying prognostic or predictive factors, or therapeutic targets. RUNX2 has potential to be predictive of response to the standard chemotherapy regimen according to studies by our lab, but further work to discover its cancer-specific function is needed. Additionally, larger cohorts of patients are necessary to definitively link RUNX2 level to treatment response in osteosarcoma tumours. In conclusion, the frequency of *RUNX2* gain and elevated RUNX2 in osteosarcoma patient specimens as well as its documented functions lends to its possible value as a predictive factor and as a therapeutic target.

Acknowledgments

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Review Article

Using Epidemiology and Genomics to Understand Osteosarcoma Etiology

Sharon A. Savage and Lisa Mirabello

Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, 6120 Executive Boulevard, EPS/7018, Rockville, MD 20892, USA

Correspondence should be addressed to Sharon A. Savage, savagesh@mail.nih.gov

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Osteosarcoma is a primary bone malignancy that typically occurs during adolescence but also has a second incidence peak in the elderly. It occurs most commonly in the long bones, although there is variability in location between age groups. The etiology of osteosarcoma is not well understood; it occurs at increased rates in individuals with Paget disease of bone, after therapeutic radiation, and in certain cancer predisposition syndromes. It also occurs more commonly in taller individuals, but a strong environmental component to osteosarcoma risk has not been identified. Several studies suggest that osteosarcoma may be associated with single nucleotide polymorphisms in genes important in growth and tumor suppression but the studies are limited by sample size. Herein, we review the epidemiology of osteosarcoma as well as its known and suspected risk factors in an effort to gain insight into its etiology.

1. Introduction

Osteosarcoma, the most common primary bone malignancy, typically during the adolescent growth spurt but there is a second, smaller peak in the elderly [1]. There are a limited number of proven risk factors associated with osteosarcoma. It occurs more frequently after therapeutic radiation for a different cancer, in individuals with certain cancer predisposition syndromes, and in those with Paget disease of the bone. However, the majority of osteosarcoma cases occur in the absence of these risk factors. Numerous studies of growth and other genetic risk factors have been conducted but strong data on risk for apparently sporadic osteosarcoma are limited. The primary goal of this paper is to examine the recent studies seeking to understand osteosarcoma etiology through epidemiology and studies of germline genetics (Figure 1).

2. Osteosarcoma Epidemiology

2.1. Incidence. Osteosarcoma represents approximately 55% of child and adolescent malignant bone tumors in the US [1]. It is rarely diagnosed before the age of five, but the incidence increases with age until around puberty [1, 3–7]. This primary peak is followed by a decrease and plateau in incidence in individuals between 25 and 60 years of age (Figure 2). A second, smaller peak is observed during the seventh and eighth decades of life; this bimodal age incidence distribution of osteosarcoma is observed worldwide [8]. This is also noted in childhood and adolescent osteosarcoma where rates are relatively consistent around the world, ranging between 3 to 4.5 cases/million population/year [6, 8–13]. The rates in older persons have been less studied; current estimates are 1 to 2 cases/million population/year for persons aged 25 to 59 years and 1.5 to 4.5 cases/million population/year for persons over the age of 60 [1, 8]. Elderly individuals have a higher incidence of osteosarcoma related to Paget disease of the bone or as a consequence of treatment for a different cancer [1, 6, 14–16]. In the US and Europe, osteosarcoma incidence has somewhat increased over time in younger cases [1, 6, 12] and decreased in elderly individuals in the US [1].

In the US, using population data from the Surveillance, Epidemiology, and End Results (SEER) program, osteosarcoma incidence has been shown to vary by race based

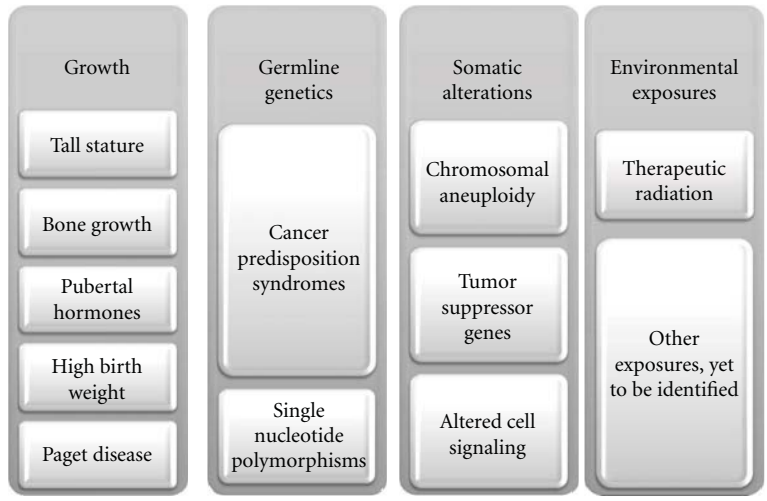


Figure 1: *Potential contributing factors in the etiology of osteosarcoma.*

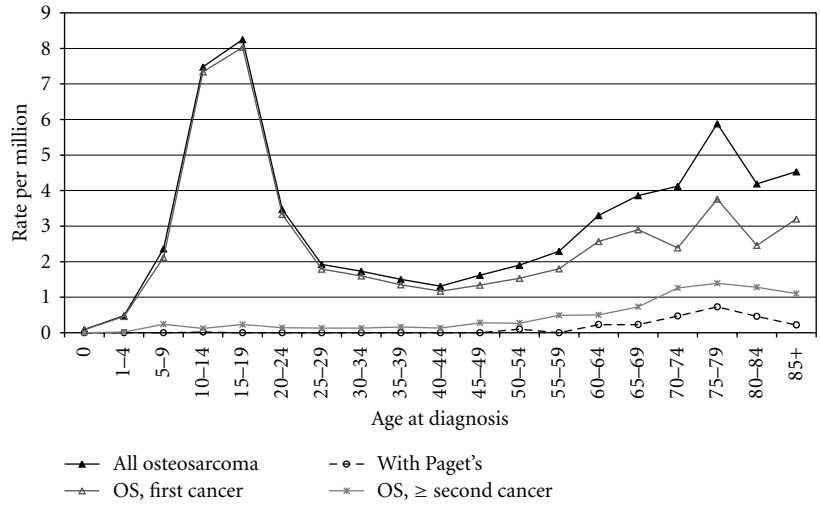
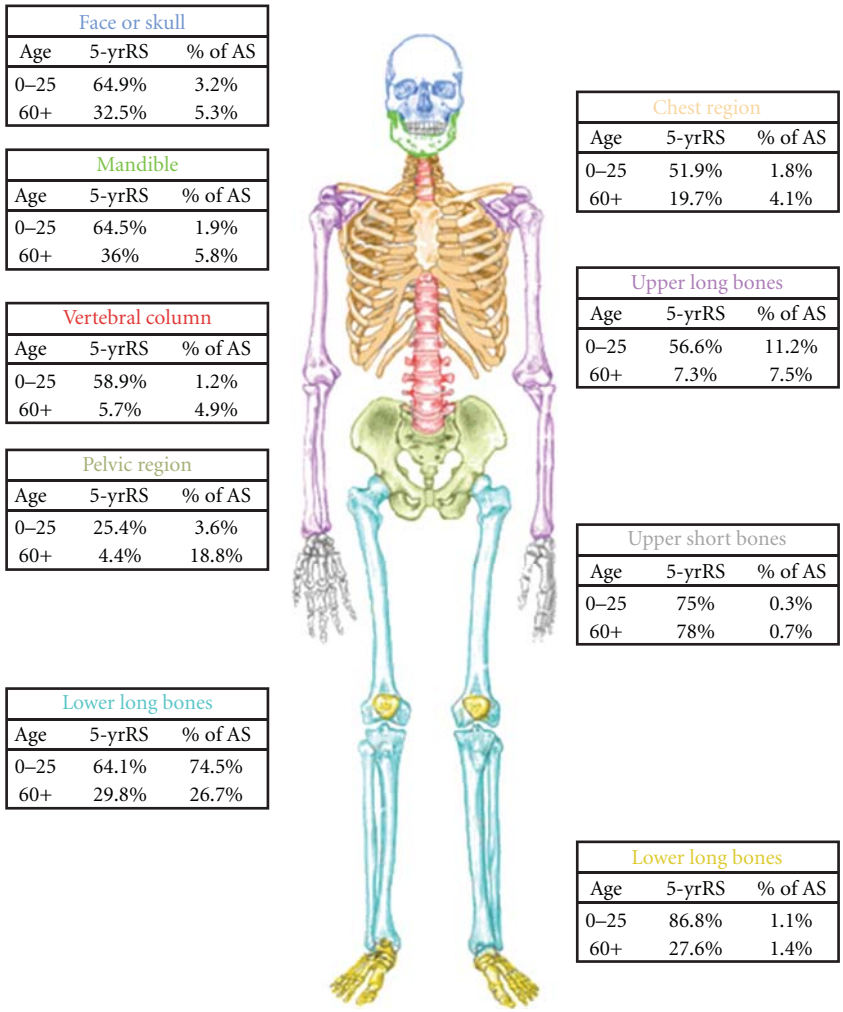


Figure 2: *Incidence of osteosarcoma per million population.* Data were derived from the Surveillance, Epidemiology, and End Results (SEER) program on the US population. Previously published by Mirabello et al. [2].

on the age of onset [17]: (1) in children and adolescents, the incidence is greatest in Asian/Pacific Islanders; (2) in individuals 25–59 years of age, the incidence is greatest in Blacks; (3) in individuals over the age of 60, osteosarcoma incidence is greatest in Whites [1]. A higher incidence of childhood osteosarcoma has been reported in Italy [18], Latin America [8], and in two African countries, Sudan and Uganda [13] compared to other populations around the world. Lower rates have been reported in Western Australia compared to the US [19]. Higher rates of osteosarcoma in the elderly have been noted in the UK and Australia [8].

It has been reported that, when a wide range of ages are combined, males are affected with osteosarcoma more frequently than females [1, 3, 4, 6, 8, 12, 19–21]. However, it has also been reported that females less than 15 years of age have slightly higher rates than males in the same age group [1, 5, 6, 14, 22–26]. In elderly patients, osteosarcoma is more common in Blacks [5] and in females, particularly those with a prior history of cancer [1]. In adolescence, incidence peaks at a later age and reaches higher rates in males (age 15–19, peak rate of 9–15 cases/million population) compared to females (age 10–14, peak rate of 6–10 cases/million population) [1, 6, 8], which suggests that bone growth, hormonal changes, and/or development associated with puberty may be involved in osteosarcoma etiology. This relationship between osteosarcoma, hormones, and growth may also partly explain the slightly higher overall incidence in males compared to females.

2.2. Tumor Location. Osteosarcoma occurs most frequently in the lower long bones [1, 5, 7, 21] (Figure 3). In young patients, it most often arises at sites of rapid bone growth,



Face or skull		
Age	5-yrRS	% of AS
0–25	64.9%	3.2%
60+	32.5%	5.3%

Mandible		
Age	5-yrRS	% of AS
0–25	64.5%	1.9%
60+	36%	5.8%

Vertebral column		
Age	5-yrRS	% of AS
0–25	58.9%	1.2%
60+	5.7%	4.9%

Pelvic region		
Age	5-yrRS	% of AS
0–25	25.4%	3.6%
60+	4.4%	18.8%

Lower long bones		
Age	5-yrRS	% of AS
0–25	64.1%	74.5%
60+	29.8%	26.7%

Chest region		
Age	5-yrRS	% of AS
0–25	51.9%	1.8%
60+	19.7%	4.1%

Upper long bones		
Age	5-yrRS	% of AS
0–25	56.6%	11.2%
60+	7.3%	7.5%

Upper short bones		
Age	5-yrRS	% of AS
0–25	75%	0.3%
60+	78%	0.7%

Lower long bones		
Age	5-yrRS	% of AS
0–25	86.8%	1.1%
60+	27.6%	1.4%

Figure 3: *Five-year relative survival rates (RS) by anatomic site (AS) for individuals with osteosarcoma age 0–25 years and 60+ years in the US.* The percent (%) of AS is the % of patients in that age group with osteosarcoma at that location. This figure was created using data from the SEER program in Mirabello et al. [2].

the metaphyses of long bones, such as the distal femur, proximal tibia, and proximal humerus [1, 21, 27]. The occurrence of osteosarcoma most frequently in the metaphyseal area adjacent to the growth plate of long bones [28], which are the sites of particularly rapid growth during the adolescent growth spurt, reinforces the relationship between bone growth and osteosarcoma formation. There may be an increased vulnerability at these physes due to the high cell turnover during puberty. The tendency of osteosarcoma to occur in the extremity bones decreases with age, although the most common site is still the lower long bones. The lower long bones account for approximately 80% of osteosarcoma in the young patients, 27–43% in middle aged and elderly persons [1, 3, 16, 29]. In elderly patients, osteosarcomas often occur secondary to Paget's disease of the bone or some other benign bone lesion [14–16].

The anatomic site distributions do not vary significantly by sex or race in young patients [1] but there is more variability in middle-aged and elderly patients. This includes a higher frequency of osteosarcoma of the mandible in Blacks compared to Whites, and higher frequency of chest and upper long bone osteosarcoma but lower rates of vertebral, pelvic, or mandibular osteosarcoma in females compared to males [1].

2.3. Survival. Survival rates vary by age, gender, disease stage, and anatomic site (Figure 3). For children and adolescents, these rates are similar in most countries, ranging from 55–75%; although, lower rates (19–39%) have been observed in Slovakia, Estonia, and Denmark [1, 8, 30–33]. The five-year survival rate in persons aged 0–39 years was 58% in northern England [12] and 53% in Great Britain [26]. The 5-year survival in Finland for the whole study population was 58% [34]. Survival at 5 years was 57% for patients of all ages, 68% for those <41 years, and 22% for patients older than 65 years at an institute in Italy [35, 36]. Data for patients of all ages from the US National Cancer Database reported a 5-year survival of 53.9% [21], similar to the 54% reported for all age cases at the M.D. Anderson Cancer Center [37]. SEER

data in the US from 1973 to 2004 showed that the relative 5-year survival rate for young-onset osteosarcoma was 61.6%; it was 58.7% for middle aged persons and 24.2% for persons over 60 years of age [1]. This paper showed a sharp decrease in survival after age 50, with rates dropping from around 50% for patients in their 50s to 17% for those in their mid-late 60s to only 11% for those in their 80s. Others have also shown that survival rates in adults over age 40 years are lower than in younger patients with rates ranging from 18–55% [16, 21, 29, 36–39].

Females have higher survival rates than males [1, 12, 21, 26, 32, 33, 40]. Disease stage is an important prognostic factor in patients with osteosarcoma at all ages, with distant disease having a much lower 5-year survival rate than localized or regional disease [1, 32]. Osteosarcoma survival rates are higher when it occurs in the short bones, and the poorest with osteosarcoma of the pelvic region and vertebral column for all ages [1, 3]. Osteosarcoma pathology has also been suggested to affect survival, though this is difficult to evaluate in most reports because many of the subtypes consist of very small sample sizes and rates are thus unstable. However, parosteal osteosarcoma has been associated with a high survival rate [21], and osteosarcoma with Paget disease a low rate [1, 21]. It has also been shown that patients with larger tumor size, metastatic disease, soft-tissue extension of the primary tumor, less tumor necrosis after neoadjuvant chemotherapy, inadequate surgical margins, or recurrence have significantly worse prognosis [7, 36–38].

Older patients may have unique tumor biology, for example, more axial tumors or other factors associated with a poorer prognosis, such as Paget disease (see below) and response to therapy due to age-related adjustments in therapeutic regimens, which could contribute to their worse overall survival [7, 29, 37]. Overall, osteosarcoma survival has improved over time with each decade until the 1990s, but little thereafter [1, 6, 26, 31, 32, 34, 40–43]. It has likely improved with advancements in patient care and the advent of chemotherapy, but there is still a need for novel treatment and patient management strategies shown by the lack of improvement in the last decade.

3. Environmental Exposures and Osteosarcoma

Studies of environmental exposures and rare cancers, such as osteosarcoma, are challenging and often limited by sample sizes. Most studies are case-control, ecologic, and/or descriptive in nature. This is because the extremely large cohorts required to study these cancers are nearly impossible to conduct. For example, even in a cohort of one million individuals, only 4 or 5 would be expected to develop osteosarcoma. Studies of environmental exposures and osteosarcoma are often combined with studies of other bone tumors, including Ewing sarcoma and others. This makes separating the potentially etiologic clues even more challenging. The reader is referred to a recent, comprehensive review of these studies [44]. Two well-studied exposures are described below.

Many years ago, it was hypothesized that fluoride could contribute to osteosarcoma risk. This was based, in part, on the fact that it is taken up by and stored in bones and on *in vitro* data which suggested that fluoride could act as a mitogen on osteoblasts [45]. Studies of fluoride exposure and osteosarcoma risk have not yielded conclusive results and have generated significant controversy. The initial ecologic studies suggested that fluoride could contribute to bone cancer etiology, but subsequent studies did not confirm this finding (reviewed by Eyre et al., 2009 [44]). A more recent study did suggest an association between fluoride and osteosarcoma in males but not in females [46] but caution was suggested in its interpretation [47].

Therapeutic radiation is a proven risk factor for osteosarcoma. It was noted to occur more frequently than expected in survivors of Hodgkin disease who received therapeutic radiation [48, 49]. Increased incidence of osteosarcoma was also noted in individuals who received Radium for ankylosing spondylitis (reviewed in [48]). However, very low doses of radiation received for medical evaluations, such as X-rays or CT scans are not associated with osteosarcoma risk (reviewed in [50]).

4. Growth and Osteosarcoma

Since osteosarcoma occurs most commonly during puberty, a time of rapid bone growth and remodeling, it is highly plausible that factors related to growth and development play a role in osteosarcoma etiology. Case reports of osteosarcoma occurring in individuals with acromegaly, a growth disorder caused by over production of growth hormone, lent further support to this hypothesis [51]. Early studies suggested that individuals who were longer at birth and/or taller than their peers were at increased risk of osteosarcoma [52–55]. These associations are further supported by the strong positive association of sporadic osteosarcoma and height in canines [56].

Osteosarcoma incidence is highest during puberty when endogenous sex hormones, growth hormones, and insulin-like growth factor 1(IGF1) levels are at their highest, so this biological pathway is likely to play an important role in osteosarcoma etiology. Insulin-like growth factors (IGFs) play critical roles in carcinogenesis and circulating levels are associated with risk of several cancers [57], including prostate, breast, colorectal, and lung cancer [58]. IGF1 is a potent mitogen for human osteosarcoma cell lines [59, 60]. The overexpression of insulin-like growth factor 2 (*IGF2)* and loss of IGF2 imprinting occurs in diverse cancers [61], further suggesting a role for this pathway in carcinogenesis. In addition, one small study identified single nucleotide polymorphisms (SNPs) in *IGF2R* as potential risk factors for osteosarcoma (see below) [62].

4.1. Height. The association between taller stature and increased risk of developing osteosarcoma was first reported in 1967 [63]. That study compared the height of 85 individuals with osteosarcoma to 202 controls between 1945 and 1965 and found that the cases were taller than controls.

Two subsequent studies of 54 and 18 cases each which used percentiles of height also noted that the osteosarcoma cases were taller than expected [54, 64]. Five additional studies [53, 55, 65–67] confirmed the association of increased height and osteosarcoma risk but eight others [52, 68–74] did not find an association between height and osteosarcoma risk. The discrepancies among these studies could be a result of limited sample sizes, variable methods and control selection procedures, and thus limited statistical power. However, the largest study, a cohort study of 962 patients with osteosarcoma which used standard deviation scores to evaluate the relative height of patients, found that patients with osteosarcoma were taller than average but the association was primarily in those less than 18 years of age [53].

A recent meta-analysis of height and osteosarcoma compiled individual osteosarcoma patient data on 1067 osteosarcoma cases derived from 5 published [52, 54, 66, 67, 73] and 2 unpublished studies of height (Mirabello et al., Under Review). Cases were compared to age- and gender-matched 1000 simulated controls per case based on the 2000 US National Center for Health Statistics Growth Charts. That study showed that "taller-than-average" (51st–89th percentile) and "very tall" individuals (≥90th percentile) had an increased risk of osteosarcoma (odds ratio 1.40, 95% CI 1.13–1.73, and odds ratio 2.63, 95% CI 1.98–3.49, resp., $P_{\text{trend}} < 0.0001$).

The meta-analysis (Mirabello et al., Under Review), and a separate study of 962 patients with osteosarcoma [53], which was not included in the meta-analysis, confirm that taller stature is associated with osteosarcoma. However, the specific basis for this association is not known. For example, there are currently no data in the literature on osteosarcoma and patient height that also consider parental height. The incidence of osteosarcoma does not vary widely around the world but the average adult height varies based on country of origin [75]. Individuals with a more rapid growth velocity during puberty could potentially have increased risk of osteosarcoma because cell division is occurring more rapidly. Attaining a greater height than expected based on parental heights could also be a risk factor because of the increased bone growth required. Future studies of parental height and growth velocity will be helpful in understanding these differences.

4.2. Birth Weight. Numerous epidemiologic studies have evaluated associations between high birth weight and cancer. This is based on the hypothesis that high birth weight may be the result of multiple factors that are also associated with cancer. For example, IGFs are important in fetal development [76] and are also associated with cancer risk [57]. Higher birth weight has been associated with several childhood cancers, including acute lymphoblastic leukemia (ALL) [77–79], primary brain tumors [80], rhabdomyosarcoma [81], and Wilms' tumor [82, 83]. Interestingly, recent studies of ALL and Wilms' tumor suggest that the strongest associations are in females with high birth weight [82, 84]. There are also several studies suggesting associations between high birth weight and adult-onset cancers, including prostate and breast cancer [85, 86].

Review of the literature identified five published studies that evaluated the potential association between birth weight and osteosarcoma; four were null [65, 74, 79, 87], and one showed an association between higher birth weight and osteosarcoma [52]. The inconsistencies in the published data on birth weight may also be due to small sample sizes and/or inconsistent methods. A meta-analysis of the raw data from two published [52, 79] and one unpublished study of birth weight and osteosarcoma compared the birth weights of 434 individuals with osteosarcoma to age- and gender-matched controls (1000 simulated controls per case) derived from US growth charts (Mirabello et al., Under Review). In that study, individuals with high birth-weight (≥4046 g) had a marginally significant increased risk of osteosarcoma (OR 1.35, 95% CI 1.01–1.79). Females with high birth-weight, but not males, had an increased risk of OS (OR 1.49, 95% CI 1.00–2.22). Overall, the association between birth weight and osteosarcoma is not as strong as the height association, but it is similar in magnitude to other cancers. It remains conceivable that prenatal growth and factors that influence it, such as growth factors and hormones, contribute to osteosarcoma risk.

4.3. Paget Disease and Osteosarcoma. Paget disease of bone is a relatively common metabolic bone disorder that typically occurs in older individuals [88, 89]. It is characterized by highly exaggerated bone remodeling caused by abnormalities in osteoclast regulation. Sarcomatous transformation is rare but associated with a high mortality rate. The incidence of osteosarcoma secondary to Paget disease is not precisely known, but studies estimate that about 1% of patients with Paget disease will develop osteosarcoma [90]. In elderly persons, about half of the osteosarcomas reported are estimated to be associated with Paget disease.

The co-occurrence of osteosarcoma in the setting of abnormal bone remodeling due to Paget disease of the bone suggests that osteosarcoma may be etiologically related to abnormal bone remodeling [90, 91]. This could appear to be the case in elderly individuals but the role of abnormal bone remodeling in osteosarcoma adolescents is not known. It is conceivable that a subset of younger patients have increased genetic risk and that there could be overlap with genes that contribute to the etiology of Paget disease. Paget disease is genetically heterogeneous but recent studies implicate the RANK-NF-κB signaling pathway [89]. Mutations in *SQSTM1*, a downstream scaffold protein in this pathway, are associated with familial Paget disease [92]. Many, but not all, of the associated mutations occur in the ubiquitin-associated domain of the p62 protein which is encoded by the *SQSTM1* gene [88]. Ubiquitin-associated proteins, such as p62, are important in the RANK-NF-κB signaling pathway which promotes osteoclastogenesis and formation.

5. Genetic Risk Factors

Chromosomal aneuploidy is common in osteosarcoma cells which suggests that somatic or germline chromosomal instability could potentially predispose an individual to

Table 1: Inherited disorders associated with increased rates of osteosarcoma.

Disorder	Gene	Chromosome	Autosomal inheritance pattern
Li-Fraumeni Syndrome	*TP53,* tumor protein p53	17p13.1	Dominant
Retinoblastoma	*RB1,* retinoblastoma 1	13q14.2	Dominant
Rothmund Thomson Syndrome	*REQL4,* RecQ protein-like 4, DNA helicase	8q24.3	Recessive
Werner Syndrome	*WRN,* Werner syndrome, RecQ helicase-like	8p12	Recessive
Bloom Syndrome	*BLM,* Bloom syndrome, RecQ helicase-like	15q26.1	Recessive
Diamond Blackfan Anemia	Ribosomal protein genes, including *RPS19, RPL5, RPL11, RPL35A, RPS24, RPS17,* and *RPS7*	multiple	Dominant

osteosarcoma [93, 94]. There are numerous studies of the somatic changes present in osteosarcoma cells but a common somatic defect has not yet been identified. Osteosarcoma is associated with several cancer predisposition syndromes that are caused by highly penetrant germline mutations as described in Table 1. These disorders are extremely rare and not a common cause of osteosarcoma. However, they may provide important insights into osteosarcoma etiology because the same genes that are associated with these disorders are often also disrupted in osteosarcoma tissues. Common germline genetic variants, such as SNPs, are associated with risk of numerous diseases, including cancer. The role that they play in sporadic osteosarcoma is not known, but several pilot studies have sought to understand this (Table 2).

5.1. Inherited, Cancer-Prone Disorders. Inherited cancer predisposition syndromes are a heterogeneous group of disorders. There are several disorders in which higher rates of osteosarcoma are noted (Table 1). Studies of these disorders have provided important clues to understanding osteosarcoma etiology.

The careful characterization of families with high rates of breast cancer, sarcomas, and other cancers by Li and Fraumeni Jr. in 1969 led to the recognition of the syndrome now known as Li-Fraumeni syndrome (LFS) [101, 102]. The classic LFS is clinically diagnosed based on family history which includes a personal history of a sarcoma diagnosed under the age of 45, a first-degree relative with cancer under age 45, and another first- or second-degree relative with cancer diagnosed under age 45 or sarcoma at any age. LFS is caused by autosomal dominant germline mutations in *TP53* [102, 103] although approximately 30% of individuals who meet clinical criteria for LFS do not have a *TP53* mutation. Additional clinical descriptions and criteria for mutation testing in individuals with suspected LFS are reviewed in [102]. The p53 protein, encoded by *TP53*, is crucial for normal cell growth, apoptosis, DNA repair, and numerous other cellular processes. The p53 gene is mutated in a majority of somatic tumor tissues, many of which disrupt the DNA-binding domain and result in a loss of tumor suppressor function [104]. Many, but not all, osteosarcomas have *TP53* mutations but these have not consistently been correlated with disease stage or prognosis [105].

Retinoblastoma is a malignant retinal tumor that typically occurs prior to the age of 5. It is caused by mutations in the *RB1* tumor suppressor gene [106]. The *RB1* gene encodes the Rb protein which is critical in normal cell cycle and differentiation processes. Loss of normal Rb function is noted in several sporadic human tumors, including apparently sporadic osteosarcoma. In addition, osteosarcoma is the most common second tumor in patients with retinoblastoma. It occurs more frequently than expected in individuals with *RB1* mutations whether or not they had radiation therapy [107, 108]. The standardized incidence ratio (SIR) for osteosarcoma occurring after retinoblastoma was 406-fold over expected for individuals who had radiation and 69-fold over expected for those who had not received radiation therapy. This suggests that both primary genetic and gene/environment interactions contribute to osteosarcoma development in the setting of a germline *RB1* mutation, and this may also be the case in apparently sporadic osteosarcoma.

Increased rates of osteosarcoma are also present in individuals with germline mutations in DNA helicase genes, including Rothmund Thomas syndrome (RTS), Werner syndrome, and Bloom syndrome. RTS is a rare, autosomal recessive disorder caused by mutations in the DNA helicase *RECQL4* (reviewed in [109, 110]). It has a characteristic sun-sensitive rash which presents in infancy and then enters a chronic phase with poikiloderma through adulthood. Individuals with RTS may also have small stature, skeletal dysplasias, sparse hair, or cataracts. Osteosarcoma is the most common cancer in RTS; one study of 41 patients found that 32% had osteosarcoma [111]. The role of *RECQL4* in sporadic osteosarcoma is not well understood. Since the DNA helicases are critical for normal DNA structure and function, it is feasible that proteins in this family are likely to be important in carcinogenic processes and could contribute to the DNA damage and chromosomal aberrations seen in osteosarcoma cells.

Table 2: Association studies of single nucleotide polymorphisms and osteosarcoma risk. Abbreviations: SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval.

First Author, Year, Reference	No. cases/no. controls	Study Design	Gene	Main Finding(s)
Patiño-Garcia, 2000, [95]	63/111	Case-Control	Tumor Necrosis Factor-α (*TNF*)	Evaluated 3 SNPs in the promoter. *TNF*-α -238G>A was inversely associated with risk (OR 0.17, 95% CI 0.04–0.76, $P = 0.0095$)
Ruza, 2003, [66]	72/143	Case-Control	Vitamin D Receptor (*VDR*)	3 SNPs (FokI, ApaI, TaqI) studied. FokI *Ff* genotype associated with increased risk (OR 1.78, 95% CI 1.0–3.16, $P = 0.048$)
			Estrogen Receptor (*ESR1*)	2 variants (Pvu II and XbaI) evaluated were not associated with osteosarcoma
			Collagen 1α1 (*COL1A1*)	1 variant studied (Msc 1) was not associated with osteosarcoma.
Savage, 2007, [96]	104/74	Hospital-based Case-Control	Tumor Protein p53 (*TP53*)	12 tag-SNPs in *TP53* genotyped. Recessive model noted potential increased risk with rs1642785 (IVS+38C>G; OR 6.7, 95% CI 1.06-41.6, $P = 0.04$) and rs1042522 (Ex4+119C>G, P72R; OR 7.5, 95% CI 1.2–46.3, $P = 0.03$).
Savage, 2007, [62]	104/74	Hospital-based Case-Control	Insulin-like Growth Factor 2 Receptor (*IGF2R*)	Evaluated 52 SNPs in 13 growth-related genes. Two linked *IGF2R* SNPs, rs998075 (Ex16+88G>A) and rs998074 (IVS16+15C>T), associated with increased risk (haplotype OR 2.04, 95% CI 1.29–3.24, $P = 0.006$).
Koshkina, 2007, [97]	123/510	Case-Control	Fas (TNF receptor superfamily, member 6; *FAS*)	4 SNPs in *Fas* studied. Increased risk with exon 3, 18272A>G, most pronounced in non-Hispanic whites (OR 2.3, 95% CI 1.2–4.6, $P = 0.014$)
Toffoli, 2009, [98]	201/250	Case-Control	Mdm2 p53 binding protein homolog (*MDM2*)	1 SNP in *MDM2* studied, rs2279744 (SNP309T>G), was associated with high-grade osteosarcoma in females
			Tumor Protein p53 (*TP53*)	1 SNP evaluated, rs1042522 (Ex4+119C>G, P72R), was associated with survival.
Hu, 2010, [99]	168/168	Case-Control	Transforming growth factor beta receptor 1 (*TGFBR1*)	1 variant evaluated (TGFBR1*6A) was associated with increased susceptibility (OR 4.6, 95% CI 2.3–7.9, $P = 0.002$)
Mirabello, 2010, [100]	99/1430	Hospital-based Case-Control	8q24 region	Evaluated 214 SNPs, including 9 previously associated with cancer. Strongest association noted at rs896324 in additive model (OR 1.75, 95% CI 1.13–2.69, $P = 0.01$)

Bloom syndrome, caused by autosomal recessive inheritance of mutations in the *BLM* helicase, also has a characteristic rash, but not true poikiloderma [112]. Individuals have severe pre- and postnatal growth retardation, learning disabilities, and high rates of cancers. The most common cancers are epithelial, hematopoietic, lymphoid, connective tissue, germ cell, nervous system, and kidney cancers. Three out of 168 individuals with Bloom syndrome listed in the Bloom syndrome registry were reported to have a sarcoma between 1954 and 2000 [112]. While osteosarcoma is still rare in Bloom syndrome, it is more common in this disorder than in the general population. The role of *BLM* mutations in osteosarcoma somatic cells is not well described.

Werner syndrome is a premature aging syndrome which typically presents after the first decade of life [113, 114]. It is caused by mutations in the *WRN* DNA helicase and inherited in an autosomal recessive manner. Individuals with Werner syndrome typically have characteristic "bird" facies, short stature, parental consanguinity, cataracts, atrophic skin, and signs of premature aging such as atherosclerosis. They are at increased risk of osteosarcoma as well as other malignancies [115, 116].

Diamond Blackfan anemia (DBA) is another inherited disorder associated with increased risk of osteosarcoma [117]. DBA is an inherited red blood cell aplasia with a broad phenotypic spectrum. Patients have variable degrees of anemia, normal leukocytes and platelets, occasional physical malformations, and increased risk of acute myelogenous leukemia, myelodysplastic syndrome, and solid tumors. Approximately 40% of patients have an identifiable mutation in a gene important in ribosomal function (*RPS19, RPL5, RPL11, RPL35A, RPS24, RPS17,* or *RPS7*). Osteosarcoma was noted in three of the 354 patients in the DBA registry in 2001 [118]. The role of these ribosomal proteins in

osteosarcoma biology is unexplored. However, the higher than expected occurrence of osteosarcoma in patients with DBA is notable and warrants further study of ribosomal function in osteosarcoma.

5.2. Inherited, Cancer-Prone Disorders. The inherited disorders caused by rare, highly-penetrant mutations and associated with osteosarcoma described above explain only a very small percentage of all osteosarcoma cases. It occurs more often in individuals without a family history of cancer or other medical problems. Several studies have been conducted in an effort to understand the contribution of common genetic variants, such as SNPs, to osteosarcoma risk (Table 2) although the vast majority await replication. SNPs are the most common form of genetic variation in the genome; approximately 10 million with minor allele frequencies of at least 1% are thought to be present in the genome. Most SNPs do not alter gene expression or protein function, but a subset can have subtle, yet important, biological effects. For example, an SNP in the promoter of the *MDM2* gene increases the affinity of the Sp1transcription factor which results in higher MDM2 levels and p53 pathway attenuation [119].

Most of the studies of SNPs and osteosarcoma conducted to date have been limited by sample size and therefore should be considered exploratory in nature (Table 2). These studies were based on *a priori* hypotheses that the genes of interest were potentially important in osteosarcoma biology. The first such study evaluated three SNPs in the promoter of the Tumor Necrosis Factor-α (*TNF*) gene in 63 osteosarcoma cases and 111 controls from Spain [120]. The TNF protein is a proinflammatory cytokine that has important roles in cellular proliferation and differentiation. It is also involved in bone remodeling and is a component of the RNKL pathway described above. SNPs in the TNF promoter have also been noted to affect protein expression. That study suggested that the *TNF*-238G>A was inversely associated with osteosarcoma. The *TNF*-308G>A variant was not associated with osteosarcoma. The authors also evaluated these genotypes in 47 individuals with Ewing sarcoma but did not find an association.

In a second study, the same group hypothesized that variants in the estrogen receptor (*ESR1*), vitamin D receptor (*VDR*), and/or collagen 1α1 (*COL1A1*) gene could be osteosarcoma risk factors. Variants in *ESR1* could be important in osteosarcoma since estrogen is critical during puberty which is the key time of risk for osteosarcoma. The *VDR* and *COL1A1* genes are required for proper bone formation and thus, if aberrations are present, could be associated with osteosarcoma. A total of 72 osteosarcoma cases and 143 controls were evaluated. Ruza et al. found that the F*f* genotype of *VDR* was associated with increased risk of osteosarcoma (odds ratio [OR] 1.78, 95% confidence interval [CI] 1.0–3.16, $P = 0.048$) [66]. Variants in *ESR1* or *COL1A1* were not associated with osteosarcoma.

Since mutations in *TP53* cause LFS and osteosarcoma is a defining tumor of the syndrome, SNPs in *TP53* were evaluated as potential osteosarcoma risk factors in the Bone Disease and Injury Study of Osteosarcoma (BDISO), a hospital-based study of 104 cases and 74 controls [96]. Subjects genotyped were whites from the US Twelve tag-SNPs were genotyped and several inheritance models evaluated. The recessive inheritance model suggested that rs1642785 (IVS+38C>G) and rs1042522 (Ex4+119C>G, Pro72Arg) were associated with osteosarcoma risk. However, these genotypes were quite rare and this study, like those described above, was limited by its small sample size.

In a different study of the p53 pathway, Toffoli et al. genotyped the Pro72Arg (rs1042522, Ex4+119C>G) SNP in *TP53* and the *MDM2* -309 promoter SNP (rs2279744, T>G) in 201 osteosarcoma cases and 250 controls from Italy [98]. The Pro72Arg SNP in *TP53* has been associated with risk of several cancers, including lung and breast cancer (reviewed in [121]). In addition, the presence of the 72Arg allele was correlated with earlier age of cancer onset in individuals with LFS [122]. The MDM2 protein is an important regulator of TP53 function, and the -309 T>G SNP is associated with altered *MDM2* expression. LFS patients with the G allele have an earlier age of onset of cancer [122]. In addition, this *MDM2* SNP is also associated with risk of several cancers [121]. This osteosarcoma study noted that the *MDM2*-309 SNP was only associated with high-grade osteosarcoma in females. The *TP53* Pro72Arg SNP was not associated with osteosarcoma risk but an association with survival was suggested. This study did not report results of a recessive genetic model so direct comparison with the BDISO *TP53* findings in osteosarcoma was not possible.

The first study to evaluate SNPs in growth-related genes did so based on the hypothesis that since osteosarcoma most commonly occurs during a period of active growth, that variants in genes that regulate pubertal growth could be important osteosarcoma risk factors. Common SNPs in 13 growth-related genes were also evaluated as candidate risk modifiers in the BDISO. Of the 52 SNPs evaluated, two correlated SNPs in insulin-like growth factor receptor 2 (*IGF2R*, rs998075 and rs998074) were associated with increased risk of osteosarcoma (OR 2.04, 95% CI 1.29–3.24) [62]. One of those SNPs, rs998075 (Ex16+88G>A), resulted in loss of methylation in a CpG island but the impact of this alteration on *IGF2R* protein function is not known. As noted above, the IGFs are potential regulators of carcinogenesis in several cancer types and IGF1 levels have been associated with cancer risk. Followup of these findings in osteosarcoma is needed to better understand how genetic variation in *IGF2R* contributes to its etiology.

SNPs in the 8q24 chromosomal region are being intensely studied because genome-wide association studies (GWAS) have consistently found them to be associated with risk of adult onset cancers, including prostate, breast, colon, and others [123–125]. Therefore, we recently evaluated 214 SNPs in 8q24 with a focus on the 9 SNPs which were previously associated with cancer in GWAS [100]. Ninety-nine cases and 65 controls plus an additional 1365 controls from the Prostate, Lung, Colorectal, Ovarian (PLCO) cancer screening trial were genotyped. All subjects were self-identified whites. Associations with the 9 SNPs previously associated with cancer were not noted in this study. Overall, seven SNPs

were associated with osteosarcoma; the strongest result was noted for SNP, rs896324 (OR 1.75, 95% CI 1.13–2.69). These SNPs are in slightly different locations than the SNPs associated with other cancers. The details of 8q24 are still being explored, but a long-range regulator of the MYC proto-oncogene may be present in this region [126]. MYC inhibition was suggested to cause differentiation of osteosarcoma cells into mature osteocytes in a mouse model [127]. The combination of these findings suggests that further study of the 8q24 locus may yield important insights into the regulation of MYC and its role in osteosarcoma pathogenesis.

The Fas protein (gene name *FAS*, or *TNFRSF6*) is a member of the TNF receptor superfamily and plays a central role in programmed cell death. Genetic variants in FAS have been associated with increased risk of several cancers, such as melanoma, gastric, and renal cell cancer [128, 129]. Based on this, Koshkina et al. hypothesized that SNPs in *FAS* may be osteosarcoma risk factors. They evaluated four SNPs in *FAS* in 123 osteosarcoma cases and 510 controls from the US [97]. An important limitation of this study is the fact that the study subjects were of variable ethnicity; 51.2% of cases (63) and 78% of controls (398) were described as non-Hispanic whites. An SNP in exon 3 (18272A>G, dbSNP number not given) was associated with increased risk of osteosarcoma in non-Hispanic whites (OR 2.3, 95% CI 1.2–4.6).

TGF-β signaling is important in the regulation of cellular proliferation. A functional polymorphisms, referred to as TGFBR1*6A, is caused by the deletion of 3 GCG triplets which code for alanine in exon 1. It is a hypomorphic variant that results in reduced TGF-β growth inhibitory signaling. The TGFBR1*6A variant has been associated with breast and ovarian cancer, but not consistently associated with other cancer types [130]. Thus, the potential role of this variant was explored in a study of 168 osteosarcoma patients and 168 controls [99]. The authors found that both homozygosity and heterozygosity for the TGFBR1*6A variant resulted in increased risk of osteosarcoma in the Chinese population, in a gene-dose response pattern (OR 4.6, 95% CI 2.2–7.97 and OR 2.9, 95% CI 1.59–5.34, resp.).

As a whole, the studies conducted, to date, of common genetic variants and osteosarcoma risk have yielded promising results. Their strength lies in the fact that they have evaluated genes which have a high biologic likelihood of being related to osteosarcoma etiology based on laboratory and/or other epidemiologic studies. However, the results of all the studies described above and in Table 2 should be interpreted with caution because they all have small sample sizes and limited statistical power. Future, large, multi-institutional, collaborative studies are required to obtain the necessary sample size and adequate statistical power to follow up these findings.

6. Summary and Future Directions

Some progress has been made in understanding the cause of osteosarcoma, but we still have much to learn. The biggest clue generated in the study of osteosarcoma epidemiology is its association with either rapid or abnormal growth. Its occurrence primarily during the adolescent growth spurt and association with tall height at diagnosis show that bone growth is clearly an important factor. It is not known whether or not tall stature, in and of itself, is the key, or if it is taller stature than expected based on parental heights or due to height velocity during puberty. An ongoing Children's Oncology Group epidemiology study which will investigate parental height and growth charts of children and adolescents with osteosarcoma will help shed light on this question. Future clinical and laboratory studies should also carefully evaluate the complex hormonal changes that occur before, during, and after puberty.

The association of osteosarcoma with the abnormal bone remodeling present in Paget disease also warrants more careful examination. The role of variants in genes of the RANKL-NF-κB signaling pathway, which are strongly associated with Paget disease, have not been thoroughly studied as potential osteosarcoma risk factors. The case reports of the occurrence of osteosarcoma in the setting of acromegaly, a state of abnormal growth hormone production, also warrant followup. Is the literature biased by these case reports, or is there an increased risk of osteosarcoma amongst individuals with acromegaly?

The studies of rare, but highly penetrant, cancer predisposition syndromes can shed some light on the biological mechanisms of osteosarcoma. In general, the cancers that occur in individuals with the cancer predisposition syndromes described above occur at much younger ages than in the same cancer types in the general population. The fact that several of these syndromes include osteosarcoma in the phenotype suggests that there may be common genetic mechanisms which also contribute to the apparently sporadic occurrence of osteosarcoma. It is also likely that the genetic contribution to cancers which occur in the first two decades of life, such as osteosarcoma, is greater than in cancers which do not occur until many decades later. In childhood cancer, there has been considerably less time for exposure to known and unknown environmental carcinogens.

The contribution of environmental exposures to osteosarcoma and to other cancers of children and young adults is not known. The heterogeneity and relative rarity of these cancers create significant complexity in study design and interpretation. In addition, it is likely that a combination of environmental exposure and genetic risk factors contribute to cancer risk. Large, longitudinal, cohort studies of the cancers of children and young adults are required to address these study design issues and likely contribution of multiple factors. The International Childhood Cancer Cohort Consortium (I4C) is a multi-institutional, international collaborative group of childhood cohort studies that is working to better understand the etiology of childhood cancer [131]. However, even this large-scale effort will not be able to address osteosarcoma risk factors in detail, because of its rarity.

Like many cancers, the etiology of most osteosarcoma remains unknown. Epidemiology studies have provided many important clues, such as associations with puberty,

height, and disorders of bone growth and remodeling. The genetic clues derived from the occurrence of osteosarcoma in the setting of germline mutations in genes such as *TP53* and *RB1* suggest that the genetic contribution to what appears to be sporadic osteosarcoma may also be important. Understanding potential environmental contributions to osteosarcoma risk is very challenging because of its rarity and the fact that a single environmental exposure is not likely to be the primary cause. Numerous studies are underway which seek to improve our understanding of osteosarcoma etiology and through this understanding we will be better equipped to counsel patients and refine treatment strategies.

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Research Article

Tyrosine Phosphorylation in the C-Terminal Nuclear Localization and Retention Signal (C-NLS) of the EWS Protein

Ruzanna P. Leemann-Zakaryan,[1,2] Steffen Pahlich,[1] Doris Grossenbacher,[1] and Heinz Gehring[1]

[1] *Department of Biochemistry, University of Zurich, Winterthurerstraße 190, 8057 Zurich, Switzerland*
[2] *Division of Experimental Pathology, Institute of Pathology, CHUV, Faculty of Biology and Medicine, University of Lausanne, Rue du Bugnon 25, 1005 Lausanne, Switzerland*

Correspondence should be addressed to Heinz Gehring, gehring@bioc.uzh.ch

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Ewing sarcoma (EWS) proto-oncoprotein, an RNA-binding protein, is involved in DNA recombination and repair, gene expression, RNA processing and transport, as well as cell signalling. Chimeric EWS oncoproteins generated by chromosomal translocations between *EWSR1* and the genes of transcription factors cause malignant tumors. To understand the loss of function by these translocations, the role of the intact EWS protein has to be investigated. The predominantly nuclear localization of the EWS protein via a transportin-1-mediated mechanism is dependent on the recently identified C-NLS (also known as PY-NLS). Among other residues in the C-NLS, Y656 interacts with transportin-1 and is essential for its nuclear localization. Here, we show that Y656 is phosphorylated, which seems to be a critical factor for transportin-1-mediated nuclear import. If Y656 was mutated cytosolic aggregates of the EWS protein, colocalized with transportin-1, were observed, similar to those described with mutants of the closely related FUS/TLS protein that had amino acid substitutions in the PY-NLS causing familial amyothrophic lateral sclerosis.

1. Introduction

The EWS protein is mainly located in the nucleus, accumulated in Cajal bodies and central regions of nucleoli, but it is also present in cytoplasm and associated with cell membrane [1, 2]. We have identified and characterized a nuclear localization and retention signal at the C-terminus of the EWS protein (C-NLS) (Figure 1(a)), which assures nuclear accumulation of the protein [3]. The EWS protein has been shown to be a ligand of transportin-1, a mediator in nucleocytoplasmic protein transport, among many others, including related RNA-binding proteins such as FUS/TLS, hnRNP A1, hnRNP M, and Sam68 [4–6]. The C-NLS of the EWS protein has been classified as PY-NLS, a consensus sequence recognized by transportin-1 [5]. R648, R652, P655 and Y656 have been found to be essential residues in the C-NLS for the nuclear transport of the EWS protein [3].

Brk (breast tumour kinases) phosphorylate tyrosine residues present in the NLS of Sam68 [7], which is highly homologous to that of the EWS protein (Figure 1(a)). Y440 of Sam68 corresponds to Y656 of the EWS protein. The residues P and R at position -1 and -4 (from Y) correspond completely, and both proteins have positive charges at position -2 (H/R) and -8 (K/R) as well as a negative charge at -3 (E/D). Phosphorylation and dephosphorylation regulate subcellular localization of numerous proteins [7]. In the present study, we investigated and found that Y656 in the EWS protein occurs in a phosphorylated state and if phosphorylation is abolished, it accumulates in the cytosol colocalized with transportin-1.

2. Results and Discussion

Expression of the EWS-YFP fusion protein resulted exclusively in nuclear accumulation, with high concentration in nucleoplasmic speckles and a fraction in the subnuclear central region (Figure 1(b)), thereby interacting with particular proteins such as the RNA helicases p72 and 68 [8].

SAM68 412 GQDDWNGTRPSLKAPPARPVKGAYREHPYGRY 443

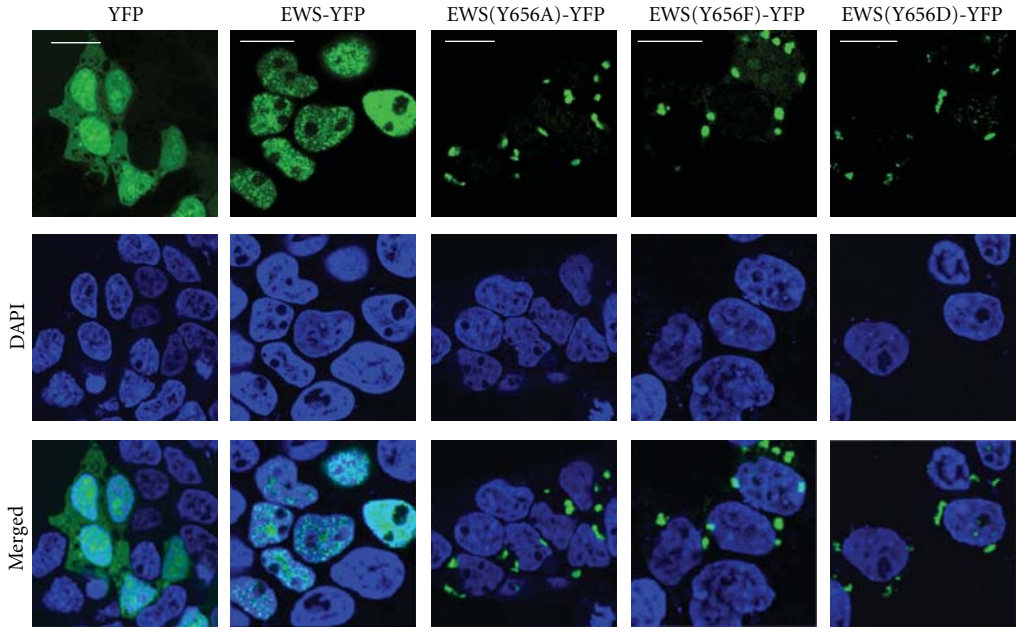
EWS 639 PGKMDKGEHRQERRDRPY 656

FUS 498 RGGGDRGGFGPGKMDSRGEHRQDRRERPY 526

hnRNP A1 316 GNYNNQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY 357

hnRNP M 38 GEGERPAQNEKRKEKNIKRGGNRFEPY 64

(a)



(b)

Figure 1: (a) Sequence alignment of PY-NLSs. The homologous regions of C-NLS of the EWS protein, NLS of Sam68 and FUS/TLS protein and M9 NLS of hnRNP A1 and hnRNP M, classified as PY-NLS are in yellow boxes. Phosphorylated Y656 of the EWS protein and Y440 of Sam68 are indicated (in red). Positions of identical residues in SAM68 and EWS C-NLS are indicated in bold and residues with identical charges are underlined. Known positions of the FUS/TLS mutations in ALS are in bold. (b) Subcellular localization of YFP, the C-terminally tagged EWS-YFP, EWS(Y656A)-YFP, EWS(Y656F)-YFP, and EWS(Y656D)-YFP (in green). Nuclei are shown by DAPI staining (in blue). Bars, 15 μm.

YFP alone is diffusively distributed between the nucleic and cytoplasmic compartment. Single amino acid substitutions of the C-terminal Y656 by alanine, phenylalanine, and aspartic acid revealed a drastic redistribution of the EWS protein with cytoplasmic accumulations in the perinuclear region (Figure 1(b)). The resulting cytoplasmic aggregation pattern demonstrates that none of these amino acids could successfully substitute the tyrosine residue. Phenylalanine substitution does not fulfill the function of the Y656, implying the importance of the hydroxyl group of tyrosine. Thus, a possible phosphorylation of this amino acid residue in nuclear import function seems likely. However, not even an aspartic acid, which, due to its negative charge, is often used as phosphomimetic of phosphorylated residues, could restore the nuclear localization of the protein.

To demonstrate a possible phosphorylation of Y656, GFP-Zf protein was constructed by fusion of His-GFP with a part of EWS protein (aa 525–656). This part of the C-terminal RNA-binding domain of the EWS protein consists of the Zinc finger (Zf) motif followed by the arginine-glycine rich box 3 (RGG3) and C-NLS (Figure 2(a)). This fragment of the EWS protein (aa 525–656), hereafter called Zf, contains Y656 as the only tyrosine residue and at the same time is large enough to avoid diffusive nuclear import of the GFP-Zf fusion protein. Additionally, the construct GFP-Zf(Y656A), having alanine as the single amino acid substitution for Y656, was produced as a negative control. GFP-Zf shows the subcellular localization pattern of the full-length EWS protein. However, its subnuclear partition is different from that of the full-length protein, as previously described [3] (Figure 2(b)). GFP-Zf(Y656A) reveals the characteristic cytoplasmic distribution as is typical for Y656 mutations of the full-length EWS protein (Figure 2(b)). To show that the cytoplasmic accumulations of the GFP-Zf(Y656A) are not aggregation and precipitation of an insoluble mutant protein, but the result of specific inactivation of NLS

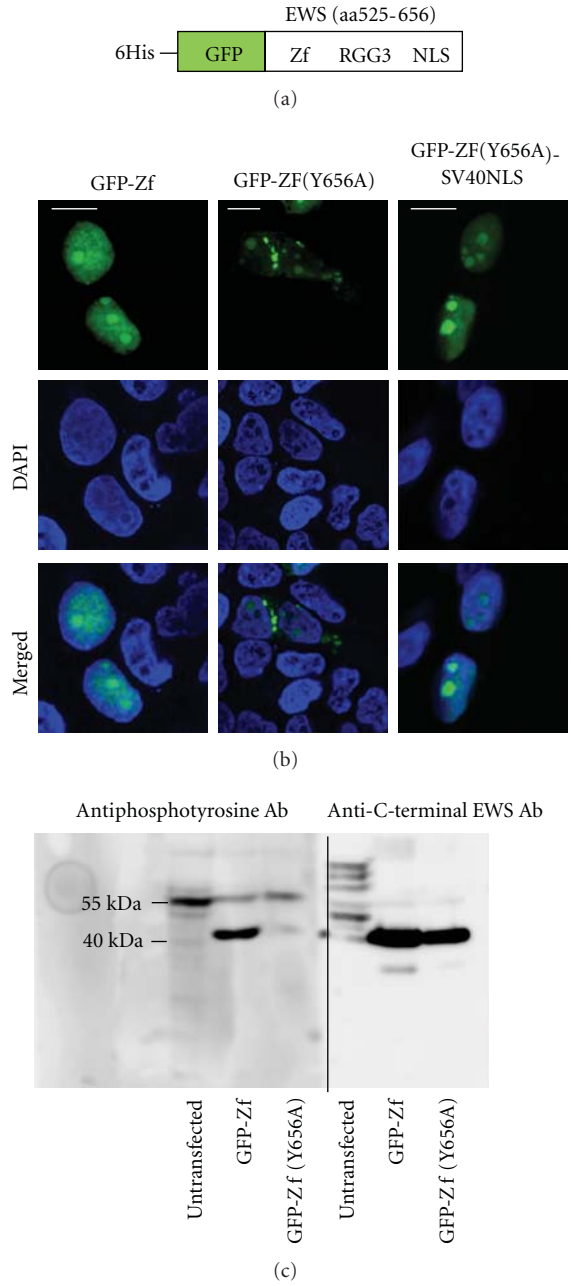


Figure 2: (a) Schematic representation of the N-terminally tagged GFP-Zf fusion protein. 6His-GFP is fused to the EWS mutant protein (aa 525–656). (b) Representative examples of the subcellular localization (all cells expressing the construct are showing the indicated localization) of GFP-Zf, GFP-Zf(Y656A), and GFP-Zf(Y656A)-SV40NLS (in green). Nuclei are shown by DAPI staining (in blue). Bars, 15 μm. (c) Phosphorylation analysis of GFP-Zf and GFP-Zf(Y656A) using antiphosphotyrosine antibodies. GFP-Zf and GFP-Zf(Y656A) fusion protein bands are detectable at ~40 kDa. The additional phosphorylated protein band detected at ~55 kDa was identified as p54nrb.

function, GFP-Zf(Y656A) was fused with the canonical SV40 NLS. GFP-Zf(Y656A)-SV40NLS shows complete nuclear localization similar to the GFP-Zf without any detectable cytoplasmic aggregates (Figure 2(b)).

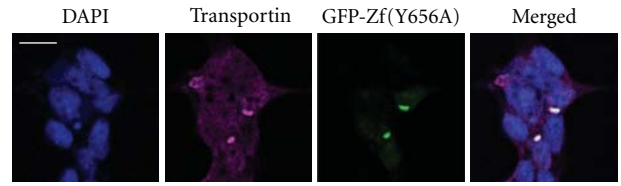


Figure 3: Colocalization of transportin-1 (in magenta) and GFP-Zf(Y656A) (in green). Nuclei are shown by DAPI staining (in blue). Bars, 15 μm. Representative examples of the subcellular localization are shown (all cells expressing the constructs are showing the indicated localization).

GFP-Zf and GFP-Zf(Y656A) fusion proteins containing 6His-tag at the N-terminus of GFP were expressed in eukaryotic HEK 293(T) cells, extracted, subjected to SDS-PAGE, and analyzed on Western blots by using antiphosphotyrosine antibody. Phosphorylation was detected in GFP-Zf (band at ~40 kDa) but not in GFP-Zf(Y656A) and not in the untransfected sample (Figure 2(c)). The Western blots with the same samples but with anti-C-terminal EWS antibody, that recognized both fusion proteins, demonstrate similar expression level of both proteins (Figure 2(c)). The multiple protein bands detected by anti-C-terminal EWS antibody in the lysate of untransfected HEK 293(T) cells reflect different degradation fragments of endogenous EWS protein (unpublished observations). Phosphorylation of a protein at 55 kDa was also detected. This protein was identified by mass spectrometry as the nuclear RNA- and DNA-binding protein p54nrb. Whatever reasons might be responsible for binding to the resin (possibly endogenous histidine residues located in close proximity), p54nrb can serve here as an internal standard for equal loading.

As nuclear import of the EWS protein is mediated by transportin-1 [5], we have visualized the subcellular localization of transportin-1. In cells expressing GFP-Zf(Y656A), transportin-1 colocalizes with the mutant GFP-Zf(Y656A) in cytoplasmic accumulations, apart from its characteristic homogeneous nucleocytoplasmic distribution (Figure 3). Conceivably, the part of transportin-1 bound to GFP-Zf(Y656A) is spatially restricted to these cytoplasmic structures and cannot fulfill its functions in nucleocytoplasmic transport due to the missing phosphorylated tyrosine. This finding indicates that phosphorylation is not required for transportin-1 binding which is in accordance with previous data showing that unphosphorylated PY-NLS of EWS or the mutated peptides containing Y656A still bind recombinant transportin-1 [5]. Why are these mutations then causing loss of nuclear import function? Possibly, binding in the absence of a phosphorylated C-NLS cannot induce a conformational change in transportin-1, which might be essential for recruiting or binding to other partners in the nuclear import process and leads, thus, to cytoplasmic accumulations of the transportin-1-EWS complex.

Recently, mutations in FUS/TLS have been shown to be responsible for familial amyotrophic lateral sclerosis (ALS), and mutants of the FUS/TLS protein accumulate in the cytoplasm of cortical neurons and lower motor neurons in the brain of ALS patients [9, 10]. These results indicate that

mutant FUS/TLS has a tendency to be insoluble compared to the wild type. Remarkably, mutations are found in the C-NLS (PY-NLS) of FUS/TLS (Figure 1(a)), and the cytoplasmic accumulation is similar with that of the EWS mutant GFP-Zf(Y656A). Our present data indicate that these cytoplasmic aggregations (or, alternatively, accumulations of protein complexes) of FUS/TLS are formed, as in case of the EWS mutants, due to a disturbed nuclear import leading to an increased cytoplasmic concentrations of these proteins. In the normal steady state, the predominant amount of these proteins is transported into the nucleus. Knowledge about the mechanisms of nuclear import, including the role of tyrosine phosphorylation for the function of C-NLS, might have an impact particularly in better understanding the pathogenesis of ALS in order to be able to develop a strategy for its treatment. In addition, it is of interest to confirm the presence of transportin-1 in these cytoplasmic aggregations and to further test the functionality and the role of this restricted protein in progression of the disease.

It is possible that different kinases are able to phosphorylate the EWS protein individually in a cell cycle-dependent and a cell compartment-dependent manner, as it has been found with Sam68 containing PY-NLS. Sam68 is phosphorylated in the nucleus by Brk (breast tumour kinases) and at the cell membrane by Src kinases [7]. Remarkably, kinases of the Src-subfamily are localized in the cytoplasm and can be bound, due to N-terminal myristoylation, to the inner face of the plasma membrane [11], where the EWS protein has also been found [2, 12]. The members of the Brk family, related to the Src family, lack a myristoylation site, and cytoplasmic and nuclear localization is more typical for these kinases [13, 14]. Tyrosine kinases, known to interact with the EWS protein, are Pyk2 and Bruton tyrosine kinase, as well as Lck, a member of Src kinases [15, 16]. Src kinases and Pyk2 recognize a similar tyrosine phosphorylation motif, which suggests that the latter might phosphorylate Y656 of the EWS protein. Conceivably, tyrosine phosphorylation might regulate, apart from its role in nucleocytoplasmic transport, interactions with other proteins or with RNA, as was observed with the related Sam68 and QKI [7, 13, 17].

Although phosphorylation of Y656 in the EWS protein seems to be essential, it might not be the exclusive regulating factor for nuclear localization, since other known or predicted mechanisms can be recruited to cooperatively or sequentially control nucleocytoplasmic distribution of a particular protein. SUMO-ylation, another reversible post-translational modification of Sam68 [18] and of hnRNP M, has been described to play a role in nucleocytoplasmic shuttling of mRNA-binding protein complexes [19]. Remarkably, the SUMO-ylation motif GKMD is predicted with high probability also in the C-NLS region of the EWS protein (http://us.expasy.org/, SUMOplot) and is a potential subject for further investigation.

3. Materials and Methods

3.1. Expression Constructs. The eukaryotic vectors for expression of the EWS-YFP, EWS(Y656A)-YFP, and EWS(Y656F)-YFP fusions were constructed as described [3]. To produce the EWS(Y656D)-YFP mutant, the Y656D reverse primer was used. The vectors for expression of His-GFP-Zf and His-GFP-Zf(Y656A) fusions were constructed as follows. For construction of pcDNA3.1(−)B-Zf and pcDNA3.1(−)B-Zf(Y656A) expression vectors, Zf and Zf(Y656A) fragments were amplified by traditional PCR using *EWSR1* cDNA as template and Zf-XhoI-NotI-forward and EcoRI-stop-reverse or Y656A-stop-reverse primers 5′-TCTCTCGAGCGGCCG-CGCCACCATGAATCCGGGTTGTGGAAACCAGAA-3′ and 5′-CCGAATTCTCAGTAGGGCCGATCTCTGCGC-TCCTG-3′, or 5′-CCGAATTCTCAGGCGGGCCGATCT-CTGCGCTCCTG-3′, respectively. The PCR products were treated with restriction enzymes XhoI and EcoRI and subcloned into pcDNA3.1(−)B/myc-His (Invitrogen) to generate in-frame fusions. Finally, the His-GFP-Zf and His-GFP-Zf(Y656A) vectors were constructed by amplification of the His-GFP fragment from EWS-Myc-6xHis/pEGFP-N2, a derivative of pEGFP-N2 plasmid (Clontech) using the NheI-His-forward and NotI-GFP-reverse primers 5′-TCTGCTAGCGCCACCATGGCCGTC-GACCATCATCATCATCATCAT-3′ and 5′-TGCGTC-GCGGCCGCTCTTGTACAGCTCGTCCATGCCGAG-3′, respectively, and digestion with the appropriate restriction enzymes, and by cloning the resulting product into the pcDNA3.1(−)B-Zf and pcDNA3.1(−)B-Zf (Y656A) plasmids. To obtain His-GFP-Zf(Y656A)-SV40NLS, His-GFP-Zf(Y656A) vector was fused with an NLS from SV40 large T antigen (PKKKRKV). The oligonucleotides 5′-AAT-TCCCAAAAAAGAAGAGAAAGGTCAGG-3′ and 5′-AGC-TTCTGACCTTTCTCTTCTTTTTTGGG-3′ were annealed, and the resulting fragment was digested with restriction enzymes EcoRI-HindIII and was inserted into His-GFP-Zf(Y656A).

3.2. Cell Culture and Transfections. Human embryonic kidney (HEK) 293 (T) cells, kindly provided by Professor P. Sonderegger (Department of Biochemistry, University of Zurich, Switzerland), were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Life technologies) and 1% (w/v) each of penicillin and streptomycin (Life technologies) in a humidified 10% CO_2 atmosphere at 37°C. For visualization analysis, HEK 293 T cells were cultured on glass cover slips to 40% confluency and transiently transfected with mammalian expression constructs using the method of calcium phosphate precipitation. For protein visualization, the cells were fixed 24 h after transfection with 4% paraformaldehyde (PFA) for 5 min and stained with 4′,6-diamidino-2-phenylindol (DAPI) (Roche). Cover slips were mounted using Vectashiled medium (Vector) onto glass slides, and the cells were analyzed by fluorescence and confocal microscopy.

3.3. Antibodies, Protein Purification, and Western Blotting. Primary rabbit anti-C-terminal EWS antibody SE 680, kindly provided by Dr. O. Delattre (Institut Curie, Pathologie Moléculaire des Cancers, Paris Cedex), was used with

1 : 5000 dilution. Primary mouse anti-transportin-1 antibody (TNPO1, ab10303, Abcam) was kindly provided by Professor I. Stamenkovic (Department of Experimental Pathology, Institute of Pathology, CHUV, Lausanne, Switzerland). Primary rabbit antiphosphotyrosine antibody (Zymed) was used with 1 : 2000 dilution. The results were confirmed by using primary mouse antiphosphotyrosine antibody (P-Tyr-100, Cell Signaling) with 1 : 2000 dilution (data not shown). The secondary goat antirabbit and goat antimouse antibodies, respectively, coupled to horseradish peroxidase (Sigma) were used with 1 : 2000 dilution. His-GFP-Zf and His-GFP-Zf(Y656A) protein purification by His-SELECT Nickel affinty gel (Sigma) was performed as described [2]. In all the purification buffers, sodium orthovanadate was added as protein phosphatase inhibitor.

Western blotting was performed as described [12] with some modifications. In case of phosphotyrosine detection, 3% BSA instead of nonfat milk powder was used as blocking agent and in antibody solutions.

3.4. Confocal Microscopy. Laser-scanning confocal fluorescence microscopy was performed using a Leica SP2 AOBS UV CLSM microscope and HCX PL APO lbd.BL 63.0x NA 1.40 OIL UV objective. Images were acquired using excitation wavelengths of 405 nm and 514 nm and the emission wavelengths of 470 nm and 528 nm for DAPI and YFP, respectively. Images were captured digitally using Leica software and processed using Adobe Photoshop 8.0. The stacks of images were imported into Imaris (Bitplane) software for the 3D rendering of the images.

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Review Article

Dr. Jekyll and Mr. Hyde: The Two Faces of the FUS/EWS/TAF15 Protein Family

Heinrich Kovar

Children's Cancer Research Institute, St. Anna Kinderkrebsforschung, 1090 Vienna, Austria

Correspondence should be addressed to Heinrich Kovar, heinrich.kovar@ccri.at

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FUS, EWS, and TAF15 form the FET family of RNA-binding proteins whose genes are found rearranged with various transcription factor genes predominantly in sarcomas and in rare hematopoietic and epithelial cancers. The resulting fusion gene products have attracted considerable interest as diagnostic and promising therapeutic targets. So far, oncogenic FET fusion proteins have been regarded as strong transcription factors that aberrantly activate or repress target genes of their DNA-binding fusion partners. However, the role of the transactivating domain in the context of the normal FET proteins is poorly defined, and, therefore, our knowledge on how FET aberrations impact on tumor biology is incomplete. Since we believe that a full understanding of aberrant FET protein function can only arise from looking at both sides of the coin, the good and the evil, this paper summarizes evidence for the central function of FET proteins in bridging RNA transcription, processing, transport, and DNA repair.

1. Introduction

The *Strange Case of Dr. Jekyll and Mr. Hyde,* a novel by the Scottish poet Robert Luis Stevenson (1850–1894), screened multiple times worldwide, describes the struggle between the good and evil sides of one individual [1]. At daylight, Dr. Jekyll is an honorable member of the society, but when the light fades he turns into an evil beast. The coexistence of two faces of one individual has inspired more than poetry and psychology. The question of which circumstances favor the surfacing of one or the other and how it may be influenced is relevant to all areas of life, including economy, technology and medicine. Cancer unravels the "Hyde" side of genes and their biology, but we can learn about how to tame fierce Mr. Hyde by understanding the Dr. Jekyll behind, the normal function of cancer genes.

FET (FUS, EWS, TAF15) proteins are a ubiquitously expressed family of similarly structured proteins predominantly localizing to the nuclear [2]. *FET* genes have attracted broad attention since all known members are found involved in deleterious genomic rearrangements with transcription factor genes in a variety of human sarcomas and acute leukemias. Chimeric FET proteins are considered and mostly studied as aberrant transcription factors. This paper aims at summarizing the good sides of FET proteins and looking at the characteristics of aberrant FET proteins as Dr. Jekyll's second face which surfaces only upon gene rearrangement or mutation.

2. Dr. Jekyll

2.1. The FET Family of Proteins. The prototype FET protein EWS was identified in 1992 as the gene product encoded by the Ewing's sarcoma breakpoint region 1 (*EWSR1*) on chromosome 22q12 constituting the first identified member of a family of putative RNA-binding proteins [3], including also FUS/TLS/Pigpen/hnRNP P2 [4–7], TAF15/hTAF$_{II}$68/TAF2N/RPB56 [8, 9], and Drosophila Cabeza/SARFH [10, 11] that share distinct structural characteristics (Figure 1). This protein family is frequently referred to as the FET (previously TET) (FUS/TLS, EWS, TAF15) family of proteins. Our restricted knowledge about the molecular functions of FET proteins derives mainly from protein interaction studies which identified more than 30 associated proteins mostly as part of protein/RNA complexes [12] (Table 1). Of note, pull-down experiments using EWS as bait revealed that all three FET proteins interact with each

other and are therefore likely to be part of the very same protein complexes. As demonstrated for EWS, the association with most interacting proteins depends on the presence of RNA and is destroyed upon RNaseA treatment (Table 1). The functional roles of interacting proteins suggest a general bridging role for FET proteins coupling RNA transcription, processing, transport, and DNA repair.

2.2. RNA Binding of FET Proteins. Several functional FET domains were defined (see Figure 1): the N-terminal domain is largely composed of a highly repetitive primary sequence containing multiple copies of a degenerate hexapeptide repeat motif similar to the C-terminal domain of RNA polymerase II. The C-terminal domain (CTD) contains a conserved nuclear import and retention signal (C-NLS) [13], a putative zinc-finger domain, and a conserved RNA recognition motif (RRM) flanked by 3 arginine-glycine-glycine (RGG) boxes [14] compatible with RNA binding of FET proteins. FUS has been demonstrated to bind preferentially to GGUG-containing RNAs [15]. EWS might have similar sequence specificity since it was demonstrated to bind strongly to both poly G and poly U, but not to poly A and poly C RNA, homopolymers [16]. Although it is only the zinc finger domain of FUS that makes physical contact with the GGUG motif, all three RGG boxes together with the RRM contribute to this activity [15]. Intriguingly, a recent study identified strong binding of FUS to human telomeric RNA [17] and to small low-copy-number RNAs tethered to the promoter of cyclin D1 [18]. Nothing is known about the RNA binding specificity TAF15.

2.3. A Role for FET Proteins in RNA Transcription. The FET N-terminal domain (NTD) resembles the activation domain of certain transcription factors such as SP-1 rich in glutamine and proline residues. When fused to a DNA-binding domain (DBD), as is the case in oncogenic FET derivatives, the NTD strongly activates reporter gene activity in a DNA-binding-dependent way [19–23]. The critical determinants for this transactivation activity are dispersed throughout the NTD [24], which is intrinsically disordered [25]. It is comprised of a variable number of a degenerate hexapeptide repeat motif (DHR) with the consensus SYGQQS, with homologies to the C-terminus of RNA polymerase II [3]. Mutation analysis of the EWS NTD revealed a critical dependence of the transactivation activity on the aromatic side chain of the conserved tyrosine residue present in the DHR [21].

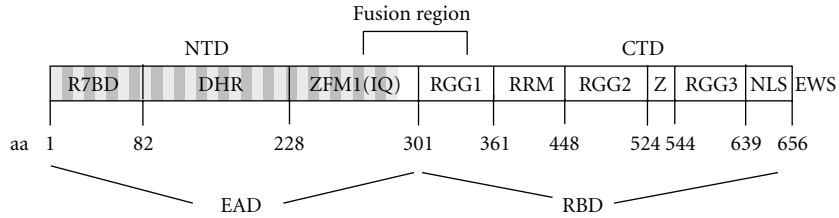
The function of the NTD in the context of germline FET proteins remains largely unexplored. When included into artificial FET-DBD fusion proteins, the CTD inhibited transcriptional activation by the NTD [26]. More recent data demonstrated that the RGG motifs of the FET-CTD repress a range of transcriptional activation domains [27]. The context-dependent difference in the transactivation potential of the NTD might be explained by different structures and accessibility of the NTD for protein interactions in the presence and absence of the CTD [28]. Protein interaction between the very N-terminus of EWS and the RNA PolII holoenzyme component hsRPB7 was only observed for EWS-FLI1 and C-terminal truncated EWS, while interaction with hsRPB5 and hsRPB3 was restricted to germline EWS [29, 30]. EWS has been reported to support CREB-binding-protein-(CBP/p300-) dependent activation by the transcription factors HNF-4 and OCT-4 [31, 32] which is inhibited by the EWS-interacting protein STRAP (serine-threonine kinase receptor-associated protein) [33]. Similarly, FUS acts as a positive cofactor for NFkappaB-mediated transcription [34]. In contrast, EWS repressed BRN3A-dependent transcription [35].

All three FET proteins were found to associate with RNA polymerase II and subpopulations of the $TF_{II}D$ complex, respectively [8, 29, 36]. Consistent with an evolutionary conserved role of FET proteins in RNA transcription, SARFH was found to be associated with transcribed chromatin in Drosophila [10]. Interactions of the NTD with various transcription factors were described (FUS with steroid, thyroid hormone, and retinoid receptors [37], EWS with Brn3A and via CBP/p300 with HNF4 and OCT4 [31, 32, 35, 38]). Interestingly, EWS and FUS were found to bind directly to the proximal elements of the macrophage-specific promoter of the CSF-1 receptor (*CSF1R*) gene and also to high-affinity sites recognized by myeloid zinc finger protein 1 (Mzf1) suggesting a role in transcriptional start site selection of TATA-less promoters [39].

Besides their role in RNA-polymerase-II-mediated transcription, the recent finding of FUS repressing RNA polymerase III-dependent transcription of small untranslated RNAs implies a more general role for FET proteins in the orchestration of the transcriptome [40].

2.4. A Role for FET Proteins in mRNA Maturation. The RNA-binding specificity of FUS for the GGUG motif found in 5′splice sites suggests a role in RNA processing. EWS and FUS were identified within the same RNA-splicing complex together with polypyrimidine-tract-binding-protein-associated factor (PSF) [41]. In addition, EWS and FUS associate with a variety of splicing factors such as U1C, SR, SF1, and YB1 [15, 42–47]. Further, EWS NTD and FUS bind to novel RNA helicases [48, 49]. Moreover, interaction of the EWS NTD with BARD1, a protein playing an important role in the inhibition of RNA maturation at sites of stalled transcription upon DNA damage, was reported [31, 50–53]. Together, these results suggest that FET proteins couple RNA transcription to processing. The mechanism and specificity of this activity remain largely unknown.

2.5. A Role for FET Proteins in the Processing of Small Noncoding RNAs. EWS was recently identified in a protein complex with the nuclear RNase III DROSHA [54]. While DROSHA is known to be central to the cleavage of the pre-micro-RNA (miRNA) precursor from the primary miRNA transcript thereby initiating miRNA processing and transport to the cytoplasm, evidence for a functional role of the EWS containing DROSHA complex is missing. Therefore, a general role for EWS in the metabolism of noncoding RNAs remains to be demonstrated. Since about a quarter of miRNA genes are encoded in the introns of protein-coding



NTD:	N-terminal domain
CTD:	C-terminal domain
R7BS:	R7BS: RPB7-binding domain
ZFM1(IQ):	IQ domain binding to ZMF1
RGG1, 2, 3:	Arginine/Glycine/Glycineboxes 1, 2, 3
RRM:	RNA recognition motif
Z:	Zinc finger domain
NLS:	Nuclear localization signal
EAD:	EWS activation domain
RBD:	RBD: RNA-binding domain
Fusion region:	Domain disrupted by fusion to transcription factor moieties when EWS is rearranged
DHR:	Degenerate hexapeptide repeats

Figure 1: Structure of the prototype FET protein EWS.

genes [55], it is intriguing to speculate that EWS links not only transcription to RNA splicing but also to the generation of miRNAs from gene introns. This, so far hypothetical, activity may gain importance in the light of frequent negative posttranscriptional regulation of miRNA processing at the DROSHA level in cancer [56].

2.6. A Role for FET Proteins in RNA Transport. Consistent with their proposed function in gene regulation, FET proteins are mostly nuclear, localizing to inclusions such as the coiled body and the nucleolus (demonstrated for EWS, FUS, and pigpen in [9, 57, 58]). There is also evidence that FET proteins shuttle between the nucleus and the cytoplasm raising the possibility that they play a role in RNA transport [59, 60]. In mouse hippocampal neurons, FUS is localized to neuronal dendrites and, upon activation, translocate to the spines, where local translation takes place, carrying along specific mRNA transcripts [61, 62]. This finding implicates FET proteins in localizing cytoplasmic determinants for the local control of protein synthesis and secretion, at least in neurons.

For EWS, RNA binding, subcellular localization, and consequently transcriptional activity have been found to be regulated by extensive asymmetric dimethylation of the RGG motifs, mediated by protein arginine methyltransferases 1 and 8 (PRMT1, PRMT8) [67–70], which likely impacts on self-association of intact EWS required for nuclear localization [71, 72]. Extensively methylated EWS has even been identified on the cell surface [73]. So far, the functional relevance of these findings has yet to be determined.

2.7. A Role for FET Proteins in Genome Surveillance and DNA Repair. FUS deficiency in mice resulted in defective B-lymphocyte development and activation, high levels of chromosomal instability, and perinatal death [74]. EWS knock-out mice also displayed disrupted B-cell development and were extremely sensitive to ionizing radiation. Together with a defect in homologous recombination impairing meiosis and the observation of premature senescence of embryonic fibroblasts, these results suggest a role for EWS in recombination repair [75]. In the zebrafish, silencing of *EWS* genes during embryogenesis led to mitotic defects followed by p53-dependent apoptosis [76].

Consistent with the phenotype of FET deficiency in genetically modified mice, the interaction of EWS (and EWS-FLI1) with the BRCA1-associated ring finger domain protein BARD1 may point to a role of FET proteins in DNA double-strand break repair [53]. This hypothesis is strengthened by high genomic instability in FUS knock-out mice [74] and radiation sensitivity and impaired homologous recombination in EWS knockouts [75]. The recently discovered homologous DNA-strand-pairing activity of all four FET proteins may functionally contribute to this role [77].

Intriguingly, the RNA binding activity of FUS was reported to act as a sensor for DNA damage and to elicit transcriptional repression; as exemplified for cyclin D (*CCND1*) promoter regulation, DNA damage was demonstrated to induce the expression of single-stranded, low-copy-number ncRNA transcripts tethered to the 5′ regulatory regions of *CCND1* which recruit FUS and allosterically modify it to bind to and repress CREB-binding protein (CBP) and p300 histone acetyltransferase activities [18].

Activation of gene transcription by many, if not all, sequence-specific transcription factors requires DNA-topoisomerase-II-beta-dependent, transient, site-specific dsDNA break formation [78]. One may speculate that the proposed role of FET proteins in recombination repair is linked to their association with transcription initiation complexes at promoter regions.

Table 1: EWS interacting proteins: *not bound by methylated EWS; **not bound by methylated EWS upon RNaseA treatment.

RNase A sensitive	
hnRNP A0 [12]	Pre-mRNA processing, RNA metabolism, RNA transport
hnRNP A1 [12]	Pre-mRNA processing, RNA metabolism, and RNA transport may modulate splice site selection
hnRNP A2B1 [12]	Pre-mRNA processing, RNA metabolism, RNA transport
hnRNP A3 [12]	Regulation of age-related gene expression, binds to telomeric RNA
hnRNP A/B [12]	Binds to multiprotein editosome complex
hnRNP A18* [12]	Stabilization of transcripts, genotoxic stress response, translational activator, binds to 3′UTR
hnRNP D0 [12]	Regulation of mRNA stability
hnRNP F [12]	Binds G-rich sequences
hnRNP G [12]	Regulation of splice site selection, DNA double-strand break repair
hnRNP H [12]	Pre-mRNA alternative splicing regulation
hnRNP H2 [12]	Involved in Fabray disease and X-linked agammaglobulinemia
hnRNP H3 [12]	Early heat shock-induced splicing arrest
hnRNP Q [12]	RNA stability, translationally coupled mRNA turnover
Small nuclear ribonucleoprotein Sm D3 [12]	Pre-mRNA splicing and small nuclear ribonucleoprotein biogenesis, histone 3′-end processing
U1 small nuclear ribonucleoprotein A* [12]	First snRNP to interact with pre-mRNA for the subsequent binding of U2 snRNP and the U4/U6/U5 tri-snRNP
Splicing factor, arginine/serine-rich 1* [12, 44]	Accuracy of splicing and regulation of alternative splicing
Splicing factor, arginine/serine-rich 3* [12]	Putative proliferation-/maturation-associated RNA processing
Splicing factor, arginine/serine-rich 9* [12]	Constitutive splicing
RRM containing coactivator activator [12]	Activation/modulation of nuclear receptors
Tubulin alpha ubiquitous chain [12]	Scaffold for cell shape and organelle movement
Vimentin[1]	Organizer of a number of critical proteins involved in attachment, migration, and cell signaling
RNase insensitive	
Protein arginine N methyltransferase 1 [12]	Epigenetic regulation, signal transduction, DNA repair
Protein arginine N-methyltransferase 8 [63]	Localized at cell membrane
hnRNP M [12]	Splicing, selective recycling of immature GlcNAc-bearing, thyroglobulin molecules, potentially involved in signalling
hnRNP U [12, 43]	Binds double- and single-stranded RNA and DNA, binds pre-mRNA
FUS** [12]	This review
TAF15* [12]	This review
EWS* [64]	This review
RNA-dependent helicase p68 (DDX5) [12]	RNA-dependent ATPase, alteration of RNA secondary structure in splicing and translation initiation
RNA-dependent helicase p72 (DDX17) [12]	RNA-dependent ATPase, alteration of RNA secondary structure in splicing, and translation initiation
ATP-dependent RNA helicase A [12]	ATP-dependent unwinding of double-stranded RNA and DNA-RNA complexes, transcriptional regulation
ATP-dependent RNA helicase DHX36* [12]	Deadenylation and decay of mRNAs with 3′-UTR AU-rich elements
Elongation factor EF1 gamma [12]	Translation elongation, role in anchoring the translational complex to other cellular components
Elongation factor EF1 alpha [12]	Translation elongation, promotes aminoacyl-tRNA binding to ribosome
Dead box protein 3 X (DDX3X)* [12]	ATP-dependent RNA helicase
Tubuline beta-2 chain [12]	Scaffold for cell shape and organelle movement
RNA dependence unknown:	
RBP3 [29]	RNA Polymerase II component
TAF5 [29]	General transcription factor $TF_{II}D$ component
TAF7 [29]	General transcription factor $TF_{II}D$ component

Table 1: Continued.

RNase A sensitive	
TAF11 [29]	General transcription factor $TF_{II}D$ component
TAF13 [29]	General transcription factor $TF_{II}D$ component
Brn-3a [35]	Transcription factor
SF1 [47]	Splicing factor
YB1 [42]	Splicing factor
Survival motor neuron protein* [65]	Essential role in spliceosomal snRNP assembly in the cytoplasm and is required for pre-mRNA splicing in the nucleus
Serine threonine kinase receptor (STRAP) [33]	Inhibits transforming growth factor beta (TGF-beta) signaling
BARD1 [53]	DNA repair, mRNA maturation
Pyk2 [66]	Tyrosine kinase, signal transduction

3. Mr. Hyde

3.1. The Role of FUS in Neurodegenerative Disease. Point mutations of FUS have recently been found in a subset of patients with familial amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder destroying motoneurons [79, 80]. Previously, this disease has been associated with mutations in either superoxide dismutase 1 (SOD1) or TDP43 (43 kDa TAR DNA-binding domain protein). TDP43 is an essential nuclear RNA-binding protein that participates in transcriptional repression, exon splicing inhibition, and mRNA stabilization. The convergent phenotypes associated with FUS and TDP43 mutations suggest that they are part of the same machinery. In fact, TDP-43 and FUS were demonstrated to function in a biochemical complex to modulate expression of HDAC6, a recently identified mRNA substrate of TDP-43 [81].

3.2. The Oncogenic Function of FET Fusion Protein. The predominant type of *FET* gene aberrations is that of fusions to various transcription factor genes by which the FET RNA-binding domain is replaced by the DNA-binding domain of the transcription factor (Table 2). FET fusion proteins are capable of transforming cells in culture dependent on the cellular context. EWS-ETS fusions, for example, transform NIH3T3 and bone-marrow-derived mesenchymal progenitor cells, but not human or rat primary fibroblasts, mouse embryonic stem cells, or embryonic fibroblasts [102, 103]. The phenotype of tumors obtained in immunodeficient mice after transplantation of EWS-ETS-transformed NIH3T3 cells clearly differs from that obtained after transformation with other EWS-transcription factor fusions and resembles that of Ewing's sarcoma [104, 105]. In the xenograft model, the amino terminal portion of EWS, as well as FUS (and presumably also TAF15), is functionally interchangeable in the fusion protein, while the transcription factor moiety determines the tumor phenotype [7]. Functional interchangeability of the FET-NTD is also reflected in human sarcomas: both *EWS-CHOP* and *FUS-CHOP* characterize myxoid liposarcoma [5, 94], and *EWS-NR4A3* and *TAF15-NR4A3* are found in extraskeletal myxoid chondrosarcoma [106]. It was therefore hypothesized that FET fusion proteins affect differentiation programs by aberrant regulation of genes specifically recognized by the transcription factor DNA-binding moiety.

The best studied example in this respect is EWS-FLI1 in Ewing's sarcoma family tumors (ESFT). Using experimental knockdown of EWS-FLI1 in ESFT cell lines and comparison to primary tumours and normal tissues, signatures of the chimeric transcription factor on the ESFT transcriptome were defined [125, 130, 131]. An almost equal number of genes were found activated and repressed by EWS-FLI1. Of the approximately 600 to 800 significantly dysregulated genes, only a fraction is directly bound by EWS-FLI1 and many EWS-FLI1 bound genes do not show aberrant regulation (our unpublished observations). Over the years a number of directly EWS-FLI1-regulated genes have been characterized in ESFT (Table 3). It is interesting to note that almost all attempts to experimentally restore the presumed "normal" expression pattern of these targets in ESFT cell lines (by ectopic reexpression of EWS-FLI1 repressed genes and knockdown of EWS-FLI1-activated genes) resulted in reduced tumor cell growth in vitro and/or reduced tumorigenicity in vivo and in several cases enhanced chemosensitivity (Table 3). These results suggest that directly EWS-FLI1-regulated genes play essential roles in the establishment and/or the maintenance of the malignant phenotype of ESFT.

Functional annotation of EWS-FLI1-regulated genes revealed that activated genes primarily annotate to proliferation-associated functions, while genes involved in developmental and differentiation processes are predominantly repressed [131], suggesting that EWS-FLI1 suppresses differentiation of the enigmatic ESFT precursor cell. In fact, sustained silencing of EWS-FLI1 restores the potential of ESFT cells to differentiate along adipogenic, neuronal, and osteogenic lineages [132], a feature shared with mesenchymal stem cells (MSC). Conversely, ectopic EWS-FLI1 expression blocks the differentiation potential of MSC and imposes an ESFT-like phenotype on them [103, 133, 134]. Consistent with the role of EWS-FLI1 in the disruption of developmental differentiation processes is the finding of skeletal malformations in mice expressing transgenic EWS-FLI1 in the mesenchymal lineage [135]. Similarly, the FUS-ERG fusion found in human myeloid leukemia with the t(16;21) translocation was demonstrated to block terminal

Table 2: FET gene fusions in cancer. TF: transcription factor.

Phenotype	FET partner	TF partner	TF type	Ref.
ESFT				
(85%)	EWS	FLI1	ETS	[3]
(10%)	EWS	ERG	ETS	[82, 83]
(1%)	EWS	ETV1	ETS	[84]
(1%)	EWS	ETV4	ETS	[85, 86]
(1%)	EWS	FEV	ETS	[87]
(1%)	FUS	FEV	ETS	[25]
(1%)	FUS	ERG	ETS	[88]
ESFT-like	EWS	NFATC2	rel related	[89]
Askin-like, CD99 neg.	EWS	ZNF278	zinc finger	[90]
Bone sarcoma	EWS	POU5F1	pou	[91]
Mucoepidermoid carcinaoma	EWS	POU5F1	pou	[92]
Hidradenoma	EWS	POU5F1	pou	[92]
	EWS	PBX1	homeobox	[92]
Low-grade fibromyxoid sarcoma	FUS	CREB3L1	Leucine zipper	[93]
Myxoid liposarcoma	EWS	DDIT3	bZIP	[94]
	FUS	DDIT3	bZIP	[5]
Clear cell sarcoma	EWS	ATF1	bZIP	[95]
	EWS	CREB1	bZIP	[96]
Desmoplastic SRCT	EWS	WT1	zinc finger	[97]
Extraskeletal myxoid chondrosarcoma	EWS	NR4A3	nuclear receptor	[98]
	TAF15	NR4A3	nuclear receptor	[99]
AML	FUS	ERG	ETS	[100]
cALL, AUL	EWS	ZNF384	zinc finger	[101]
AML, ALL	TAF15	TAF15	zinc finger	[101]

differentiation of and confer a growth advantage to human myeloid progenitor cells [136].

Consistent with early in vitro data [19–21], activated genes showed an enrichment of ETS-binding motifs in their promoters while this motif was underrepresented in repressed genes [131]. This result suggests that gene repression regulating differentiation genes might be mediated by indirect mechanisms. One such mechanism involved in blocking osteogenic differentiation is interaction and interference of EWS-FLI1 with the master regulator of bone and cartilage development, RUNX2 [137]. RUNX2 was demonstrated to bind also to intact EWS and FUS [138]. A number of different transcription factor binding motifs overrepresented in the promoters of EWS-FLI1-repressed genes may be indicative of other protein interactions that remain to be defined. Additional mechanisms of gene repression downstream of EWS-FLI1 involve the activity of transcriptional repressors whose expression is upregulated by EWS-FLI1 such as NKX2.2 [139] or the epigenetic modifier EZH2 [120] and the regulation of microRNAs [140]. An alternative intriguing mechanism may involve the binding of EWS-FLI1 to microsatellites outside of promoter regions even at distances of several megabases from the transcriptional start sites [141–143]. While these elements can activate transcription when juxtaposed to a promoter, their activity and mechanism of action from distant sites remains elusive.

Interestingly, there is evidence that EWS and EWS-FLI1 form a fatal liaison in that genes targeted by the FLI1 DNA-binding domain encode for proteins that interact with the EWS N-terminal domain in both the intact EWS protein and the chimeric protein. This is the case for NR0B1, a protein known to form large complexes with the stem cell factors OCT3 and OCT4, as well as EWS [32, 144, 145]. Intriguingly, the translocation t(6;22)(p21;q12) found in some undifferentiated sarcomas and neoplasms of skin and salivary glands directly fuses *OCT4* to *EWSR1*. Among EWS-FLI1-repressed genes is also hsa-mir-145, a microRNA targeting OCT4 and other stem cell factors and feeding back on EWS-FLI1 expression [133]. These findings provide evidence that EWS and EWS-FLI1 form a functional network in the regulation of tumor cell stemness.

3.3. A Transcription-Independent Role for the EWS-FLI1 Fusion Protein. The first indication that malignant transformation by FET fusion proteins may involve functions other than direct transcriptional activation of target genes recognized by the DBD came from functional dissection of the EWS-FLI1 fusion protein in NIH3T3 transformation assays. These studies suggested that the minimal transforming and the minimal transcriptional activation domains can be separated from each other [146]. Specifically, the 83 N-terminal amino acids were sufficient to transform NIH3T3

Table 3: Validated direct EWS-FLI1 target genes.

EWS-FLI1 activated genes	Consequences of target suppression
Id2 [107]	*Not known*
GLI1 [108]	Reduced anchorage independent growth [109]
VEGF [110]	Decreased osteolysis [111]
STYXL1 [112]	Not known
PLD2 [113]	Inhibition of PDGF BB signalling
PTPL1 [114]	Reduced growth and increased chemosensitivity [114]
CAV1 [115]	Reduced anchorage independent growth, reduced tumorigenicity
GSTM4 [116]	Abrogation of oncogenic transformation, increased chemosensitivity [116]
NR0B1 [117, 118]	Abrogation of oncogenic transformation [119]
EZH2 [120]	Reduced anchorage independent growth, reduced tumorigenicity [120]
AURKA, AURKB [121]	Not known
Tenascin C [122]	Not known
EWS-FLI1 repressed genes	Consequences of target restoration
TGFBR2 [123]	Loss of tumorigenicity [123]
CDKN1A [124]	Inhibition of cell growth [124]
IGFBP3 [125]	Inhibition of cell growth and motility [126]
FOXO1 [127]	Not known
DKK1 [128, 129]	Decreased tumorigenicity [128]

cells when fused to the FLI1 DBD. Protein interactions with this domain were found to be context dependent [28–30]. In addition, residual transforming activity of EWS-FLI1 was retained even when the FLI1-DBD was destroyed, suggesting a DNA-binding-independent function for the oncogenic fusion protein [147, 148]. Also, EWS-FLI1 was shown to inhibit the CBP-dependent transcriptional activity of the retinoid acid (RA) receptor RXR desensitizing cells to the differentiation and apoptosis inducing activity of RA by a mechanism unrelated to DNA binding [38].

Protein interaction studies revealed that the EWS-NTD and the FUS-NTD in the context of their oncogenic fusion proteins communicate with the same RNA processing factors as in germline EWS [42–44, 46, 47, 53] but interfere with serine arginine protein (SR) and YB1-mediated splicing [42, 44, 45]. In addition, it was demonstrated that EWS-FLI1, but not EWS, interfered with heterogeneous nuclear ribonucleoprotein A1-dependent 5′ splice site selection in an in vivo E1A splicing assay [149]. This result might possibly be explained by a dominant negative effect of EWS-FLI1 on the RNA processing function of EWS that remains to be investigated. In fact, we have previously demonstrated that EWS-FLI1 can interact with its germline counterpart [64]. Importantly, mutational analysis of EWS-FLI1 revealed that the ability to affect pre-mRNA splicing coincided with transforming activity [149]. These results suggest a role for EWS-FLI1 in RNA processing. However, this role may not be regarded as transcription independent. A recent study of transcriptional elongation of the direct EWS-FLI1 target gene cyclin D1 (*CCND1*) revealed that both EWS and EWS-FLI1 stimulate transcription of the gene, but elongation by EWS-FLI1 is significantly slowed down in comparison to EWS. As a result, expression of the oncogenic splice isoform D1b is favoured over the splice isoform D1a [150]. So far it remains unknown how many genes may be affected by this or a similar phenomenon.

3.4. EWS-FLI1 and Disrupted Tumor Suppression. FET fusion proteins are aberrantly expressed transcription factors driving cell proliferation. As such they impose oncogenic stress on the cell triggering the p53 checkpoint [151]. ESFT escape the oncogenic stress imposed by EWS-FLI1 by modulating p53 activity. Two mechanisms for this oncogenic property of EWS-FLI1 have recently been described: interference with tumor suppressive NOTCH signalling pathway activity through transcriptional regulation of autocrine NOTCH ligand expression [152] and direct interaction with p53 [153]. It should be noted, however, that the ability of EWS-FLI1 to modulate p53 activity is tissue dependent. In fibroblasts, EWS-FLI1 was demonstrated to elicit a p53-mediated cell-cycle arrest [151]. Most other cell types do not tolerate EWS-FLI1 expression at all and die in response to ectopic expression of the chimeric oncogene (for review [102]). The only tissue permissive to the oncogenic properties of EWS-FLI1 identified so far is mesenchymal stem cells [103]. The tissue-specific factors that steer the p53 response into the one (growth arrest/apoptosis) or the other (escape from oncogenic stress) direction remain to be elucidated.

There is also evidence for EWS-FLI1 interfering with the other central tumor suppressor pathway in oncogenesis: although the mechanism still remains to be defined, knockdown of EWS-FLI1 in ESFT cells leads to pRB-1 hypophosphorylation [154].

4. Getting Hold of Mr. Hyde

The development of small molecule inhibitors of biological macromolecules, originally in the context of chromosome translocations, has been pioneered by research on receptor tyrosine kinases. Here, the design of smart molecules is guided primarily by crystallography and structure/function analyses of the target proteins. For FET fusion proteins, this approach is not feasible because of the intrinsic disorder of their structure. However, recent landmark studies provided proof of principle for successful interference with protein interactions of intrinsically disordered proteins [155]. Guided by a peptide aptamer screen, a small molecule mimetic was described that competes with RNA helicase A for interaction with the EWS N-terminus in the context of the EWS-FLI1 fusion protein and slowed tumor formation in mice [156]. There is evidence from protein interaction studies that the faces of the EWS N-terminus look different in the context of the wildtype protein (Dr. Jekyll) and the

transcription factor fusion protein (Mr. Hyde) [28–30]. Thus, there is hope that the evil culprit for the development and progression of several sarcomas and leukemias that is still hiding in the dark can be successfully targeted in the near future.

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Review Article

Copy Number Alterations and Methylation in Ewing's Sarcoma

Mona S. Jahromi,[1] Kevin B. Jones,[2,3] and Joshua D. Schiffman[1,3,4]

[1] *Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah School of Medicine, 2000 Circle of Hope, Salt Lake City, UT 84112, USA*
[2] *Department of Orthopaedics, Huntsman Cancer Institute, University of Utah School of Medicine, 2000 Circle of Hope, Salt Lake City, UT 84112, USA*
[3] *Center for Children's Cancer Research (C3R), Huntsman Cancer Institute, University of Utah School of Medicine, 2000 Circle of Hope, Salt Lake City, UT 84112, USA*
[4] *Division of Pediatric Hematology/Oncology, Huntsman Cancer Institute, University of Utah School of Medicine, 2000 Circle of Hope, Salt Lake City, UT 84112, USA*

Correspondence should be addressed to Joshua D. Schiffman, joshua.schiffman@hci.utah.edu

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Ewing's sarcoma is the second most common bone malignancy affecting children and young adults. The prognosis is especially poor in metastatic or relapsed disease. The cell of origin remains elusive, but the EWS-FLI1 fusion oncoprotein is present in the majority of cases. The understanding of the molecular basis of Ewing's sarcoma continues to progress slowly. EWS-FLI1 affects gene expression, but other factors must also be at work such as mutations, gene copy number alterations, and promoter methylation. This paper explores in depth two molecular aspects of Ewing's sarcoma: copy number alterations (CNAs) and methylation. While CNAs consistently have been reported in Ewing's sarcoma, their clinical significance has been variable, most likely due to small sample size and tumor heterogeneity. Methylation is thought to be important in oncogenesis and balanced karyotype cancers such as Ewing's, yet it has received only minimal attention in prior studies. Future CNA and methylation studies will help to understand the molecular basis of this disease.

1. Introduction

Ewing's sarcoma is a highly malignant tumor of children and young adults. The molecular mechanisms that underlie Ewing's sarcoma development are beginning to be understood, but the genetic risk factors leading to disease susceptibility remain largely unknown. Ewing's sarcoma is the second most common pediatric bone cancer after osteosarcoma, with 30–60% survival depending on tumor site and metastases at diagnosis [1, 2]. When patients with Ewing's sarcoma relapse, it is usually fatal: less than 20% survive [3–5]. Beyond incremental improvements in cytotoxic chemotherapy regimens, there have been no major treatment advances in the last 20 years [6, 7]. Clinical features are the only markers that have been found to correlate reliably with the outcome in Ewing's sarcoma, but no risk-adapted therapy has proven successful; worse prognosis in Ewing's is predicted by metastatic disease measured by imaging and bone marrow examination, larger tumor volume, and primary tumors in the pelvis [8]. While osteosarcoma is thought to originate from bone cell progenitors [9], the cell of origin of Ewing's sarcoma is less clear with some evidence suggesting that tumors arise from a mesenchymal stem or progenitor cells [10–12]. Other researchers in the field believe instead that Ewing's sarcoma develops from a neuroectodermal origin [13–17]. The lack of a known cell of origin contributes to the difficulty in understanding exactly how Ewing's sarcoma develops or even how to design laboratory experiments to study tumorigenesis.

Nearly every case of Ewing's sarcoma contains a translocation involving the *EWSR1* gene on chromosome 22. The most common rearrangement is t(11;22)(q24;q12), which generates the *EWS-FLI1* fusion oncogene, found in ~85% of Ewing's cases [18–21]. The translocation t(21;22)(q22;q12)

is found in another 10% of cases [21, 22] and the remainder of EWS translocations utilize a variety of fusion partners from the ETS family of transcription factors [19, 20]. All of the Ewing's sarcoma fusion proteins contain a strong transcriptional activation domain fused to a DNA-binding domain and function as aberrant transcription factors that dysregulate a number of target genes and contribute to oncogenic transformation [18–21, 23–30]. The EWS-FLI1 translocation is the best understood and most well-characterized molecular aspect of Ewing's sarcoma. This translocation (or one of the alternates) is thought to be necessary but not sufficient to cause disease [31].

In addition to translocations, neoplastic development in cancer depends on other acquired molecular changes. Such changes in tumor biology include copy number alterations (CNAs), such as genomic deletions or amplifications, and methylation abnormalities. As newer technology has become available in recent years, we have learned more about CNAs and methylation in Ewing's sarcoma and possible associations with outcome, disease classification, and tumorigenesis. These molecular investigations have been limited by the rarity of Ewing's sarcoma and the small tumor samples obtained at initial diagnostic biopsy available for analysis. Nevertheless, many overlapping regions of CNAs and methylation have been described; their underlying significance is not always clear. Further exploration as to how these changes affect the outcome and their prevalence is essential to the development of future treatment options. In this paper, we describe the reported CNAs and methylation changes associated with Ewing's sarcoma and any known clinical correlations with these molecular findings.

2. Materials and Methods

Literature searches for articles containing "Ewing's sarcoma copy number" and "Ewing's sarcoma methylation" were performed via the PUBMED database. Results consisted of 15 separate journal articles for copy number and 14 for methylation. Twelve relevant publications were selected for copy number and their references explored and included when appropriate. Nine relevant promoter methylation articles were selected and their references explored.

3. Results and Discussion

3.1. Ewing's Sarcoma and Copy Number Alterations (CNAs). Specific CNAs predict prognosis in several cancers and have been introduced as part of clinical risk stratification for colorectal and breast cancer, neuroblastoma, and brain tumors [32–35]. Despite intense investigation of Ewing's sarcoma biology, very few molecular markers have been discovered for routine clinical use in this disease. In contrast, active risk stratification based on molecular cytogenetics has increased the cure rate for childhood acute lymphoblastic leukemia (ALL) from less than 50% to over 85% in only a few decades [36]. Moreover, the use of high-resolution single nucleotide polymporphism (SNP) technology has been used to identify recurring CNAs in childhood leukemia [37–42] including relapsed cohorts [43, 44]. The study of CNAs in cancer also helps to better classify and understand the development of disease. For example, *CDKN2A* homozygous deletions in pediatric gliomas were recently found to significantly associate with specific $BRAF^{V600E}$ mutations, helping to define a new subset of tumors [45]; the same deletion and mutation were also shown to work together to promote glioma formation in mice, validating the cooperation between CNAs and mutations [46].

New genomic technology has proven effective in determining copy number changes in a variety of tissue types. Previously, DNA extracted from paraffin has been too degraded to yield reliable data for analysis, but a new molecular inversion probe assay (OncoScan, Affymetrix, Santa Clara, CA) has been used successfully to identify copy number changes in formalin-fixed paraffin-embedded (FFPE) samples [65]. The ability to now interrogate FFPE samples allows the analysis of archival tissues and increases sample sizes for future Ewing's sarcoma studies. Copy number assessment in combination with clinical data could be used to identify CNAs in archival tissue and determine their link to the outcome in Ewing's sarcoma. Moving forward, candidate loci could be further studied in vitro or in pre-clinical animal models to determine their contribution to drug resistance and tumor progression.

A large number of novel and recurrent secondary abnormalities in Ewing's tumors relating to copy number already have been discovered (see summary Table 1). The vast majority of copy number studies thus far in Ewing's sarcoma have been performed with comparative genomic hybridization (CGH) technology on either cell lines or primary samples. The most commonly reported CNAs in Ewing's sarcoma are trisomies of chromosomes 8 and 12 followed by the gain of 1q [47–49, 51–57, 66, 67]. Trisomy 8 is of particular interest as it occurs consistently and often in high frequency, being reported in $\geq$50% of cases in Ewing's sarcoma [47, 56, 57]. The oncogene, *MYC*, is thought to be a possible candidate driver of trisomy 8 as it was shown to bestow a selective advantage through nonfocal amplification when studied in undifferentiated soft tissue sarcomas [67]. In contrast to these findings, many studies have not found a statically significant link to survival outcomes in Ewing's sarcoma and trisomy 8 [47, 51, 54, 56]. Despite the lack of statistical significance, some evidence does suggest a link between trisomy 8 and worse outcome or worse overall survival. Values for 5-year distant disease-free survival ($P = .16$) and overall survival ($P = .39$) were not statistically significant, but the percentage of trisomy 8 was greater in both survival categories implicating a possible, though not statistically relevant, trend [54]. Focal amplifications in both the long and short arms of chromosome 8 (opposed to the entire trisomy) have been associated with clinical outcomes in Ewing's sarcoma. Specifically, Ozaki et al. reported that 8p amplifications occurred at higher frequency in relapsed cases compared to primary tumors ($P = .04$) [48]. They also found that combinations of CNAs, including 8q amplification in conjunction with chromosome 20 amplifications, were significant for worse cumulative overall survival rates ($P = .0065$) [48]. Savola et al. have proposed *WDR67* (8q24.13) and *GSDMD1* (8q24.3) as interesting candidate genes for

Table 1: Summary of copy number alternations (CNAs) in Ewing's Sarcoma (tumor, cell line, and xenograft) in published literature.

Deletion	Gain	Ewing's sample type	Frequency (%)	Technology	Study	Clinical significance
1p		ESFT	17/184 (9%)	Karyotyping and CGH	Hattinger et al. [47]; Ozaki et al. [48]	
1p36		ESFT	5/88 (6%)	Karyotyping (G-Band)	Roberts et al. [49]	
1p36.32-p36.11		ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	1q	ESFT	77/396 (19%)	Karyotyping and CGH	Armengol et al. [51]; Brisset et al. [52]; Hattinger et al. [47]; Ozaki et al. [48]; Roberts et al. [49]; Savola et al. [53]; Tarkkanen et al. [54]	(i) Adverse event free survival (ii) Adverse overall survival (iii) Age at diagnosis $\geq$15 years (iv) Metastatic (trend)
		Cell line	5/8 (63%)	CGH	Shing et al. [55]	
	1q21-q22	ESFT	5/28 (18%)	CGH	Tarkkanen et al. [54]	(i) Adverse overall survival (trend) (ii) Adverse 5-year distant disease-free survival (trend)
	2	ESFT	38/262 (15%)	Karyotyping and CGH	Brisset et al. [52]; Hattinger et al. [47]; Roberts et al. [49], Savola et al. [53]	(i) Localized disease
	2q	ESFT	12/62 (19%)	CGH	Ozaki et al. [48]	(i) Adverse overall survival
3p		Cell line	3/8 (38%)	CGH	Shing et al. [55]	
	4p	ESFT	10/105 (10%)	CGH	Brisset et al. [52]; Ozaki et al. [48]	(i) Relapse
	5	ESFT	28/231 (12%)	Karyotyping and CGH	Brisset et al. [52]; Hattinger et al. [47]; Roberts et al. [49]	
	5p	ESFT	5/25 (20%)	CGH	Ferreira et al. [56]	
	6p21.1~pter	ESFT	3/28 (11%)	CGH	Tarkkanen et al. [54]	(i) Adverse overall survival (ii) Adverse 5-year distant disease-free survival
	7	ESFT	26/216 (12%)	Karyotyping and CGH	Hattinger et al. [47]; Roberts et al. [49]; Tarkkanen et al. [54]	
	7p21.1-p11.2	ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
7q (partial)		ESFT	6/25 (25%)	CGH	Ferreira et al. [56]	
	7q	ESFT	5/28 (18%)	CGH	Tarkkanen et al. [54]	
	8	ESFT	197/413 (48%)	Karyotyping, CGH and FISH	Armengol et al. [51]; Brisset et al. [52]; Ferreira et al. [56]; Hattinger et al. [47]; Maurici et al. [57]; Ozaki et al. [48]; Savola et al. [53]; Tarkkanen et al. [54]; Zielenska et al. [58]	(i) Local recurrences (trend) (ii) Relapse (trend) (iii) Adverse overall survival (trend) (iv) Adverse 5-year distant disease-free survival (trend)
		Cell line	8/8 (100%)	CGH	Shing et al. [55]	
	8p	ESFT	30/62 (48%)	CGH	Ozaki et al. [48]	(i) Relapse
	8q	ESFT	32/62 (52%)	CGH	Ozaki et al. [48]	
	8q11.21-q22.3	ESFT	6/9 (67%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	8q24.11-q24.21	ESFT	7/9 (78%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	

Table 1: Continued.

Deletion	Gain	Ewing's sample type	Frequency (%)	Technology	Study	Clinical significance
9p		ESFT	7/31 (23%)	CGH	Savola et al. [53]	
9p21		ESFT	50/291 (17%)	Karyotyping, CGH, FISH, Southern Blot, SNP Microarray (Affy 100 K), and MLPA	Brownhill et al. [59]; Huang et al. [60]; Kovar et al. [61]; Neale et al. [50]; Roberts et al. [49]; Savola et al. [62]; Wei et al. [63]	(i) Adverse event free survival (trend) (ii) Adverse overall survival (iii) Axial (iv) progressive disease (trend) (v) Poor chemoresponse
9p21		Cell line	24/43 (56%)	CGH (Agilent 44 K and 244 K), Taqman qRT-PCR, FISH, Southern Blot and MLPA	Brownhill et al. [59]; Kovar et al. [61]; Savola et al. [62]	
9p21.3		Xenotransplant	4/12 (33%)	dPCR, FISH	López-Guerrero et al. [64]	
10		ESFT	12/87 (14%)	CGH	Ferreira et al. [56]; Ozaki et al. [48]	
	11p	ESFT	2/62 (3%)		Ozaki et al. [48]	(i) Relapse
	11q	ESFT	2/62 (3%)		Ozaki et al. [48]	(i) Relapse
	12	ESFT	104/434 (24%)	Karyotyping, CGH and FISH	Armengol et al. [51]; Brisset et al [52]; Ferreira et al. [56]; Hattinger et al. [47]; Maurici et al. [57]; Roberts et al. [49]; Savola et al. [53]; Tarkkanen et al. [54]; Zielenska et al. [58]	(i) Adverse event free survival (ii) Adverse overall survival (iii) Relapse (trend)
	12p	ESFT	12/62 (19%)	CGH	Ozaki et al. [48]	(i) Adverse overall survival
	12q	ESFT Cell line	11/62 (18%) 6/8 (75%)	CGH CGH	Ozaki et al. [48] Shing et al. [55]	(i) Adverse overall survival
	12q14.1-q15	ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	14	ESFT	11/143 (8%)	Karyotyping and CGH	Brisset et al. [52]; Hattinger et al. [47]	
	14q11.2	ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	15	ESFT	4/43 (9%)	CGH	Brisset et al. [52]	
	16p	ESFT	2/28 (7%)	CGH	Tarkkanen et al. [54]	
16q		ESFT	69/396 (17%)	Karyotyping and CGH	Brisset et al. [52]; Ferreira et al. [56]; Hattinger et al. [47]; Ozaki et al. [48]; Roberts et al. [49]; Savola et al. [53]; Tarkkanen et al. [54]	(i) Adverse overall survival (ii) Age at diagnosis $\geq$15 years (iii) Disseminated disease at diagnosis
16q		Cell line	5/8 (63%)	CGH	Shing et al. [55]	
16q22.3		ESFT	5/9 (56%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	

Table 1: Continued.

Deletion	Gain	Ewing's sample type	Frequency (%)	Technology	Study	Clinical significance
17		ESFT and Xenotransplant	2/19 (11%)	dPCR, FISH	López-Guerrero et al. [64]	
17p		ESFT Cell line	9/62 (15%) 4/8 (50%)	CGH CGH	Ozaki et al. [48] Shing et al. [55]	(i) Adverse overall survival
17p13		ESFT	8/88 (9%)	Karyotyping (G-Band)	Roberts et al. [49]	
	17q21.31-q25.3	ESFT	6/9 (67%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	18	ESFT	6/68 (9%)	CGH	Brisset et al. [52]; Ferreira et al. [56]	
19		ESFT	4/25 (16%)	CGH	Ferreira et al. [56]	
19p		ESFT	7/62 (11%)	CGH	Ozaki et al. [48]	
19q		ESFT	11/62 (18%)	CGH	Ozaki et al. [48]	
	20	ESFT	35/248 (14%)	Karyotyping and CGH	Brisset et al. [52]; Ferreira et al. [56]; Hattinger et al. [47]; Roberts et al. [49]	(i) Adverse event free survival (ii) Adverse overall survival
	20p	ESFT	11/62 (18%)	CGH	Ozaki et al. [48]	(i) Adverse overall survival
	20q	ESFT	11/62 (18%)	CGH	Ozaki et al. [48]	(i) Adverse overall survival
	20q11.23-q13.33	ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	21q22.3	ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
	22q11.21	ESFT	2/9 (22%)	SNP Microarray (Affy 100 K)	Neale et al. [50]	
Y		Cell lines	3/5 (60%)	CGH	Shing et al. [55]	

* Modified from Toomey et al. *Oncogene* 2010. ESFT: Ewing's Sarcoma Family of Tumors, CGH: comparative genomic hybridization.

tumorigenesis and progression as part of 8q amplification that warrant future investigation based on their integrated outcome analysis ($P < .001$ and $P < .001$, resp.) [53].

Trisomy 12 has been suggested to be linked to trisomy 8. While one study found that every case with trisomy 12 was combined with trisomy 8 [51], others state the these trisomies are independent events [57]. The frequency of trisomy 12 occurring with trisomy 8 is higher than trisomy 12 alone, but both events have been shown to occur independently [47, 57]. Copy number gains of 8 and/or 12 appear more frequently in local recurrences (83% of the time) compared to primary (47%) and metastatic (42%) lesions and are hypothesized to appear with increased frequency during tumor progression or after initial translocation [57]. Much like trisomy 8, trisomy 12 has conflicting information regarding its clinical significance. However, many studies seem to suggest that trisomy 12 or focal amplifications on chromosome 12, are more important than those for chromosome 8. Trisomy 12 correlates to adverse-event-free survival ($P = .009$) for individuals with localized disease [47]. Even though other reports of this trisomy show no statistical significance for overall survival ($P = .67$) [54], evidence to the contrary links aberrations on 12p and 12q to reduced overall survival by univariate analysis ($P = .039$ and $P = .019$) [48]. In one set of Ewing's tumors, the smallest region of shared amplification on chromosome 12 contained two known oncogenes, *ERBB3* and *CDK4* [53]. These genes may be indicative of the importance of trisomy 12 and its role in tumorigenesis.

Amplifications and trisomies involving chromosomes 8 and 12 have conflicting findings regarding clinical and statistical significance. This is due to either the lack of statistical power in small sample sizes or the variable nature of the disease. In either case, neither trisomy was shown to be associated with *improved* prognostic outcome. This contrasts with descriptions of chromosome 2, which Brisset et al. reported to correlate with localized tumors rather than metastatic disease ($P = .02$) [52]. However, again illustrating the variable nature of copy number studies in Ewing's sarcoma, Ozaki et al. described the association between gains of 2q and the reduction of overall survival ($P = .022$) [48]. Perhaps the gain in chromosome 2 (specifically 2q) correlates with the more unusual localized tumors that also lead to relapse. Larger studies will be needed to clarify the importance of this amplification.

The gain of 1q is often reported with the loss of 16q. This is the presumed artifact of an unbalanced translocation in Ewing's sarcoma, der(16)t(1;16) [47, 48, 51, 52, 54, 55, 66, 68]. Though it is difficult to separate the translocation's downstream effects from the resulting CNA's impact, specific

clinical factors were linked to 16q loss such as age at diagnosis $\geq$15 years and disseminated disease at diagnosis ($P = .035$ and $P = .038$, resp.) [47]. The gain of 1q and the loss of 16q in combination with chromosome 12 gain also demonstrated an increased frequency of them occurring together ($P < .0001$) [47]. The region of 1q gain, regardless of localized or disseminated disease, was determined to be significant for both adverse overall survival (localized disease $P = .002$; disseminated disease $P = .029$) and event-free survival (localized disease $P = .018$; disseminated disease $P = .010$) [47]. While 1q amplification showed no statistical significance in other studies, a high-level focal amplification was found at 1q21-q22 [51], two genes also reported in other sarcoma samples [69], *SPRR3* with 5 copies and *FLG* with 4 copies were affected [51]. Other suspected candidates in 1q21-22 locus include *CACY* and *CAPL*, both of which have been implicated in tumor progression and metastasis [51, 70]. 1q21-1q22 amplification has also been reported in other sarcomas [69, 71]. This more focal 1q gain lacked statistical significance but still suggested association with adverse distant disease-free survival and overall survival [54].

Similar to the pairing of CNAs of 1q gain and 16q loss, combined losses of 16q and 17p, resulting from another unbalanced translocation, have been described [48, 55].The loss of concomitant 16q and 17p has demonstrated lower overall survival ($P = .0012$) [48]. 17p loss may have its major impact by encompassing the loss of the well-known tumor suppressor, *TP53* [47, 64, 72]. In addition to *TP53* deletion that is contained within 17p loss, mutation of *TP53* has been reported to show an association with poor chemoresponse and overall survival in Ewing's sarcoma ($P = .03$ and $P < .001$) [60].

Deletion of 9p21 encompassing *CDKN2A (p16-INK4a)* appeared in 10–73% of cases, including Ewing's sarcoma cell lines [50, 53, 59–62], with reported *homozygous deletions* in 8% [56] and 13% [60] of patient samples. This *CDKN2A* deletion was found to be a negative predictor of disease-specific survival ($P = .001$): 7 patients with this deletion all died of disease before 36 months, 2 of which had metastases at diagnosis [63]. The combination of *CDKN2A* deletion and *TP53* mutation was shown to be the most significant negative predictor of overall survival ($P < .001$) [60]. Our own experience has demonstrated the 9p21 deletion to be much more common in cell lines (80%) than clinical samples (5%) (unpublished). Current studies validating the prevalence and prognostic significance of *CDKN2A* deletions and TP53 mutations in Ewing's sarcoma are underway through the Children's Oncology Group (COG).

3.2. Genomic Instability. Instability of cancer genomes leads to the accumulation of CNAs. Early findings showed no statistical link between total number of CNAs and worse outcome in Ewing's sarcoma [51]. However, later data indicated that unstable karyotypes with higher numbers of CNAs in Ewing's tumors may be a correlate with worse outcome [53, 55, 56, 58]. CNAs totaling above three had worse prognosis in relation to event-free and overall survival ($P = .049$ and $P = .030$) [53]. By clustering patients into two groups of genomic instability and stable genomes, prognostic significance was determined for overall survival via univariate and multivariate analysis ($P = .017$ and $P = .034$) [56]. The group with increased genomic instability contained a reduced percentage of patients to reach complete remission, specifically 64% versus 100% [56].

3.3. Copy Number Mitochondrial Data. Mitochondrial DNA (mtDNA) copy number changes have been associated with increased risk of certain cancers. To date, breast cancer and renal cell carcinoma both have been associated with an increase in mtDNA and a decrease in mtDNA, respectively [73–75]. The displacement-(D-) loop of mitochondrial DNA (mtDNA), a noncoding region comprised of 1,124 base pairs, is more prone to mutation. These mutations, in conjunction with quantitative mtDNA changes, have been linked to Ewing's sarcoma [75, 76]. The D-loop's increased mutation stems from its vulnerability to oxidative damage and reduced reparation capacity [75]. Decreased copy number of mtDNA is more often found in samples containing D-loop mutations ($P = .04$) and could be a result of the transcriptional and replicating functions of the D-loop [75]. While both D-loop mutations and reduced content of mtDNA are at higher instance in Ewing's sarcoma, the greatest statistical significance was determined to be between low mtDNA copy number and tumor metastasis as all of the metastases in the study contained low numbers of mtDNA ($P = .029$) [75].

3.4. Ewing's Sarcoma and Methylation Data. Of the vast array of oncogenic manipulations of gene expression achieved in malignant cells, not all arise from either random mutation or cytogenetic gains and losses resulting in CNAs. Methylation is an alternate method by which gene expression is changed in cancer cells [77]. Methylation is the addition of a methyl group, usually to the $5'$ position of the cytosine pyrimidine ring, most importantly on cytosine residues contiguous to guanine residues, in what are called CpG islands. CpG sequences, in general, are relatively scarce in the human genome, as spontaneous mutation of the C to a T residue is especially common in the methylated state. Most remaining CpGs in the human genome are in the $5'$ regulatory and promoter sequences of genes.

Methylation of these promoter CpGs provides a cell-heritable means by which expression can be regulated. When a new zygote is formed, the cell is extensively demethylated. As cell division proceeds and eventually differentiation, methylation also proceeds, silencing certain genes no longer necessary along the cell's prescribed differentiation course. Methylation of promoter CpG islands affects transcription of the nearby gene via physical interruption of the binding of transcription factors and by encouraging binding of methyl-CpG-binding domain proteins. This recruits histone deacetylase and other chromatin-remodeling proteins, resulting in tight chromatin packaging of the locus and exclusion of transcriptional machinery. This silenced state of the gene is then passed on to daughter cells. Normal methylation is an important developmental program by which dangerous genes, such as viral sequences integrated into the human genome over generations, and early developmental genes can be silenced when necessary.

Cancer cells can have a variety of problems with methylation. Some powerful oncogenes from integrated viruses and developmental genes that engender a highly proliferative state are often demethylated in cancers, resulting in their aberrant and deleterious expression. In addition, the promoters of many tumor suppressor genes are over-methylated resulting in their silencing. Obviously, genomic sequencing or usual hybridization techniques will not detect promoter methylation or demethylation. These powerful epigenetic modifications of genes are only noted when specifically sought. While dedicated efforts are underway to understand methylation in many cancer types, such large scale efforts are lacking for the Ewing's sarcoma family of tumors. In fact, the relative absence of wild karyotype anomalies and rampant mutations argues that epigenetic modifications such as methylation may be a prominent mechanism of disease in sarcomas bearing balanced translocations such as Ewing's sarcoma. While methylation and epigenetics have only received limited attention in the literature thus far, they seem likely to be important genetic mechanisms for Ewing's sarcomagenesis and progression.

Two studies have assessed genetic alterations in the 9p21 locus in Ewing's sarcoma [64, 78]. One identified 4 tumors with homozygous deletion and 2 with promoter hypermethylation of *CDKN2A* among 26 tumors in total [64]. Two tumors had codeletion of *CDKN2B (p15-INK4b)* and 3 promoter hypermethylation of p15 [64]. The second study found 1 methylated, 1 point-mutated, and 2 homozygous deleted *CDKN2A* among 24 tumors, as well as 2 methylated and 2 homozygous deleted *CDKN2B* [78].

Another gene studied with respect to promoter methylation in Ewing's sarcoma is *RASSF1A*. One study interrogated *RASSF1A* along with *p16, MGMT, GSTP1, APC, DAPK, RARβ, CDH1,* and *CDH13* and found only *MGMT* and *CDH1* promoters methylated in 1 of 8 (12.5%) Ewing's sarcoma tumor samples [79]. Failure to detect *CDKN2A* promoter methylation in this study can be reconciled with the results of the larger studies described above based simply on insufficient sample size. With respect to *RASSF1A*, these results are more difficult to reconcile with the high frequency of *RASSF1A* methylation in a previously published report [80]; Avigad et al. identified 21 of 31 (68%) patient samples and 1 of 4 (25%) cell lines with hemizygous promoter methylation and 2 of 4 (50%) cell lines with homozygous promoter methylation. This larger study also correlated reduced *RASSF1A* expression with promoter methylation in 12 tumors checked. Further, they demonstrated reexpression of *RASSF1A* in the 2 homozygous methylated cell lines, upon in vitro application of 5-aza-2′ deoxycytidine, a powerful demethylating agent.

Two studies have corroborated each other in identifying 1 of 4 (25%) and 9 of 41 (22%) Ewing's cell lines with reduced caspase 8 expression, secondary to promoter methylation [81, 82]. The larger of these studies went further to confirm this reduced or lost caspase 8 expression by promoter methylation as the mechanism by which the 9 cell lines evaded TRAIL-induced apoptosis [82]. They confirmed the absence of deletions in the caspase 8 gene, as well as the reexpression of caspase 8 upon 5-aza-2′ deoxycytidine administration. Reexpression restored TRAIL and cytotoxic chemotherapy-induced apoptosis in these cell lines. They further checked 20 primary Ewing's sarcoma tumor samples, where they identified the predominance of the methylated caspase 8 promoter in 13 cases.

Finally, 5-aza-2′ deoxycytidine-driven demethylation has been tested as a means of disrupting the transformed phenotype of Ewing's sarcoma cell lines [83]. Using a clonogenic assay, demethylation alone dropped clonogenicity by 20 percent. Synergistic with a panel of histone deacetylase inhibitors, effects of 80 to 90 percent disruption of clonogenicity were detected, in addition to the reexpression of tumor suppressors such as E-cadherin and *TSLC1*.

4. Conclusions

In this paper, we focused primarily on copy number and methylation data. We also acknowledge the importance of other molecular changes potentially at work in Ewing's sarcomagenesis such as pleiotropic effects of the chromosomal translocation beyond creation of the specific fusion oncogene, somatic mutations in yet uninterrogated tumor suppressors, increased expression of oncogenes or oncogenic microRNAs, and other epigenetic mechanisms of expression regulation such as histone and chromatin packaging that could not be covered within the scope of this review. CNAs and methylation changes in Ewing's sarcoma, along with some of these yet unexplored genetic and epigenetic perturbations may be essential to Ewing's tumorigenesis as evidence suggests that the EWS-FLI1 translocation is necessary but not sufficient for Ewing's transformation in vitro [31]; CNAs and methylation changes may form some of the necessary second hits required for Ewing's sarcoma to develop. The complex cooperative relationships of these many molecular mechanisms of expression alteration have not been fully explored, and a full-system biology approach may prove to be informative in the field of Ewing's sarcoma. As explored in this review, isolated combinations of chromosomal gains and deletions already have begun to be described. Unfortunately, the results of the limited copy number studies are rarely in agreement likely due to poor statistical power in each small sample studied. In many instances, statistical significance cannot be determined, but trends still suggest that these CNAs have prognostic impact or contribute to genomic instability associated with worse outcome.

The investigation of copy number in Ewing's sarcoma will continue to advance given the rapid acceleration of high-resolution genomic technology to interrogate clinical samples, including archived FFPE specimens. Discovery of *specific genes* (rather than larger chromosomal cytobands) associated with tumor development and outcome will extend rapidly as the coverage in new SNP microarray platforms continues to become more dense and whole genome sequencing becomes more affordable. Novel and recurrent CNAs have been reported to cover nearly the entire genome. The main copy number recurrences in Ewing's sarcoma included trisomies 8 and 12, along with 1q amplification. These findings were consistent throughout the majority of studies, despite the inability of many studies to find statistical

significance related to treatment response, prognosis, outcome, or tendency to relapse. Although several recurring regions, such as, 16q deletions, have been repeatedly shown in different copy number studies to be associated with worse outcomes, these findings still await validation and incorporation into clinical trials.

For methylation as a mechanism of sarcomagenesis, two prominent tumor suppressor loci, *CDKN2A* and *RASSF1A,* as well as one important apoptosis activator, *caspase 8,* have been implicated. Further, functional assays have shown the reversibility of these expression repressions by the application of demethylating agents. For these methylation-associated genetic perturbations, therapeutic implications are very direct because the clinical drugs affecting methylation status and downstream histone deacetylation are already available for patient use. We expect that researchers have only scratched the surface of the Ewing's methylome. With the knowledge of demethylated oncogenes and other methylation-silenced tumor suppressors, the mechanisms leading to further CNAs and increased genomic instability with tumor proliferation may be elucidated. The continued investigation of copy number and methylation in Ewing's sarcoma will lead to a better understanding of tumorigenesis, more accurate risk stratification and hopefully new targets for developmental therapeutics. As genomic technology continues to improve, CNA and methylation changes detected in clinical samples can be rapidly incorporated into patient care to improve the outcome in Ewing's sarcoma.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Targeting Angiogenesis in Childhood Sarcomas

Hemant K. Bid and Peter J. Houghton

Center for Childhood Cancer, Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205, USA

Correspondence should be addressed to Peter J. Houghton, peter.houghton@nationwidechildrens.org

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Angiogenesis and vasculogenesis constitute two processes in the formation of new blood vessels and are essential for progression of solid tumors. Consequently, targeting angiogenesis, and to a lesser extent vasculogenesis, has become a major focus in cancer drug development. Angiogenesis inhibitors are now being tested in pediatric populations whereas inhibitors of vasculogenesis are in an earlier stage of development. Despite the initial enthusiasm for targeting angiogenesis for treatment of cancer, clinical trials have shown only incremental increases in survival, and agents have been largely cytostatic rather than inducing tumor regressions. Consequently, the role of such therapeutic approaches in the context of curative intent for childhood sarcomas is less clear. Here we review the literature on blood vessel formation in sarcomas with a focus on pediatric sarcomas and developments in targeting angiogenesis for treatment of these rare cancers.

1. Introduction

The generation of new capillaries from preexisting blood vessels is termed angiogenesis [1]. Angiogenesis functions as the result of a dynamic balance between proangiogenic factors, for example, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), and factors that inhibit angiogenesis such as thrombospondin-1 and angiostatin [2]. The process of regulated angiogenesis occurs during embryogenesis, the menstrual cycle, wound healing, and pathologic states. Unregulated angiogenesis may lead to numerous diseases and is thought to play an indispensable role in solid tumor growth and metastasis. Numerous investigations on tumor development have shown that an alteration in the blood supply can noticeably influence the tumor growth and its metastasis [2]. As with normal tissue, the growing tumor requires an extensive network of capillaries to provide the necessary nutrients and oxygen. Moreover, the new intratumoral blood vessels offer a way for tumor cells to enter the circulation and metastasize to distant organs. In this context, angiogenesis plays a crucial role in facilitating the growth of the primary tumor and generating metastasis. However, in the early 1900s it was recognized that "vessels showed changes, such as defective coatings, dilation, obliteration, and thrombosis" [3, 4] (cited in [5]). Extensive research in this area has indicated that the effective inhibition of blood vessel formation can result in tumor regression, although the predominant effect is the slowing of tumor growth. However, targeting the stromal elements of the tumor, rather than focusing on the cancer cells exclusively, represents a major shift in emphasis in cancer research. Unfortunately, due to the heterogeneity of the angiogenesic process within diverse neoplasms, it is difficult to generalize research findings to all tumor types. Here we have focused on the available data on angiogenesis and targeting angiogenesis as it pertains to pediatric sarcoma.

2. Angiogenesis in Childhood Sarcomas

Sarcomas present a great challenge for cancer therapy because they comprise a relatively uncommon group of diseases. Sarcomas encompass many diseases, not simply a representation of a single entity of mesenchymal origin. Pediatric soft tissue sarcomas are a group of malignant tumors that originate from primitive mesenchymal tissue and account for 7% of all childhood tumors [6]. As a result

of their diverse biology, therapeutics for pediatric sarcomas will ultimately be tailored to the specific tissue type [7–10].

Established chemotherapy regimens for advanced or metastatic sarcoma generally have low 5-year event-free survival, and current therapies have substantial toxicity. Resistance often arises quickly, making advanced sarcoma an acceptable target for alternative treatment approaches. Antiangiogenic therapies have a number of potential advantages including decreased resistance, fewer side effects, and a broad spectrum of activity. Human sarcomas express a number of proangiogenic factors that may represent potential therapeutic targets, with VEGF being the best characterized. Inhibitors of angiogenesis have demonstrated antitumor activity in animal models of childhood sarcomas, and clinical trials are in the early stages, although promising results are already being seen. Antiangiogenic and immunomodulatory therapies are gaining momentum in the pediatric arena and, when tested in combination with traditional cytotoxic agents for recurrent and high-risk primary pediatric sarcomas, may lead to more effective and tolerable therapies [11].

An example of potential antiangiogenic therapeutic targets can be observed in rhabdomyosarcoma (RMS) cell lines. These cells secrete VEGF [12, 13] as well as other angiogenic factors such as basic fibroblastic growth factor (bFGF) and interleukin 8 [14] as well as other potential angiogenic factors [15]. In most RMS cell lines VEGF stimulates proliferation or activates the PI3K/Akt pathway [12, 13], hence acting as both an autocrine growth factor and a paracrine factor involved in angiogenesis.

Microvessel density (MVD) has also been found to be a prognostic factor in the response to therapy and survival in several adult carcinomas [16–19]. Observations from different studies suggest that MVD in soft tissue sarcomas (STS) was not associated with histological type, grading, metastatic behavior, or survival [20–23]. Rather, tissue levels of VEGF were associated with local recurrence and survival [20]. In contrast MVD was correlated with survival in adult soft tissue sarcoma of the extremities [24]. Tomlinson et al. describe a different pattern of angiogenesis in STS compared to breast carcinoma. In breast cancer, the capillaries were clustered in bursts within the stroma of the tumor, while the sarcoma capillaries were homogeneously distributed throughout the tumor stroma. They credit this difference to the greater number of activated fibroblasts in carcinomas, with their own gradients of angiogenic factors in the tissues. This aspect has been studied in carcinosarcoma, which contains both tumor types. In accordance with the findings of Tomlinson et al. [22], a study by Yoshida et al. describes a significantly higher MVD in the carcinomatous areas of the tumor than that found in the sarcomatous parts [25]. However, the consequences of this in terms of antiangiogenesis therapy of STS are not yet clear.

3. Angiogenic Factors Secreted by Childhood Sarcomas

As noted above, balance between proangiogenic and antiangiogenic factors, with the involvement of different cells and stimulating factors, regulates the process of angiogenesis. Some of the cells engaged are endothelial cells (EC), lymphocytes, macrophages, and mast cells. Vascular endothelial growth factor and fibroblast growth factor (FGF) are two of the major factors involved in this process. These cells and stimulating factors play different and important roles during tumor angiogenesis. From the more than 20 proangiogenic and antiangiogenic agents identified, VEGF and bFGF have been established as the two most potent positive regulators of angiogenesis [26–28]. However, other cytokines, such as interleukin-1 receptor α (IL-1R), IL-6, and IL-8, tumor growth factor-α (TGF-α), TGF-β, tumor necrosis factor-α (TNF-α), hepatocyte growth factor (HGF), leptin, and platelet-derived growth factor (PDGF), have been reported to be involved as well, though their roles have not always been clearly defined [28–34].

As previously described, tumor cells require nutrients and oxygen to overcome hypoxia and starvation. When a condition such as hypoxia is present within the tumor tissue, the tumor cells are stimulated to promote the secretion of various angiogenic factors for the induction of angiogenesis [35]. Of clinical significance, the pretherapeutic serum VEGF levels were found to be significantly higher in patients with osteosarcoma who relapsed during the first year of treatment, providing the basis to establish further antiangiogenic therapy to target patients at high risk of angiogenesis-dependent relapse of osteosarcoma [36]. However, the prognostic significance of angiogenic factors in childhood sarcoma remains ambiguous.

4. Antibody-Based Antiangiogenic Therapy

Bevacizumab (Avastin; Genentech Inc.), a humanized antibody against that binds VEGF [37], must be regarded as the gold standard against which other antiangiogenic treatments are compared. Bevacizumab specifically binds to VEGF-A and its isoforms to counteract the proangiogenic effects of VEGF, allowing for the normalization of the tumor vasculature [38]. It is approved for several adult indications and currently being evaluated in combination treatment regimens for various adult malignancies [39–41]. The clinical experience with bevacizumab in pediatric patients is limited. A report by Benesch et al. [42] indicated that bevacizumab has activity in pediatric malignancies, but large multicenter trials are needed to quickly assess the clinical value of this drug in childhood malignancies. Ongoing trials include evaluation against osteosarcoma and Malignant Fibrous Histiocytoma (MFH) of bone. Although bevacizumab is not without toxicity, pediatric trials combining this agent with conventional chemotherapy regimens are in development.

5. Small Molecule Inhibitors of Angiogenesis in Sarcomas

There are multiple humoral factors involved in the regulation of both normal and abnormal angiogenesis. From the perspective of small molecule development VEGF signaling has been the predominant target, as VEGF was found to be

overexpressed in various malignancies [43, 44]. Preclinical studies have evaluated a wide range of strategies and compounds to inhibit angiogenesis in laboratory models. Of these agents, tyrosine kinase inhibitors (TKIs) have gained attention as a means of targeted treatment for a wide range of human cancers. At least 90 tyrosine kinase genes have been identified in human cancers [45], and several TKIs inhibitors are now approved for use in the treatment of cancer in the United States. However, only few of these have been adequately evaluated in childhood sarcoma. However "selective," small molecule inhibitors usually do not inhibit only a single kinase, but result in the targeting multiple signaling pathways. The "multitargeted" TKIs have, in general, shown the most activity against solid tumors.

Of the small molecule inhibitors with oral bioavailability, sorafenib (Nexavar; Bayer Pharmaceuticals) and sunitinib (Sutent; Pfizer Inc.) act on multiple intracellular and receptor protein kinases (e.g., VEGF receptors, PDGFR, FLT3, RET, BRAF, KIT) that are components of signaling pathways controlling tumor growth and angiogenesis. Both of these agents have similar drug profiles and overlapping targets [46] and are currently approved by the U.S. Food and Drug Administration for the treatment of advanced renal cell carcinoma (RCC) in adults [47]. In a study conducted by the Pediatric Preclinical Testing Program (PPTP), sorafenib was demonstrated to be an effective inhibitor of tumor growth across multiple histotypes in vivo [48]. Currently, sorafenib is being evaluated in high-grade osteosarcoma. Sunitinib also showed significant tumor growth inhibition against most of the PPTP's solid tumor panels, but little activity against the neuroblastoma and ALL panels. The antitumor activity of sunitinib was manifested as primarily a delay in tumor growth, consistent with an antiangiogenic effect against many of the pediatric preclinical models evaluated [49].

Cediranib (Recentin; AZD2171; Astrazeneca Inc.), another small molecule with oral bioavailability, is an indole-ether quinazoline which inhibits the tyrosine kinase activity of the vascular endothelial growth factor receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR), VEGFR-3 (Flt-4), and c-KIT. Cediranib was previously shown to prevent both physiologic and pathologic angiogenesis in vivo [50], inhibiting the growth of a number of different pediatric human tumor xenografts [50, 51]. Currently in phase II/III clinical development, early clinical studies have shown encouraging antitumor activity in patients with a broad range of solid tumors, as well as time-dependent and dose-dependent changes in pharmacodynamic markers [52]. The results from a recently completed clinical trial have shown that the daily administration of cediranib to glioblastoma patients resulted in a rapid and prolonged normalization of the tumor vasculature which subsequently led to a reduction in tumor-associated edema [53]. Promising preliminary results have also been reported for treatment of alveolar soft part sarcoma (ASPS), a tumor that responds poorly to conventional chemotherapeutic regimens [54]. Of seven patients with ASPS four had partial responses and two either marginal response or stable disease [55].

Rapamycin and its derivatives (temsirolimus, everolimus, radiforolimus) selectively inhibit a serine/threonine kinase mTOR (mammalian target of rapamycin) that controls translation and transcription. These are immunosuppressive macrocyclic lactone antibiotics that block mTOR function and produce an antiproliferative effect in a variety of malignancies. Rapamycin has demonstrated broad antitumor activity against the pediatric cancers in PPTP's in vivo tumor panels, with noteworthy activity in selected sarcoma and ALL xenografts [56]. Initial reports have suggested that the effects of rapamycin may be related to its inhibitory action against the endothelial cells, effectively blocking tumor angiogenesis [57, 58]. Rapamycin has the potential to disrupt the action of vascular endothelial growth factor (VEGF) in the growth plate and interfere with insulin-like growth factor I (IGF-I) signaling [59]. Rapalogs have been shown to inhibit hypoxia-induced induction of hypoxia inducible factor-1α (HIF-1α), the major transcription factor that reprograms cells under hypoxic stress and induces VEGF transcription. These new findings suggest potential benefits of including rapamycin as an antiangiogenic agent in the treatment regimens of pediatric cancer patients. However, at least in some sarcoma xenograft models, rapamycin treatment stimulated tumor-associated VEGF, although the mechanism for this is poorly understood [60].

6. Role of IGFs in Childhood Sarcomas

It is becoming increasingly evident that the Type-1 insulin-like growth factor (IGF-1R) and its ligands (IGF-1, IGF-2) play roles in both tumor cell proliferation and survival, and proliferation of vascular endothelial cells. IGFs are balanced by insulin-like growth factor-binding proteins (IGFBPs). IGFBPs comprise a family of secreted proteins that modulate the bioavailability of insulin-like growth factors (IGFs) in the IGF-I/IGF-I receptor (IGF-IR) signaling axis. IGF binding protein-3 (IGFBP-3) is emerging as a key regulator of cell growth and apoptosis, both as an IGF antagonist and as an independent molecule, which plays roles in the proliferation and migration of HUVEC cells. IGFBP-3 expression is generally inhibited in Ewing's sarcoma cells, as a consequence of EWS/FLI1 expression. Exposure of neoplastic cells to IGFBP-3 inhibits their growth, migratory, invasive, angiogenic, and metastatic potential, therefore demonstrating the protein as a molecule of therapeutic relevance to be considered in the treatment of patients with Ewing sarcoma [61]. In contrast, the IGFBP-3-induced endothelial cell motility and migration may suggest a direct role for this binding protein in promoting angiogenesis [62, 63]. The functional role of IGFBP-5 in retarding angiogenesis has also been described. Tumor growth and tumor vascularity were decreased in the presence of IGFBP-5 expression in a xenograft model of human ovarian cancer [64]. Insulin-like growth factor-binding protein-7 (IGFBP7) is a secreted 31-kDa protein, which is also called as IGFBP-related protein 1 (IGFBP-rP1) [65]. IGBP7 shares high homology with the IGFBPs and binds IGF-I and insulin, but its binding affinity for IGF-I is lower than those of IGFBPs 1 to 6 [66]. IGFBP7 is highly expressed in the blood vessels of various human cancer tissues, suggesting that it might suppress the pathological

action of VEGF, which is mainly derived from tumor cells. Data suggest that IGFBP7 in the blood vessels of tumors may lead to a unique tumor vasculature with characteristics significantly different from those of normal vasculature. The inhibitory effect of IGFBP7 on tumorigenicity might be partially mediated by its ability to suppress VEGF-stimulated angiogenesis, although there is so far no direct evidence to explain if IGFBP7 affects tumor blood vessels. The use of IGFBPs to limit IGF-1R signaling has been proposed as a therapeutic approach. Gallicchio et al. [67] reported that IGFBP-6 significantly inhibited monolayer RD and Rh-30 cell proliferation in a dose-dependent manner. Furthermore, the overexpression of IGFBP-6 resulted in a 74–88% reduction in Rh-30 tumor size in vivo after 18 days, showing that IGFBP-6 can be a potent antitumor agent.

For approximately two decades, the insulin-like growth factor (IGF) has been implicated in the pathogenesis of numerous pediatric malignancies, including osteosarcoma, Ewing sarcoma, and rhabdomyosarcoma (RMS). The role of IGF-1R signaling in the pathogenesis of RMS and its role in preventing apoptosis induced by a multitude of cellular stresses, including cytotoxic drugs, radiation, and hypoxia [68], indicate that targeting this pathway may have considerable utility in the therapy of RMS. IGF-II is also involved in normal muscle growth, and Northern blot analysis of tumor biopsy specimens from patients with both alveolar and embryonal rhabdomyosarcoma has demonstrated high levels of IGF-II mRNA expression [69]. This suggests the possibility that upregulation of IGF-II plays a role in the unregulated growth of these tumors. Support for this hypothesis came from the finding that RMS cell lines also secrete IGF-II, which then binds to IGF-1R, resulting in autocrine growth proliferation and increased cell motility [70].

Ewing's sarcoma, peripheral primitive neuroectodermal tumor, and Askin tumor form a group of tumors collectively termed Ewing's sarcoma family of tumors (ESFT). These tumors are characterized by specific chromosomal translocations that cause the N-terminus of EWS to be fused to the C-terminus of one member of the ETS family of transcription factors, most commonly FLI1 [71]. Expression of the fusion product has been implicated in oncogenesis. The role of IGF-1R signaling in ESFT has undergone extensive evaluation. EFST cell lines express IGF-1R and secrete IGF-I, and IGF-1R-blocking antibodies interrupt this autocrine loop [72, 73].

The importance of the IGF axis to cell growth and differentiation in both normal tissues and cancer and the aforementioned association of osteosarcoma with periods of rapid bone growth help to explain the current focus for therapies targeting the type 1 IGF receptor (IGF-1R). The peak incidence of osteosarcoma occurs during adolescence, corresponding to both the growth spurt and peak concentrations of circulating GH and IGF-1 [74]. This epidemiological correlation has led to the hypothesis that high levels of IGF-1 play an important role in the pathogenesis of osteosarcoma. This hypothesis is supported by a host of preclinical data: (a) osteosarcoma cells express functional IGF-1R on the cell surface, (b) exogenous IGF-1 stimulates osteosarcoma cells to proliferate, (c) IGF-1-dependent growth can be inhibited using monoclonal antibodies or antisense oligonucleotides against IGF-1R [75], (d) the treatment of mice with a humanized anti-IGF-1R antibody resulted in tumor regression in two osteosarcoma xenograft models [76], and (e) the majority of osteosarcoma patient samples express IGF ligands, and 45% express IGF-1R [77].

7. Role of IGF's in Angiogenesis

As described above, IGFBPs have both antitumor and antiangiogenic properties, although whether these two characteristics are linked remains to be demonstrated. However, these data suggest that antibodies that prevent ligand binding to the IGF-1R, or ligand binding antibodies *per se*, may have therapeutic utility in childhood sarcomas. Phase-1 or -2 clinical trials with eight fully human antibodies, or humanized antibodies, that target IGF-1R and prevent ligand binding have been reported [78]. For commercial reasons two agents (SCH717454 and R1507) are not being developed further. These antibodies show specificity for the IGF-IR although they may also inhibit chimeric receptors formed through heterodimerization with the insulin receptor. In preclinical models of childhood cancers, the prototypical anti-IGF-1R antibody, α-IR3, mediated downregulation of IGF-IR, significantly retarded the growth of several cell lines in vitro [70], and inhibited the growth of pediatric cancer xenografts [79]. SCH717454 significantly inhibits growth of RMS xenografts and induces regressions in several sarcoma histotypes, notably osteosarcoma and Ewing sarcoma [80]. R1507 was found to inhibit growth of osteosarcoma xenografts [81]. In some in vivo models of Ewing sarcoma and osteosarcoma targeting IGF-1R with CP751871 dramatically suppressed VEGF transcription and reduced tumor-associated VEGF within 24 hours of antibody administration [60]. Furthermore, SCH717454 treatment markedly reduced blood vessel formation in tumor xenografts, showing that the in vivo activity is derived not only from its inhibition of tumor cell proliferation, but also from its angiogenesis activity [82].

The molecular characterization of these sensitive models where IGF-IR signaling appears to be critical could identify subsets of tumors that have become "addicted" to this pathway [83]. In other preclinical models, blocking IGF-IR signaling results in significant retardation of tumor growth, although in a clinical setting this response would still be scored as progressive disease. In these models with intermediate sensitivity, such as RMS, combinations of signaling inhibitors would be a potentially more effective antitumor therapy. One strategy that is being evaluated in preclinical models is the combination of the mTOR inhibitor, rapamycin, with IGF-1R inhibitors. The basis for this combination is that inhibition of mTOR upregulates IGF-1R signaling through stabilization of IRS-1 [84] and IGF-1R signaling blocks rapamycin-induced apoptosis [85, 86]. One IGF-1R inhibitor, CP751871, caused complete IGF-1R downregulation, suppressed AKT phosphorylation, and dramatically suppressed tumor-derived vascular endothelial

growth factor (VEGF) in some sarcoma xenografts. Treatment with rapamycin alone did not markedly suppress VEGF in tumors and synergized only in those tumor lines where VEGF was inhibited by CP751871. This data suggests a model in which the blockade of IGF-1R suppresses tumor-derived VEGF to a level where rapamycin can effectively suppress the response in vascular endothelial cells [60]. Exactly how rapamycin blocks the response to VEGF in vascular endothelial cells is not clear. However, recent studies show that SCH717454 potently inhibits VEGF-induced proliferation of HUVECs, indicating that IGF-1R-mediated signaling is essential for vascular endothelial cell proliferation (H. K. Bid, PJH, unpublished data). As discussed above, rapamycin has been shown to potently inhibit IGF-1-stimulated proliferation of tumor cells [87].

Alternative approaches to inhibiting IGF-1R signaling comprise the development of ligand binding antibodies. Targeting the receptor ligand rather than the receptor *per se* has proven to be a valuable approach for the antiangiogenic antibody (bevacizumab), and high-affinity, fully human antibodies have been developed against IGF-II [88].

Phase I and phase II trials using many of these IGF-1R-targeted antibodies are currently in progress. To date, there have been very few serious side effects resulting from this treatment. Hyperglycemia, when present, has been mild and has only been seen in some of the antibodies tested [89–93]. Because of the important role of the IGF pathway in normal growth, there is concern about the impact of IGF blockade in patients who are still growing. Details of recent clinical trials are provided in Table 1. Unfortunately, in these tumor types, many of the patients are teenagers or younger children. The hypothetical concern of disrupting normal growth must be taken under consideration, but also weighed against the pressing issue of tumor progression. The only way to assess the impact of IGF-1R-directed therapy on normal growth is to monitor young patients who have been treated with the antibody for a prolonged period over the course of their growth. On the positive side, this would indicate that the patient is responding or the disease is not progressing, on therapy. Data have not emerged suggesting that one antibody is more effective than another. However, slight differences in regards to whether the antibody is fully human or humanized, their relative affinity to the IGF-1R and IR, and their ability to block the binding of either IGF-I or IGF-II ligand have not resulted in markedly different side effects or tolerability, but could lead to differences in clinical activity.

8. Vasculogenesis in Childhood Sarcoma

Vasculogenesis and angiogenesis are the fundamental processes by which new blood vessels are formed [35, 94, 95]. Vasculogenesis is defined as the differentiation of precursor cells (angioblasts) into endothelial cells and the de novo formation of a primitive vascular network, whereas angiogenesis is defined as the growth of new capillaries from preexisting blood vessels [35]. Several studies have now been published that suggest that not only angiogenesis but also vasculogenesis may be involved during postnatal life in situations that require an expanded vessel network. Solid tumors require development and expansion of a vascular network for nourishment to support their growth. Angiogenesis was initially regarded as the sole mechanism by which tumor vessels expand. However, other mechanisms are also involved in the expansion of the tumor vascular network such as vasculogenesis.

Both angiogenesis and vasculogenesis contribute to the formation and expansion of the tumor vasculature that supports the growth of Ewing's sarcoma [96]. Data from Lee et al., 2006 [96] support the same hypothesis because they demonstrated that not only local endothelial cells but also bone marrow (BM)-derived cells are involved in the generation of the new tumor vasculature during the growth of Ewing's sarcoma. Several cytokines, such as Granulocyte Colony-Stimulating Factor, Placental Growth Factor, and Stromal cell-derived Factor-1 (SDF-1), have been shown to induce BM stem/progenitor cell mobilization and chemotaxis. With respect to tumor growth, both stimulatory and inhibitory roles of SDF-1 have been reported. Disruption of the SDF-1/CXCR4 axis was found to inhibit tumor growth, microvessel density, and intratumoral blood flow without affecting VEGF levels [97]. Reddy et al., 2008 [98] suggest that the effects of SDF-1 on tumor neovascularization include augmented chemotaxis of BM cells, retainment of BM-derived pericytes in close association with the vessel endothelial lining, enhanced overall pericyte coverage of tumor neovessels, and remodeling of vascular endothelium into larger, functional structures. All these processes together support the growth of Ewing's tumors, with distinctly reduced $VEGF_{165}$. Overall these reports suggest that BM-derived cells play a critical role in the expansion of the Ewing's tumor vasculature, and that vasculogenesis may be one of the mechanism by which tumors can evade the effects of antiangiogenic therapy targeted at VEGF. Similarly, vasculogenesis is likely in other sarcomas. With inhibitors of CXCR4 in development, the therapeutic potential for simultaneous inhibition of angiogenesis and vasculogenesis may be tested.

9. Summary and Perspective

Growth of sarcoma, like other solid tumors, is dependent on angiogenesis and vasculogenesis, hence understanding the basic mechanisms and factors that influencing these processes has the potential to reveal additional targets for intervention. To date, the effectiveness of angiogenesis-directed treatments has not been particularly striking. In adults these agents extend event-free survival by weeks or months in the majority of malignancies. Thus, the role of such therapeutic approaches must be considered in the context of childhood cancer where the intent is cure. One can see the value of essentially cytostatic therapy in the context of young children where delay in tumor progression may be of value in delaying radiation treatment (particularly in CNS malignancies). However, is it realistic to anticipate that antiangiogenic treatments will convert childhood cancer

Table 1: Summary of select recent clinical studies for sarcoma.

Compounds/Agents in Clinical trials	Tumor type	Identifier
A Phase II trial of dasatinib	Advanced sarcoma	SARC009
A Phase II trial of R1507, a recombinant human monoclonal antibody to the insulin-like growth factor-1 receptor	Recurrent or refractory ewing's sarcoma, osteosarcoma, synovial sarcoma, rhabdomyosarcoma, and other sarcomas	SARC011
A randomized, double-blinded, placebo-controlled, multiinstitutional, phase II.5 study of AZD0530, a selective Src kinase inhibitor	Recurrent osteosarcoma localized to the lung	SARC012
IMC-A12 and doxorubicin hydrochloride	Patients with unresectable, locally advanced, or metastatic soft tissue sarcoma	NCT00720174
IMC-A12	Young patients with relapsed or refractory ewing sarcoma/peripheral primitive neuroectodermal tumor or other solid tumor	NCT00609141
R1507	Patients with recurrent or refractory sarcoma	NCT00642941
CP751871 figitumumab combined with pegvisomant	Advanced solid tumors	NCT00976508
Bevacizumab and AZD2171	Patients with metastatic or unresectable solid tumor, lymphoma, intracranial glioblastoma, gliosarcoma, or anaplastic astrocytoma	NCT00458731
Cediranib (tentative trade name recentin), also known as AZD2171	Young patients with refractory or recurrent solid tumors or acute myeloid leukemia	NCT00354848
Temozolomide, cixutumumab, and combination chemotherapy	Treating patients with metastatic rhabdomyosarcoma	NCT01055314
Cixutumumab IMC A12	Treating patients with relapsed or refractory solid tumors	NCT00831844
Cixutumumab and temsirolimus	Treating young patients with solid tumors that have recurred or not responded to treatment	NCT00880282
A study of SCH 717454 in combination with different treatment regimens	Pediatric subjects with advanced solid tumors (Study P05883)	NCT00960063
SCH 717454 in combination with different treatment regimens	Advanced solid tumors (P04722)	NCT00954512
Temsirolimus and valproic acid	Treating young patients with relapsed neuroblastoma, bone sarcoma, or soft tissue sarcoma	NCT01204450
Temsirolimus, irinotecan hydrochloride, and temozolomide	Treating young patients with relapsed or refractory solid tumors	NCT01141244
PCI-24781 in combination with doxorubicin	Treat sarcoma	NCT01027910
Angiogenesis inhibitor SU5416	Treating patients with soft tissue sarcoma	NCT00023738
Sorafenib and bevacizumab	Treating patients with refractory, metastatic, or unresectable solid tumors	NCT00098592
Sunitinib	Treating patients with metastatic, locally advanced, or locally recurrent sarcomas	NCT00474994
Radiation therapy with or without SU5416 (TK inhibitor antiangiogenesis compound)	Treating patients with soft tissue sarcoma	NCT00023725
Phase II study of doxorubicin and bevacizumab (anti-VEFG monoclonal antibody, NSC 704865)	Patients with advanced or metastatic soft-tissue sarcoma	NCT00052390
Phase I/II study of gemcitabine, docetaxel, and bevacizumab	Patients with soft tissue sarcoma	NCT00276055
Phase II study of neoadjuvant bevacizumab and radiation therapy	Resectable soft tissue sarcomas	NCT00356031

*Source: http://www.clinicaltrials.gov/.

into a chronic disease? Genomic plasticity is the hallmark of cancer, thus one would anticipate evolution of cancer cells that circumvent such treatments. Thus, the role of antiangiogenic therapy is most likely in the context of conventional chemotherapy, or in combination with other signaling inhibitors. Whether addition of small molecule inhibitors of angiogenesis will permit the maintenance of dose intensity of current cytotoxic regimens remains to be determined. For example, results from adult clinical trials suggest that dose intensity of cytotoxic agents or the dose of cediranib has to be reduced [99]. Available results from trials of IGF-1R-targeted antibodies suggest a low response rate even in Ewing sarcoma, suggesting that these agents poorly suppress tumor cell proliferation or tumor cells are able to circumvent the antiangiogenic activity of these antibodies. One aspect of antiangiogenic therapy that holds some promise is the effect of vascular normalization that allows reoxygenation [100, 101] (and hence increased radiation sensitivity) or increased uptake of drugs into tumor tissue [102–104].

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Review Article

Immune-Based Therapies for Sarcoma

Seth M. Pollack, Elizabeth T. Loggers, Eve T. Rodler, Cassian Yee, and Robin L. Jones

Fred Hutchinson Cancer Research Center, University of Washington, 825 Eastlake Avenue East, G3630, Seattle, WA 98109-1023, USA

Correspondence should be addressed to Robin L. Jones, rjones@seattlecca.org

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Immunotherapy has shown promise in a number of tumor types, but its exact role in sarcoma remains to be defined. Advanced bone and soft tissue sarcomas are challenging diseases to treat with an unmet need for effective systemic therapy. Previous reports have suggested that immune-based treatments may be effective in sarcoma, but such approaches have not yet become part of standard clinical practice. A number of sarcoma subtypes express targets known as cancer testis antigens and hence may be excellent targets for immunotherapy. This paper will focus on the recent advances and understanding of cancer testis antigens in sarcoma and also clinical data of immunotherapeutic approaches in these diseases.

1. Introduction

Immunotherapy has recently had significant well-publicized successes. Placebo controlled, randomized Phase III trials have demonstrated a survival benefit for vaccine-based therapy in follicular lymphoma [1] and prostate cancer [2]. Ipilimumab, an antibody that blocks the inhibitor of T-cell activation, CTLA-4, has been shown to improve survival in patients with metastatic melanoma [3].

Progress in the systemic treatment of sarcoma has been frustratingly slow. Immunotherapy has long been discussed as a promising method for the treatment of patients with metastatic sarcoma [4, 5]. Sadly, despite a number of ambitious early phase immunotherapy trials, no immunological treatments have become part of standard clinical management. However, because of significant strides in our understanding of cancer immunology and because of progress in other disease types, immunotherapy remains a source of hope that exciting new therapies are on the horizon for patients with sarcoma. We now know that many of the most promising targets for immunotherapy are frequently expressed in certain sarcoma subtypes. Lessons learned from other diseases, such as melanoma, can guide a new generation of immunotherapy trials with the aim of preventing recurrent disease in resected sarcoma and improving the survival of patients with advanced disease.

Here we discuss some of the potential targets for immunotherapy trials with a focus on the cancer testis antigens (CTAs) and their expression in individual sarcoma subtypes. We also review prior trials of immunotherapy including nonspecific immunomodulators, vaccines, and adoptive immunotherapy.

2. Completed Immunotherapy Trials

Immunotherapies can be divided into the following three categories: nonspecific immunomodulation, vaccines, and adoptive cellular therapy. Nonspecific immunomodulation induces antitumor immunity without exposing the patient to a target molecule. By contrast, vaccines expose patients to antigens in order to provoke an antitumor immune response usually in the presence of adjuvant and occasionally in combination with immunomodulation [3, 6]. Some of these vaccines have been targeted to sarcoma-specific fusion products such as SYT-SSX in synovial sarcoma, whereas other vaccines are less antigen directed such as those that have used irradiated autologous tumor cells. Finally, adoptive cellular therapy involves the *ex vivo* expansion of immune effector cells (often T cells and/or NK cells) from a patient for later reinfusion. This may be nonspecific, as in the case of leukocyte-activated killer cells or tumor-infiltrating

lymphocytes, or may use antigen-specific cultures *ex vivo* or genetically engineered to have tumor-directed specificity.

3. Nonspecific Immunomodulation

Some of the first trials demonstrating the potential of immunotherapy in cancer used high-dose interleukin-2 (IL-2) in patients with metastatic melanoma and renal cell carcinoma. Six sarcoma patients were included in these early high-dose IL-2 trials used in combination with leukocyte-activated killer cells. None of these patients responded [7]. More recently, however, high-dose IL-2 was given in a pediatric population including several patients with osteosarcoma [8]. In total, 10 pediatric patients with heavily pretreated, progressive, or metastatic solid tumors were treated with high-dose IL-2. The cohort included 4 patients with osteosarcoma and 2 patients with Ewing's sarcoma. Two of the four osteosarcoma patients had complete responses that were durable with median followup of 28 months (range 11–36 months for the 10 patients treated on study). Given that in long-term follow-up studies of adult patients with metastatic solid tumors treated with high-dose IL-2, patients who are disease-free 30 months following treatment are considered extremely unlikely to relapse [7], this pediatric study represents an encouraging finding that warrants more investigation focused on osteosarcoma.

Muramyl tripeptide phosphatidylethanolamine (MTP) is a synthetic analogue of a bacterial cell well that has been studied clinically as a nonspecific immune modulator. Early studies demonstrated that peripheral blood mononuclear cells taken from patients following treatment with liposomal MTP demonstrated increased tumor cell killing *in vitro* compared with baseline samples [9, 10]. Furthermore, the drug was associated with increased serum levels of TNF alpha and IL-6 [10].

The Children's Oncology Group's Intergroup-0133 studied MTP using a 2×2 design. In the first randomization, patients either received or did not receive ifosfamide with a chemotherapy backbone of cisplatin, doxorubicin, and high-dose methotrexate. In the second randomization, patients either received or did not receive liposomal-MTP. Analysis of this study has been complicated; the first analysis published in 2005 showed a trend towards improved outcomes for the MTP-containing arm that was not statistically significance. With more mature followup, a 2008 report demonstrated a statistically significant improvement in overall survival with a strengthening of the event-free survival trend for the MTP-containing arm. A 2009 report in cancer suggested that improvements in outcomes may also be seen in patients with metastatic disease although this analysis was not powered to demonstrate a statistically significant benefit in either event-free or overall survival [11–13]. To date, liposomal MTP has not secured FDA approval but is available at a number of centers for compassionate use.

Alpha interferon has also been used in several sarcoma subtypes, particularly osteosarcoma, with varying success. There have been case reports of responses to interferon in osteosarcoma [14, 15] and complete responses in clear cell sarcomas [16, 17]. From 1971 to 1990, 89 consecutive patients with localized high-grade osteosarcoma received adjuvant therapy with interferon-α. Between 1971 and 1984, 70 patients were treated with a dose of 3×10^6 IU once a day for one month, and subsequently 3 times weekly for further 17 months. Nineteen patients were treated between 1985 and 1990 with a dose of 3×10^6 IU daily, with treatment extending for 2–5 years. With a median followup of 12 years (range 2–16), the observed 10-year metastases-free and sarcoma-specific survival rates were 39% and 43%, respectively. Detailed toxicity data was not available for the period following 1979, but excellent compliance with treatment implies no major additional toxicity [18, 19]. Contrasting results have been observed by other investigators. The German/Austrian cooperative study COSS-80 randomized 158 patients with localized osteosarcoma to receive methotvexate and doxorubicin with either cisplatin alone or the combination of bleomycin, cyclophosphamide, and dactinomycin. Patients were also randomized to receive or not receive 22 weeks of interferon-β. Interferon-β was commenced at week 16, consisting of 2 injections weekly for 2 weeks, then daily injections for 4 weeks and then 2 injections weekly for further 16 weeks. The dose of interferon-β was 100,000 U/kg. No significant difference in 30-month continuous disease-free survival was observed between patients treated with and without interferon-β (77% versus 73%, resp.) [20]. The differing results observed in the Scandinavian and German/Austrian studies may be due to the relatively low interferon dose and duration of therapy in the COSS-80 trial. The current European and American Osteosarcoma Study Group (EURAMOS 1) trial randomizes patients with localized osteosarcoma, who have had a good histological response to neoadjuvant chemotherapy, to receive postoperative systemic therapy consisting of methotrexate, doxorubicin, and cisplatin with or without pegylated interferon α-2b. The pegylated preparation of interferon α has an extended half life and consequently can be administered less frequently with higher dose delivery. The results of this large randomized trial will, it is hoped, define the role of interferon in the adjuvant treatment of osteosarcoma.

Ito and colleagues reported decreases in size of lung metastases in 2 out of 3 osteosarcoma patients treated with interferon. Edmonson et al. reported on a Phase II trial of recombinant interferon α-2a in 20 patients with advanced bone sarcomas, 17 of whom had osteosarcoma. Partial tumor regression was documented in 2 patients with osteosarcoma and one with malignant fibrous histiocytoma, for 1, 3, and 2 months, respectively. Three other patients had stable disease (each for 2 months), but all other patients had disease progression.

4. Targeted Immunotherapy

Potential targets for immunotherapy have been divided into five categories: mutated, shared tumor specific, differentiation antigens, overexpressed antigens, and viral antigens [21].

"Mutated" antigens involve a mutation in the cancer not present in normal tissues, thus making the target inherently specific. An example of this in sarcoma is the SYT-SSX fusion protein. This epitopes from this mutant protein have been targeted in two Phase I trials (described below).

"Shared tumor specific" antigens are frequently expressed by a number of malignancies but rarely are expressed by normal tissue. This category includes the cancer testis antigens (described below). These antigens are highly immunogenic and are important for early development. They are frequently seen in the developing embryo but are not found in significant quantities in adults except in the testis and occasionally the placenta.

"Differentiation antigens" are antigens involved in the normal differentiation of a specific tissue type. MART-1 is an example of this type of antigen that has been successfully targeted in melanoma. This protein is expressed as part of the normal differentiation of melanocytes and certain other cells from neural crest tissue. This differentiation antigen appears to be expressed in clear cell sarcoma as well [22].

"Overexpressed" targets are expressed in normal tissue but greatly overexpressed in tumors. This category includes HER2 which is frequently expressed in synovial sarcoma [23]. Some of these overexpressed antigens have been described as "universal antigens," as they may be more uniformly expressed by tumors such as telomerase (hTERT) and survivin; these antigens may be associated with tumorigenic advantage thus targeting these antigens may circumvent the potential for outgrowth of antigen-loss variants [24, 25].

Viral antigens from viruses such as EBV have been shown to present immunogenic epitopes. This strategy may be applicable to Kaposi's sarcoma which is associated with HHV8 [26].

4.1. Cancer Testis Antigens. As described above, the cancer testis antigens (CTAs) are a group of proteins considered to be some of the most exciting potential targets for immunotherapy. Investigators have long sought to characterize specific tumor-associated antigens that would be considered "immunogenic," that is, capable of inducing an immune response. Pioneering work by Thierry Boon and colleagues at the Ludwig Institute for Cancer Research in Brussels uncovered distinct antigens recognized by cytotoxic T lymphocytes (CTLs). This group first described 4 distinct antigens in mice (A, B, C, and D), two of which were products of the same gene, P1A [27, 28]. Following on the heels of this discovery, the Boon group identified the first human tumor-associated T-cell-defined antigen, MAGE-1 (Melanoma Antigen-1, subsequently renamed MAGE-A1) by screening target cells transfected with the cDNA library of a tumor line using autologous tumor reactive antigen-specific CTL.

More T-cell-defined antigens were discovered, and MAGE-1 was eventually recognized to be part of a family of MAGE antigens which represent a broader class of antigens ultimately described by Lloyd Old as "cancer-testis" antigens. These antigens have expression restricted to germline tissues, placental trophoblasts, and a broad range of cancers. To date there are more than 70 CT gene families, many of which are being developed as T-cell targets for vaccine and adoptive cellular therapy [29].

4.2. Cancer Testis Antigen Expression in Specific Sarcoma Subtypes. Only a handful of articles have described cancer testis antigen expression in specific sarcoma subtypes. Complicating matters is that while all cancer testis antigens are by definition immunogenic, they are not all necessarily immunogenic for all individuals. Each CTA has epitopes described for at least one HLA type but many HLA types are quite rare. Since the class I HLA type A*02.01 is relatively common, expressed by about half of the Caucasian population, targeting A*02.01 associated epitopes in pilot immunotherapy trials for sarcoma is a reasonable approach. Some of the commonly expressed cancer testis antigens, for which A*02.01 epitopes have been identified, are NY-ESO-1, LAGE-1, PRAME, MAGE-A3, MAGE-A4, MAGE-A9, and SSX-2. The expression of these antigens in the most common sarcoma subtypes is illustrated in Table 1.

Currently, there is more data available on the expression of these antigens for synovial sarcoma than any other sarcoma subtype. It is well documented that the majority of these tumors express the cancer testis antigen NY-ESO-1, particularly those with monophasic histology where it is frequently expressed homogenously [30]. The biphasic type also expresses NY-ESO-1 in the majority of cases, although not always and occasionally these tumors may only express NY-ESO-1 in one of the biphasic compartments. Synovial sarcomas tend not to express MAGE-A1 or CT7, though little is known about the prevalence of other CT antigens in this histological subtype. One gene microarray study found that all four cases of synovial sarcoma included in that study expressed PRAME [31]. This study included 7 cases of myxoid liposarcoma and 5 nonmyxoid. All the nonmyxoid liposarcoma cases and 1 of the myxoid subtype expressed PRAME. LAGE-1 was expressed in over 70% of myxoid liposarcomas and in 60% of nonmyxoid liposarcomas [31].

One study by the Ludwig group in New York assessed CT antigen expression for a number of different sarcoma subtypes and included 6 liposarcomas [32]. Three expressed LAGE-1.

Less is known about leiomyosarcoma and it is possible that uterine and nonuterine leiomyosarcoma have distinct patterns of CT antigen expression. In the study by Ayyoub et al., for example, four of six uterine leiomyosarcomas examined expressed MAGE-A3, while only one of the seven nonuterine leiomyosarcomas expressed MAGE-A3 [32]. Three of the six uterine leiomyosarcomas expressed NY-ESO and 2 expressed LAGE-1. No nonuterine leiomyosarcomas expressed NY-ESO and only 1 of 7 expressed LAGE-1.

Many leiomyosarcomas, particularly uterine leiomyosarcomas, may express CTAs from the SSX family including SSX-2 which has an A*02.01 epitope. In the study of SSX antigens by Ayyoub et al. 3 of 4 expressed SSX-2 [33].

Among the skeletal sarcomas, osteosarcoma is known to express several CT antigens. One study of CT antigen expression in pediatric solid tumors included 9 osteosarcoma patients. All of these osteosarcoma samples expressed

Table 1: Selected CT antigen expression (all with A*02.01 epitopes) in selected sarcomas.

Sarcoma subtype	Reference	Method	NY-ESO-1	LAGE-1	PRAME	MAGE-A3	MAGE-A4	MAGE-A9	SSX-2
MFH/pleomorphic spindle cell	[32]	RT-PCR	1/6	0/6		1/6	0/6		
	[33]	RT-PCR							1/2
	[31]	Microarray		0/16	1/16			1/16	
Liposarcoma	[32]	RT-PCR	2/2	1/2		1/2	2/2		
	[33]	RT-PCR							1/2
Myxoid	[31]	Microarray		5/7	6/7			5/7	
Nonmyxoid	[31]	Microarray		3/5	5/5			2/5	
Leiomyosarcoma	[31]	Microarray		0/9	3/9			1/9	
Uterine leiomyosarcoma	[32]	RT-PCR	3/5	2/5		3/5	4/5		
	[33]	RT-PCR							3/4
Nonuterine leiomyosarcoma	[32]	RT-PCR	0/7	1/7		1/7	2/7		
	[32]	RT-PCR							0/1
Synovial sarcoma	[32]	RT-PCR	2/2	1/2		1/2	2/2		
	[30]	IHC	20/25						
	[31]	Microarray		3/4	4/4			3/4	
Skeletal sarcomas									
Osteosarcoma	[32]	RT-PCR	0/1	0/1		0/1	0/1		
	[33]	RT-PCR							0/1
	[34]	qRT-PCR	8/9 (NY-ESO + LAGE)			9/9	4/9		
Ewings Sarcoma	[34]	qRT-PCR	0/18 (NY-ESO + LAGE)			5/18	4/18		
Chondrosarcoma	[32]	RT-PCR	2/2	2/2		2/2	1/2		

MAGE-A3 by real-time PCR and all but one expressed LAGE-1/NY-ESO [34]. By contrast, few of the Ewing's sarcoma patients in that study expressed cancer testis antigens.

4.3. Vaccine-Based Trials. A number of small trials have immunized patients against sarcoma achieving varying levels of success using a variety of different vaccines. Some of these trials have targeted well-defined antigens, others have targeted tumor lysate. In one such trial, sarcoma patients received an intradermal injection of irradiated autologous tumor cells grown in culture to vaccinate against antigens that would be released from these dying cells. Almost all of the patients also received either interferon gamma or GM-CSF as an adjuvant. An immune response was demonstrated using a delayed-type hypersensitivity (DTH) skin test against autologous tumor which converted from negative to positive in 8 of 16 evaluable patients. Median survival was 16.6 months among patients who were DTH responders compared with 8.2 months in those who were nonresponders. This was a statistically significant difference that is hypothesis generating but is of questionable causality. There were no objective responses among the study participants with measurable disease. Of note, the study included one patient with resected pulmonary metastatic disease (without measurable disease at the time of vaccination) who was disease-free over 3 years following vaccination [6].

One vaccine trial gave intradermal injections of dendritic cells pulsed with autologous tumor lysate [35]. Ten pediatric patients were treated; one patient with fibrosarcoma had a partial response to the treatment which included the complete regression of several sizable pulmonary sites of metastatic disease.

The largest dendritic cell vaccine trial to date for the treatment of patients with sarcoma targeted recurrent or metastatic Ewing's sarcoma family tumors or alveolar rhabdomyosarcoma having a t(2;13) or t(11;22) translocation. Patients were treated with dendritic cells pulsed with tumor-specific peptides derived from the fusion proteins as a consolidative therapy after patients achieved a complete remission. Improved survival was seen in the group of patients receiving vaccination compared with those undergoing leukapheresis but not receiving vaccination. However, this was a nonrandomized study in which patients not receiving vaccination were more likely to have progressive disease or declining performance status [36]. In the Phase I trial of these vaccines 16 patients with bulky metastatic disease were treated, one patient had a mixed response and three patients had stabilization of disease [37].

In the posttransplant setting, a dendritic cell vaccine trial was administered to 5 children with residual tumors following autologous transplantation [38]. Three patients received dendritic cells pulsed with tumor lysate. Two patients received dendritic cells, pulsed with three synthetic tumor-specific peptides related to either the SYT-SSX2 translocation sometimes seen in synovial sarcoma or the EWS-FLI-1 fusion gene often seen in Ewing's sarcoma. One patient had a complete response that was durable for over 77 months and was ongoing at the time of the report. This was the only patient with Ewing's sarcoma receiving DCs pulsed by EWS-FLI-1-related synthetic peptides and suggests

that these peptides may be worthy of further study. Two other patients had stabilization of disease but ultimately progressed.

Several studies have used vaccines of peptide alone. One trial focused on the study drug 105AD7, a vaccineagainst the complement regulatory protein CD55 frequently overexpressed in osteosarcoma, was able to induce cytokine production and antibody production in patients although clinical response was modest [39, 40].

A peptide encompassing the SYT-SSX fusion region of the gene resulting from the t(*X*;18) translocation has been used to vaccinate six HLA-A*24.02 positive patients. The peptide vaccine succeeded in generating peptide-specific CTLs that were successfully detected from four patients following vaccination although all patients had negative DTH skin testing. None of the patients experienced an objective clinical response although one patient's disease stabilized [41]. The same group has produced interesting *in vitro* data showing that while CTL generated to the wild-type peptide killed tumor relatively poorly (the peptide used for the vaccine), a one amino acid substituted K9I peptide (also an A*2402 associated epitope) produced CTL which killed tumor far more effectively [42].

There is an on-going randomized placebo controlled multicentered Phase II trial of a trivalent peptide vaccine to the gangliosides GD2, GD3, and GM2 in patients with stage IV sarcoma who have no evidence of disease following resection. These gangliosides are thought to play a role in cell adhesion and cell-cell interactions. They are usually expressed by melanomas and also may be expressed by some sarcomas [43, 44], and in Ewing's and osteosarcoma in particular [45, 46]. Moreover, soft tissue sarcoma patients frequently develop an antibody response to GD2 compared with healthy controls [47]. However, the promise of this vaccine must be tempered by the fact that a randomized trial in melanoma failed to demonstrate improvement in patient-related outcome measures [48].

4.4. Adoptive Immunotherapy. In adoptive immunotherapy, patients are treated with autologous lymphocytes taken from a patient and expanded *ex vivo*. Some of the most impressive clinical results have come from studies using tumor-infiltrating lymphocytes (TILs) in patients with melanoma. In these studies, tumor is taken from a patient and the lymphocytes are separated and expanded *ex vivo* and then reinfused following patient lymphodepleting conditioning. The most promising results were those patients whose condition involved an intensive regimen requiring autologous transplant with total body irradiation, cyclophosphamide and fludarabine conditioning followed by high-dose IL-2 postinfusion. The median survival for metastatic melanoma is less than a year, however, a 2-year survival rate of over 40% has been reported using this adoptive immunotherapeutic approach. It should be noted that considerable toxicity has been reported in these trials [49]. Though some early work did seem to demonstrate that TIL could often be grown in culture from patients with sarcoma, although with lower yield compared with other tumor types, little follow-up work has been done [50, 51]. However, given that a number of sarcoma subtypes do often have tumor infiltrating lymphocytes (unpublished data), this may be an area deserving further study.

Furthermore, as more has been learned about the potential targets for adoptive immunotherapy, greater interest has been given to developing T cells targeted towards specific antigens either by isolating rare tumor targeted cells from a patient's peripheral blood or by genetically modifying T cells to target a specific antigen. Given the frequent expression of CT antigens in certain sarcoma subtypes, sarcoma may be an ideal target for antigen-specific adoptive immunotherapy. The Rosenberg group at the NCI has begun treating synovial sarcoma patients with lymphocytes using a transduced T-cell receptor specific for NY-ESO-1.

5. Conclusion

While past attempts to use immunotherapy have failed to dramatically shift the paradigm of care for the treatment of patients with sarcoma, a great opportunity exists to shape the future. Nonspecific immunomodulation with the use of muramyl tripeptide phosphatidylethanolamine in resected osteosarcoma has shown a significant survival benefit. Other immune approaches have shown signals of potential in isolated patients with dramatic responses to immunotherapy.

A greater understanding of the immune system and the ability to harness more potent approaches to utilize the ability of the immune system to fight cancer could result in advances in the treatment of sarcoma. There remains a need for novel effective therapy in advanced soft tissue sarcoma, particularly in chemoresistant subtypes where no conventional systemic therapy is available. Emphasis on the immunological characteristics of individual sarcoma subtypes and the consequent tailoring of therapy could increase the therapeutic options available. The exact role of immunotherapy in sarcoma is yet to be delineated. It is hoped with well-designed, multiinstitutional clinical trials that this treatment approach will result in improvements in survival in this challenging group of diseases.

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Review Article

Targeting the Insulin-Like Growth Factor Pathway in Rhabdomyosarcomas: Rationale and Future Perspectives

Ana Sofia Martins,[1] David Olmos,[1,2] Edoardo Missiaglia,[1,3] and Janet Shipley[1]

[1] *Molecular Cytogenetics, The Institute of Cancer Research, 15 Cotswold Road Sutton, Surrey SM2 5NG, UK*
[2] *Sarcoma Unit, The Royal Marsden NHS Foundation Trust, London SW3 6JJ, UK*
[3] *Bioinformatics Core Facility, Swiss Institute of Bioinformatics, 1015 Laussane, Switzerland*

Correspondence should be addressed to Janet Shipley, janet.shipley@icr.ac.uk

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Rhabdomyosarcomas (RMS) are a heterogeneous group of tumors that share features of skeletal myogenesis and represent the most common pediatric soft tissue sarcoma. Even though significant advances have been achieved in RMS treatment, prognosis remains very poor for many patients. Several elements of the Insulin-like Growth Factor (IGF) pathway are involved in sarcomas, including RMS. The IGF2 ligand is highly expressed in most, if not all, RMS, and frequent overexpression of the receptor IGF1R is also found. This is confirmed here through mining expression profiling data of a large series of RMS samples. IGF signaling is implicated in the genesis, growth, proliferation, and metastasis of RMS. Blockade of this pathway is therefore a potential therapeutic strategy for the treatment of RMS. In this paper we examine the biological rationale for targeting the IGF pathway in RMS as well as the current associated preclinical and clinical experience.

1. Introduction

Rhabdomyosarcomas (RMS) are the most common soft tissue sarcoma of childhood [1, 2] with an incidence of 4.5 cases per million children/adolescents per year in the United States [3]. They are divided in two main histological variants: Embryonal (ERMS, 60–70% of all RMS cases) and Alveolar (ARMS, approximately 30%). Other minor variants include botryoid RMS, considered a subgroup of ERMS, and pleomorphic RMS, that occur in adults [1, 2, 4]. ERMS are predominant in younger patients and are generally associated with a good outcome in nonmetastatic cases, while ARMS are considered to be a tumor of adolescents and young adults that generally have a worse prognosis [2, 3, 5, 6]. The majority of ARMS are characterized by specific translocations between the DNA binding encoding domain of either the *PAX3* or *PAX7* genes and the transactivation encoding domain of *FOXO1* [7–9]. Rare variants involve fusion of the *PAX3* gene to members of the nuclear receptor transcriptional coactivator family of genes [10]. An estimated 30% of all histopathologically defined ARMS do not have these fusion transcripts [11] and recent gene expression profiling studies have indicated that these tumors biologically and clinically are more similar to ERMS than fusion gene positive ARMS [12, 13]. Other genetic events are associated with these tumors including those considered to cooperate with the fusion gene product in ARMS such as *MYCN* amplification and overexpression, and mutation of *TP53* [14–19]. ERMS are not characterized by specific fusion genes but are aneuploid with frequent gain of chromosome 8 and have activating mutations of *RAS* genes [20, 21]. Another frequent genetic alteration present in RMS is loss of heterozygosity (LOH) at the 11p15.5 locus. The region includes the genes *IGF2*, *H19*, and *CDKN11C* that are all subject to parental imprinting which can be aberrant in RMS and result in loss of imprinting (LOI) [22, 23]. In both ARMS and ERMS loss of heterozygosity or imprinting is thought to lead to overexpression of the gene encoding the insulin-like growth factor 2 (IGF2). Furthermore, overexpression of a receptor for this growth factor, IGF1R, is frequently found in RMS, occasionally associated with genomic amplification events [24]. Evidence supports IGF1R signaling in the genesis, growth, proliferation and metastatic

behavior of RMS [25–27]. As the prognosis of RMS patients with metastatic or recurrent disease is still very poor, with only 30–40% achieving a cure, there is an urgent need to develop better therapies to treat these patients. In this paper we describe the evidence that implicates components of the IGF pathway in RMS development and examine the biological rationale for therapeutically targeting this pathway. We also consider the current preclinical and clinical experience with targeted approaches for treating RMS and suggest potential improvements that may be possible with combination strategies.

2. IGF Signaling in RMS

Components of the IGF pathway consist of 3 ligand molecules (IGF1, IGF2 and insulin), 6 binding proteins (IGFBP1 through to IGFBP6), and 4 receptors (IGF1R, IGF2R, IR and hybrid receptors). These orchestrate a cascade of signals (Figure 1) involved in numerous developmental and mitogenic pathways that lead to cellular processes such as activation of cell proliferation, invasion, and angiogenesis as well as inhibition of apoptosis [28, 29]. IGF2 and IGF1R are two components of the signaling pathway that are known to play a significant role in RMS oncogenesis.

2.1. IGF2 in RMS. IGF2 is normally expressed in the liver and other extrahepatic sites, similar to IGF1. Unlike IGF1, IGF2 expression in mammals is not just regulated by growth hormone (GH). However, the mechanisms regulating IGF2 expression remain uncertain. IGF2 is the predominant circulating IGF, with plasma levels 3- to 7-fold higher than IGF1 [31, 32].

In RMS, several studies have shown overexpression of IGF2 in both cell lines and primary tumors [25, 33]. This is confirmed by our analysis of expression profiling data for a panel of RMS patient samples (Figure 2). LOH and LOI are the principal mechanisms underlying these IGF2 expression levels [22, 34]. In most nonmalignant tissues, *IGF2* is transcribed from the paternal allele, with the maternal allele being imprinted and consequently silenced by methylation. The imprinting of *IGF2* is influenced by the product of the downstream *H19* gene, with these two genes showing opposite imprinting patterns and transcription from *H19* occurring from the maternal allele. The process of LOI leads to biallelic expression (both paternal and maternal alleles) of the *IGF2* gene and IGF2 overexpression [23, 35]. LOH has been shown for ERMS in particular, with loss of the maternal 11p15.5 locus and duplication of the paternal *IGF2* allele (paternal isodisomy) that results in expression from the two paternal genes [36].

It has also been shown that increased IGF2 expression could be due to enhanced expression of transcriptional initiators such as AP-2 [37]. Potential AP-2-binding sites have been identified in the promoters of both the *IGF2* and *IGF1R* genes with an increase in AP-2-dependent *IGF2* mRNA expression found in RMS cases compared to normal skeletal muscle. In addition, loss of p53 has been shown to be associated with increased expression of IGF2 in RMS, even though the mechanisms supporting this are not fully elucidated [38]. The consistent overexpression of IGF2 in both ERMS and ARMS [25, 39] has led to the suggestion that IGF2 could be used as a marker for their differential diagnosis [25].

El-Badry and colleagues first demonstrated that IGF2 was acting as an autocrine and paracrine growth factor stimulating cell line growth and motility in RMS [27]. Later on, the same group investigated the potential of IGF2 to activate IGF1R and IGF2R and showed that the mitogenic response was primarily mediated though IGF1R [26].

Based on the fact that PAX3, PAX7, and IGF2 are involved in growth and differentiation, Wang and colleague's investigated the potential oncogenic cooperation between IGF2 and PAX3 or the PAX3-FOXO1 fusion protein. Mouse myoblasts transfected to express IGF2 alone or cotransfected to also express either PAX3 or PAX3-FOXO1 were transformed *in vitro* and could form tumors *in vivo* [40]. Only cells expressing both IGF2 and PAX3-FOXO1 developed invasive, poorly differentiated tumors with low rate of apoptosis. It has also been shown that the PAX3-FOXO1 fusion protein can induce both IGF2 and IGF1R expression that results in enhanced IGF signalling [41, 42].

IGF2 appears to be consistently overexpressed and acts as an autocrine/paracrine growth factor signaling through IGF1R in RMS. Its likely key role in the development and progression of both ARMS and ERMS is consistent with therapeutically targeting this pathway for the treatment of patients with RMS.

2.2. IGF1R in RMS. IGF1R is a transmembrane receptor with two extracellular ligand-binding α-subunits and two β-subunits forming the transmembrane and tyrosine kinase catalytic domains that are linked by disulfide bonds. It is primarily activated by its cognate ligands, IGF1 and IGF2 (IGF2 with 2- to 15-fold lower affinity) and by insulin with a lower affinity [28, 43–45]. The binding of the ligands to the cysteine-rich domain of the α-subunits leads to a conformational change of the β-subunit, stimulating the tyrosine kinase activity. This is followed by autophosphorylation of a cluster of tyrosine residues on the β-subunits of the intracellular domains. Subsequently, insulin receptor substrates (IRSs) 1 to 4 and the Src homology collagen-like adaptor proteins (Shc) bind to the juxtamembrane domain of the β-subunit, initiating alternative intracellular signaling cascades [46–48]. One of these pathways leads to PI3K-AKT-mTOR activation, while another results in MAPKs (Mitogen-Activated Protein Kinases) activation (Figure 1). Depending on the cellular context, the activation of these pathways results in cell proliferation, protein synthesis, and/or inhibition of apoptosis. IGF1R signaling can also lead to disregulation of cellular adhesion and motility, and the stimulation of myogenic differentiation in RMS [26, 27, 49, 50].

Both RMS tumors and cell lines express IGF1R [27], with IGF1R protein detected in more than 80% of all RMS cases without significant differences between ARMS and ERMS [51]. This is consistent with expression at the RNA level in our analysis of primary RMS patient data (Figure 3). An elevated level of receptor expression has been found to be

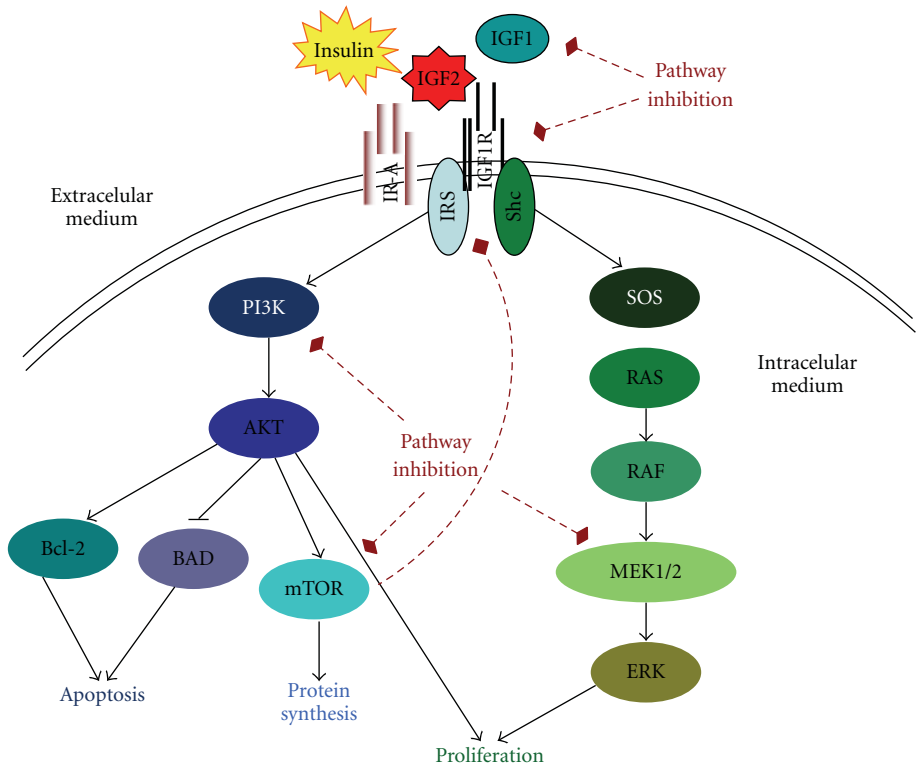

Figure 1: A simple schema of the IGF pathway and approaches to its inhibition. Insulin, IGF2 and IGF1 bind to their specific receptors including IGF1R, IGF2R, IR and hybrid receptors. Ligand binding results in the autophosphorylation of the tyrosine residues on each receptor, leading to recruitment of the adaptor proteins IRS and Shc to the receptor β-subunits intracellular domains. This process activates different signaling cascades through the PI3K-AKT and the RAS/RAF/MEK/ERK pathways resulting in stimulation of translation and cell cycle progression, increased proliferation and growth and inhibition of apoptosis. The dashed arrows indicate potential feedback mechanisms and points for strategic intervention to inhibit IGF1R signaling using anti-IGF1R mAbs or tyrosine kinase inhibitors (TKIs). Relevant downstream intracellular tyrosine kinase proteins to inhibit include PI3K, AKT, RAF, MEK and mTOR.

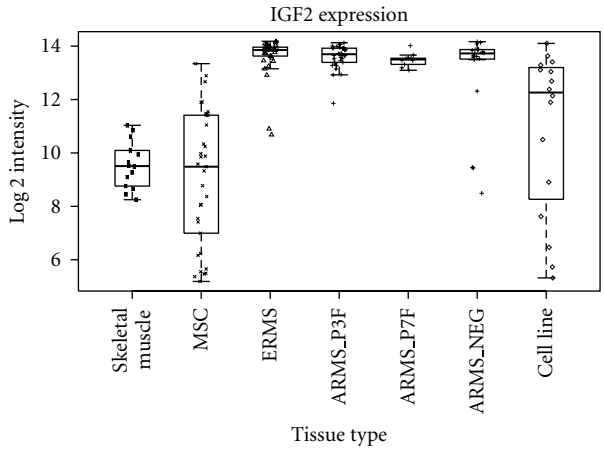

Figure 2: Levels of RNA expression for IGF2 derived from expression profiling data (Affymetrix HGU133plus2) in a panel of different tissues samples. These include normal skeletal muscle (Skeletal muscle), mesenchymal stem cells (MSC), ERMS, ARMS (*PAX3-FOXO1* and *PAX7-FOXO1* fusion positive, ARMS_P3F and ARMS_P7F and fusion gene negative ARMS_NEG) cases [13] and RMS cell lines (RH3, SCMC, RMS, RH30, RD, RMS-YM, RH18, Ruch3, T91-95, RH41, TE617T, Hs729T, T174, TE441T, Ruch2, and RH4) [30].

associated with inferior survival rates [52] and has been used as biomarker for response to targeting the pathway in RMS preclinical models [53]. In this work it has been shown that, even though IGF1R was expressed in almost all samples studied, there was a large variation in expression levels that correlated with different levels of dependence on IGF1R prosurvival signaling. This led to proposing the notion of addiction to IGF1R in some tumor cells.

2.3. IR-A in RMS. The Insulin Receptor (IR) and IGF1R have evolved from a common ancestral gene encoding proteins with related functions and a very similar tetrameric structure; 2 α-subunits containing ligand-binding domains and 2 β-subunits with tyrosine-kinase domains [54, 55]. Cells and tissues coexpress both receptors and hybrid receptors can be formed by one α- and one β-subunit IR heterodimer, and one α- and one β-subunit IGF1R heterodimer [56, 57]. Furthermore, IR has two different isoforms: IR-A (or fetal) and IR-B (classic), which are determined by alternative splicing mechanisms (IR-A lacks exon 11) [58, 59]. Even though IR-A expression in adult cells is much lower than IR-B, this is not the case for cancer cells [60], but the factors

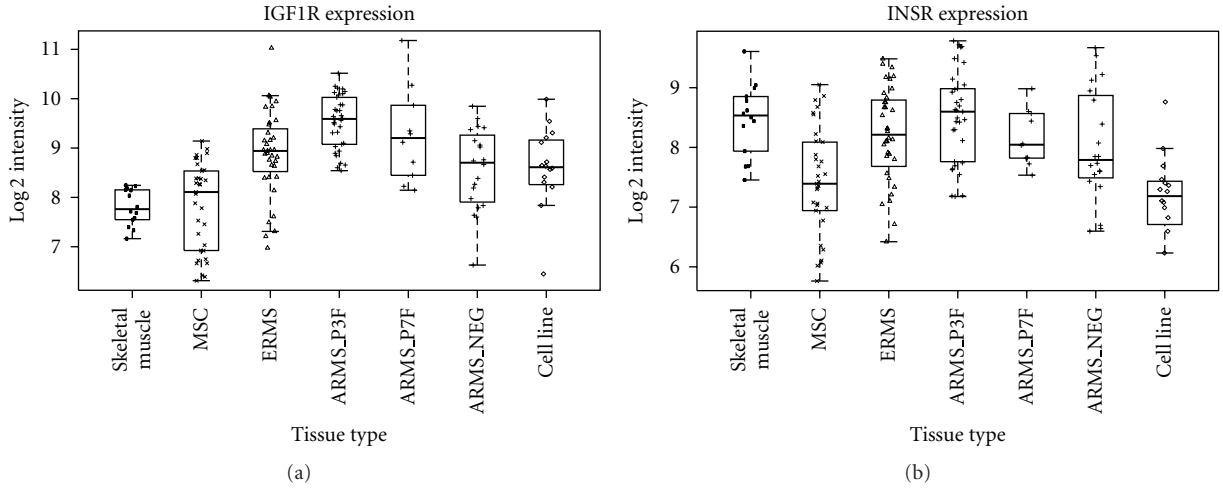

Figure 3: Levels of RNA expression of IGF1R and INSR (both isoforms of the insulin receptor combined) derived from Affymetrix HGU133plus2 expression profiling data of a panel of different tissues samples. These include normal muscle (Skeletal muscle), mesenchymal stem cells (MSC), ERMS, ARMS (*PAX3-FOXO1* and *PAX7-FOXO1* fusion positive, ARMS_P3F and ARMS_P7F and fusion gene negative ARMS_NEG) cases [13] and RMS cell lines (RH3, SCMC, RMS, RH30, RD, RMS-YM, RH18, Ruch3, T91-95, RH41, TE617T, Hs729T, T174, TE441T, Ruch2, and RH4) [30].

contributing to the switch from isoform B to A expression in cancer are poorly understood [59, 61]. Increased expression of IR-A has been reported in carcinomas of breast, colon, lung, thyroid, and ovary [59]. Similarly, an elevated level of IR-A expression has been seen in osteosarcoma [62] and leiomyosarcoma [63] cell lines although the situation in RMS is currently unknown. In addition, IR-A is frequently expressed in solitary fibrous tumors samples (whilst IGF1R is not usually detected) [64] and is essential for virus-induced malignant transformation in Kaposi's sarcoma [65].

Phosphorylated IR in RMS has been described *in vitro* [66]. An increase in tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) has also been reported in RMS. In poor prognosis patients this IRS-1 activation seems refractory to a negative feedback loop mediated by increased phosphorylated mTOR and 70S6 levels [52] which are observed in normal cells and RMS with a favourable prognosis. Thus, these facts support a persistent activation of the IR-IGF1R-mediated survival signaling in RMS patients, which may contribute to a worse prognosis in this malignancy.

3. Targeting the IGF Pathway in RMS

IGF1R has been acknowledged as a biologically relevant target in pediatric sarcomas for some time, but it has been difficult to, target it therapeutically due to its similarity to the IR and the toxicities associated with nonspecific inhibition. Nevertheless, in the last few years, new agents have emerged and have shown promising results. Essentially, the strategies for blocking or disrupting IGF1R include (a) the reduction of ligand levels or bioactivity, (b) the inhibition of receptor function using receptor-specific antibodies or small-molecule tyrosine kinase inhibitors (TKIs), or (c) inhibition of its downstream signaling molecules [67].

3.1. Targeting the Ligands. The disruption of the hypothalamus-hypophysis axis, and thus the clinical inhibition of GH release, can result in a decrease of circulating levels of IGF. Thus the disruption of this axis has been proposed as a potential strategy to reduce IGF in those cases where there is a background of elevated endocrine IGF release such as Beckwith-Wiedemann Syndrome which is associated with high rate of tumors in childhood, including RMS [68]. Another approach consists in reducing the concentrations of free active ligands using monoclonal antibodies against IGFs. DX-2647 is an antiligand monoclonal antibody which blocks IGF2, and also, but with less affinity, IGF1. Recently, this antibody has shown potential antitumor activity in human hepatocarcinomas xenografts [69], a tumor where upregulation of IGF2 expression is a common alteration. Even though there is not yet data available in sarcomas, this seems a plausible option for investigation in RMS where IGF2 is commonly upregulated. Other novel strategies to lower the ligand bioactivity may include recombinant IGFBPs [70]. *In vivo* experiments using the RMS cell line RH30 have shown that IGFBP-6 overexpression resulted in a marked delay in tumor growth in nude mice [71]. IGFBP-6 is unique among other binding proteins because of its binding specificity for IGF2. IGF2 has a higher affinity for IGFBP-6 than for IGF1R [72] suggesting that IGFBP-6 can reduce the levels of free active IGF2, preventing its binding to the receptor.

3.2. Targeting IGF1R. At the time of this paper, mAbs against IGF1R represent the most tangible clinical option, but there are also numerous small molecule tyrosine kinase inhibitors (TKIs) against IGF1R currently undergoing clinical evaluation [73]. Some of these small molecules also inhibit IR-A [74].

RMS cell lines secreting IGF2 have been shown to be able to grow in serum-free media. Under the same conditions, treatment of these cells with an antibody against IGF1R significantly inhibited cell growth suggesting that IGF2 functions as an autocrine and paracrine growth factor in RMS [27].

Overall, inhibition ligand binding using competitive antibodies and TKI have both been shown to block IGF1R activity resulting in inhibition of RMS cell proliferation, increased apoptosis, and cell cycle arrest [75, 76]. Furthermore suppression of vasculogenesis has also been demonstrated *in vitro* and *in vivo* xenograft models [77]. *In vivo*, tumor formation and growth of RMS cells was inhibited by treating mice with an antibody antagonistic against IGF1R [53, 78, 79] or with TKIs [75, 76, 80]. The most effective antibodies against IGF1R include αIR3, which detects the α-subunit of IGF1R [78], and IMCA-12 [81]. The latter has shown promising results in the Pediatric Preclinical Testing Program [81]. Regarding the TKIs, we can highlight NVP-AEW541 [75, 76] and BMS-754807 [80] as two promising molecules to move towards testing at the clinical level.

Other approaches for investigating the role of IGF1R have also been optimized recently, including using antisense RNA to reduce levels of expression and expression of a kinase-deficient form of this receptor [82, 83]. Both approaches resulted in tumor suppression.

3.3. Targeting Pathways Downstream of IGF1R. Recently, it has been shown, both *in vitro* and *in vivo*, that IGF1R survival signaling in RMS is primarily maintained through the AKT pathway, and that effective disruption of the IGF1R survival signaling results in decreased AKT activation [84]. However, activation of the PI3K pathway downstream of IGF1R and IR is subject to a negative feedback loop by mTOR through inhibition of IRS1 [85] (Figure 1). This is especially important in view of the fact that the combination of an antibody targeting IGF1R combined with an mTOR inhibitor, such as rapamycin, is predicted to inhibit RMS cell growth more effectively than either agent used alone. Indeed, an increase in AKT activation was found in RMS cells after rapamycin treatment with a more efficient inhibition of RMS growth both *in vitro* and *in vivo* when combined with an IGF1R antagonistic antibody [86, 87]. It has been described that patients with an increased phosphorylation of AKT, that result from a disruption in the feedback mechanism between mTOR and IRS, have a poorer survival [52]. Preclinical studies have also recently shown that targeting MEK/ERK (using the MEK/ERK inhibitor U0126) also leads to growth arrest of RMS tumors in an *in vivo* xenograft model [88]. All of these results provide preclinical evidence to support the use of signal transduction-based targeting of AKT/MEK in strategies for treating RMS.

4. Clinical Targeting of IGF1R in RMS: Evidence and Trends

In recent years, several agents against IGF1R have entered clinical trials of various tumor types, including sarcomas and RMS. A small number of clinical responses in patients with sarcomas have been reported across the different phase I clinical trials using IGF1R antibodies [89–91] and have raised hope for the success of this therapeutic modality. However, objective radiological responses were generally limited to patients with Ewing's sarcoma [89–91], with occasional prolonged (>6 months) disease stabilisation and clinical benefit in other sarcomas subtypes [89]. To our knowledge, only 2 patients with RMS were enrolled in these early trials. Both cases were heavily pretreated metastatic ARMS and both progressed within 6 weeks of starting treatment on figitumumab (a monoclonal antibody against IGF1R) [89]. More recently, in a preliminary report of the SARC011, a phase II trial in multiple sarcoma types, described 3 objective radiological responses in patients with RMS treated with the anti-IGF1R antibody R1507 [92]. However, more mature data in Ewing's sarcoma has shown that many responses only lasted for a finite period of time [93, 94].

Despite the difficulties of drawing conclusions from small numbers of RMS patients treated with anti-IGF1R antibodies, it is plausible to suggest that such single agent therapy in RMS might be insufficient to cause a clinically significant and persistent disruption in the IGF-mediated survival signalling, as seen in other neoplasias where IGF2 plays a relevant role [95]. Some preclinical studies have indicated that there are different binding epitopes on IGF1R that have differing biological activities [96] and different antibodies with distinct mechanisms of action to these epitopes [97]. Furthermore, combination strategies focused on blocking both IGF1 and IGF2 with two different inhibitory antibodies which resulted in enhanced inhibition of intracellular signalling through the IGF1R axis *in vitro* and *in vivo*, when compared to the activity of either single antibody alone. This effect was even more evident at high ligand concentrations where efficacy of monotherapy was relatively reduced [98]. A similar effect could be achieved by small molecule TKIs although few are currently in clinical development [73]. However, only prolonged disease stabilisation is reported in sarcoma patients treated within the OSI 906 (a TKI) phase I trial, although RMS patients were not included [99].

The efficacy of clinical strategies targeting IGF1R alone in RMS may be compromised due to the potential of cells to bypass the requirement for IGF2. Recently, it has been shown that IGF2 signaling can directly promote carcinogenesis in transgenic pancreatic neuroendocrine xenograft (an IGF2 dependent model) through IR binding [100]. Thus, RMS clinical alternatives could include the inhibition of both IGF1R and IR, using TKIs such as OSI-906 with activity against both IGF1R and IR-A [99]. However, this would potentially result in a higher metabolic toxicity.

An alternative approach is to inhibit the IGF1R/IR downstream signaling cascade with PI3K/AKT/mTOR and/or Raf/Ras/MEK/ERK inhibitors. There are several molecules against these targets that have been recently tested in patients with various tumor types. Some of these, as single or combinations of agents, are currently undergoing pivotal phase III trials for regulatory approval in solid tumors other than sarcoma [101, 102]. Many agents have shown an adequate

toxicity profile in phase I dose-finding studies and phase II trials, but to date, the clinical results with novel drugs in sarcomas, and specifically RMS in children, are limited. The largest experience in sarcomas has been provided with the study of mTOR inhibitors, particularly with compounds similar to rapamycin such as ridaforolimus, everolimus, and temsirolimus. These have shown some activity in adult soft tissue sarcomas [103]. Combining inhibitors of IGF1R/IR downstream signaling cascades, such as mTOR inhibitors, with an inhibitor of IGF1R also represents an attractive approach. A preliminary phase I trial report for figitumumab in combination with the mTOR inhibitor everolimus has shown activity in various sarcomas, including solitary fibrous tumors [104], which are characterised by the expression and secretion of high molecular weight proforms of IGF2 ("big"-IGF2) [105, 106] and constitutive activation of IR-A but not IGF1R [64]. Similar trials that include RMS are either ongoing or planned.

Another strategy to consider is decreasing the levels of bioactive ligands using anti-IGF antibodies. Reducing circulating IGF has been unsuccessfully with somatostain analogues such as octreotide [107]. Recently, a human recombinant GH receptor antagonist, called pegvisomant, has been successful in tests for the treatment of acromegaly [108]. This pegylated recombinant human analogue of GH can decrease production and release of both IGF ligands [109]. Neither octreotide nor pegvisomant would impact on the paracrine IGF2 levels when they are genetically upregulated within the tumor—which is the case in RMS, but there is epidemiological evidence to support a role of the GH-regulated IGFs secretion in the promotion, progression, and maintenance of tumors in childhood and adolescence. Currently, a phase I clinical trial of figitumumab in combination with pegvisomant (NCT00976508) [110] is active in adults patients with solid tumors, but it will also enroll patients 10 years or older with refractory sarcomas.

A final clinical strategy in RMS could be sequential or parallel IGF1R pathway blockade combined with inhibition of the Erb2 [53] or PDGFRα [111] axes, that are potentially involved in resistance to IGF1R therapies. These pathways in themselves may also be of therapeutic benefit to inhibit in some RMS [112–116]. One way to address the issue of controlling drug sensitivity, as well as pathway cross talk, is to control the response to stress response mechanisms associated with drug treatment. Heat shock stress is a cellular response to stress induced by drug treatment in which the cell increases the expression of several key molecules, called heat shock proteins (HSPs), in order to protect against the effects of treatment. HSPs are chaperone proteins that help to maintain protein stability, renature unfolded proteins, or target their degradation [117, 118]. Several of these HSP client proteins are involved in signal transduction pathways that lead to proliferation, apoptosis, or cell cycle progression in several cancers, which is precisely the case for IGF1R [119, 120]. Therefore, HSP inhibition is a therapeutic strategy to inhibit multiple receptor pathways. IGF1R chaperoning by HSP90 and its possible relationship with resistance to IGF1R targeting has been shown in Ewing's sarcoma. HSP90 was differentially expressed between Ewing's sarcoma cell lines sensitive versus resistant to treatment and HSP90 inhibition reduced Ewing's sarcoma cell line growth and survival, especially in the cell lines resistant to IGF1R inhibitors [121]. An analogous situation may be the case for RMS. It has been shown that HSP90 inhibitors, geldanamycin, and its analogs, can profoundly affect the proliferation of RMS cells, including inducing apoptosis and downregulating the expression of AKT [122].

5. Conclusions

There is a large amount of preclinical, clinical, and epidemiological data supporting targeting the IGF1R pathway in sarcomas, and specifically RMS. The activity of IGF1R monoclonal antibodies has been confirmed by the early reports of clinical activity in Ewing sarcoma [89–91, 94]. However, in RMS patients, despite some responses observed with R1507 [92], targeting IGF1R alone does not seem the optimal strategy due to the complexity of this pathway and the key role of IGF2 in this pathology. To extend the benefits of these therapeutic approaches there is an urgent need to identify predictive biomarkers to improve patient selection and facilitate the development of rational combination regimens. It is likely that a suite of biomarkers, both in the host and tumor [73] will be required rather than single biomarker selection, with some candidates for study in RMS including IGF2, pIGF1R/IGF1R, IGF1, pIRS-1/IRS-1, pIR-A/IR-A, IGFBP-6, and maybe others such as HSPs, PDGFR and Erb2.

Conflict of Interests

The authors have no conflict of interest to declare.

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Review Article

Receptor Tyrosine Kinases as Therapeutic Targets in Rhabdomyosarcoma

Lisa E. S. Crose[1] and Corinne M. Linardic[1,2]

[1] *Department of Pediatrics, Duke University Medical Center, Durham, NC 27710, USA*
[2] *Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA*

Correspondence should be addressed to Corinne M. Linardic, linar001@mc.duke.edu

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Rhabdomyosarcomas (RMSs) are the most common soft tissue sarcomas of childhood and adolescence. To date, there are no effective treatments that target the genetic abnormalities in RMS, and current treatment options for high-risk groups are not adequate. Over the past two decades, research into the molecular mechanisms of RMS has identified key genes and signaling pathways involved in disease pathogenesis. In these studies, members of the receptor tyrosine kinase (RTK) family of cell surface receptors have been characterized as druggable targets for RMS. Through small molecule inhibitors, ligand-neutralizing agents, and monoclonal receptor-blocking antibodies, RTK activity can be manipulated to block oncogenic properties associated with RMS. Herein, we review the members of the RTK family that are implicated in RMS tumorigenesis and discuss both the problems and promise of targeting RTKs in RMS.

1. Introduction

The most common soft tissue sarcomas of childhood and adolescence are rhabdomyosarcomas (RMSs). These malignancies express skeletal muscle markers but are believed to be the result of dysregulated skeletal muscle differentiation of mesenchymal precursors. Like other sarcomas, RMS tumors are molecularly diverse; histological classification separates RMS into two major types, embryonal (eRMS) and alveolar rhabdomyosarcoma (aRMS). As the name implies, eRMS tumors consist of cells morphologically similar to embryonic muscle precursors. The histology of aRMS tumors is distinctive, with clusters of primitive, round cells and open spaces between cell sheets developing upon fixation in formalin, vaguely resembling lung alveoli [1]. The eRMS and aRMS subtypes differ not only in histological appearance but also in prognosis. Patients with eRMS have a generally favorable prognosis, while patients with aRMS do significantly worse, with a five-year survival rate of less than 50% [2]. Furthermore, aRMS can be specified by the presence of a chromosomal translocation resulting in a *PAX3-FOXO1* (or the less frequent *PAX7-FOXO1, PAX3-NCOA1*, or *PAX3-NCOA2* [3]) gene product. When metastatic, *PAX3-FOXO1*-positive aRMS patients survive in fewer than 10% of cases [4]. Although staging of RMS still utilizes histology, recent gene profiling studies have suggested that a more accurate classification of RMS might be as fusion gene positive or negative [5, 6]. Thus, modified classification of RMS may lead to better risk stratification at diagnosis and direct appropriate therapy.

Treatment for RMS has depended on a multimodal approach of surgery, chemotherapy, and radiation. This team strategy has resulted in an overall survival of RMS at about 70% [7]. But as described above, high-risk patients have a poor prognosis, and treatment options are limited. It is believed that without targeted therapies specific for genetic abnormalities associated with RMS, the survival rate will not improve.

Over the past two decades, research into the molecular mechanisms of RMS has identified key genes and signaling pathways involved in disease pathogenesis. Opportunely, many groups have identified favorable molecular targets for

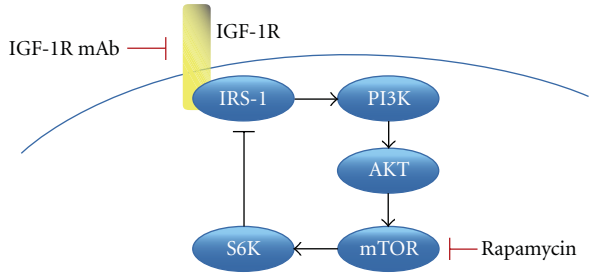

Figure 1: Rationale for dual treatment targeting the IGF-1R signaling pathway in RMS. Rapamycin inhibits mTOR signaling, preventing inhibitory feedback on IRS-1 which allows proliferative signals from IGF-1R to IRS-1, PI3K, and AKT. Dual treatment using rapamycin in combination with IGF-1R inhibition, such as monoclonal blocking antibodies, prevents signaling to these critical progrowth signaling nodes.

inhibition, such as cell surface receptors. In this review, we will describe the receptor tyrosine kinases (RTKs) associated with RMS and subsequently discuss the therapeutic potential of these targets.

2. Receptor Tyrosine Kinases Associated with Rhabdomyosarcoma

2.1. IGF-1R. The IGF-1R is a 150-kDa transmembrane RTK expressed on almost all mammalian cells. It is a classical RTK, signaling through ligand occupancy, dimerization, and transmembrane signaling to the cytoplasm through the IRS-1 and IRS-2 adaptor proteins. Present during both embryogenesis and postnatally, IGF-1R is critical for the growth of a variety of mammalian tissue types [8]. In myogenesis, IGF-1R is essential for myoblast proliferation, and IGF ligands induce a strong proliferative response in myogenic precursors. IGF-1R signaling is also necessary for myogenic differentiation through upregulation of the myogenic cascade [9]. There are two known IGF-1R ligands, IGF-1 and IGF-2. While both of these ligands have a ubiquitous tissue distribution, IGF-1 is considered to exert its effects postnatally, while IGF-2 is thought to be dominant during embryogenesis [10]. Through numerous *in vitro* and *in vivo* studies performed by many groups, it is well established that IGF activation of IGF-1R is critical for both proliferation and differentiation of muscle cells.

The original evidence for upregulation of IGF-1R signaling in RMS came from early studies of IGF ligands in pediatric tumors. As such, IGF-2 was found to be upregulated in both primary RMS tumor samples and cell lines [11, 12], mechanistically the result of loss of imprinting of the maternal or duplication of the active *IGF2* allele [13, 14]. IGF-1R was later found to be upregulated in aRMS by the *PAX3-FOXO1* fusion gene [15]. In this way, increased expression of both IGF-2 and IGF-1R leads to a strong mitogenic feed-forward signaling loop within the tumor.

The role of the IGF-1R signaling pathway in RMS has been examined through experimental loss of function using multiple approaches. Antisense constructs, small molecule inhibitors, and receptor blocking antibodies to IGF-1R have all shown antiproliferative effects in preclinical studies of RMS cell lines and xenografts [12, 16–25]. The mechanism of action appears to be through inhibition of cell proliferation by arrest in the G1 stage of the cell cycle due to downregulation of CDK1 [19, 21]. Interestingly, cell lines that were the most sensitive to IGF-1R blockade were those with the highest levels of IGF-1R expression [16].

An understanding of the signaling pathways downstream of IGF-1R has been enhanced through studies using the small molecule inhibitor, rapamycin. Rapamycin inhibits mTOR, a PIKK family member kinase that responds to changes in nutrient availability and cellular stresses. RMS sensitivity to rapamycin is mediated by IGF-1R signaling, demonstrating that the mTOR pathway is downstream of IGF-1R [17, 26]. As shown in Figure 1, in the IGF-1R signaling pathway, IGF-1R signals to IRS-1 and AKT, which then signals to mTOR. Paradoxically, treatment of cancer cells with rapamycin activates AKT, due to blockade of a feedback loop via ribosomal S6 kinase (S6K) that normally inhibits IRS-1 [27]. This effect can be reversed by inhibiting IGF-1R. Through dual treatment of RMS tumors with rapamycin and IGF-1R inhibitors, the proliferative IGF-1R signaling cascade can be dramatically reduced. In this way, IGF-1R blockade has become an attractive proposed treatment for RMS and other IGF-driven cancers [16, 28, 29].

IGF-1R inhibitors are one of many classes of compounds tested in the Pediatric Preclinical Testing Program (PPTP). This NCI-funded program provides a preclinical screening platform to test new agents that may have activity against pediatric cancers. As shown in Table 1, the IGF-1R inhibitor IMC-A12 showed effectiveness in RMS xenografts, while SCH 717454 had a partial effect. These studies, in addition to the preclinical data described above, provide a strong rationale to pursue IGF-1R inhibitors in clinical trials for pediatric RMS patients.

As shown in Table 2, phase I and phase II trials of RTK small molecule inhibitors, monoclonal antibodies against RTK ligands, and monoclonal antibodies against RTKs have been under investigation since the year 2000. Notably, inhibition of IGF-1R with monoclonal antibodies has been the most recent focus of these trials and, if successful, will be the first FDA-approved RTK-targeted therapy for RMS.

2.2. MET. MET is a proto-oncogene RTK necessary for cell proliferation, motility, and epithelial-mesenchymal transition. Similar to IGF-1R, MET is also 150 kDa and shows broad tissue expression in embryonic and postnatal tissues. In contrast to IGF1-1R, MET has only one ligand, termed hepatocyte growth factor (HGF). In the context of myogenesis, limb mesenchyme secretes HGF, which directs myogenic precursors to the limb bud. In this way, MET signaling regulates delamination and migration of muscle precursors from the embryonic dermomyotome [30]. MET also promotes cell proliferation in muscle precursors when activated with HGF *in vitro* [31]. When these cells stop proliferating and induce differentiation, HGF and MET expression decreases [32]. Thus, MET promotes both myoblast proliferation and migration processes in normal cells.

Table 1: Results of RTK inhibitors used in Pediatric Preclinical Testing Program.

Intended target	Inhibitor	Additional targets	*In vivo* response, RMS xenografts*	Conclusions	Ref.
EGFR, ErbB2	Lapatinib	Erk1/2, Akt	1/5	Limited effectiveness in all xenografts tested	[30]
IGF-1R	IMC-A12	N/A	6/6	Tumor growth inhibition in most solid tumor xenografts, most effective in RMS xenografts	[25]
IGF-1R	SCH 717454	N/A	2/4	Tumor growth inhibition in many solid tumor xenografts	[31]
PDGFR	Sunitinib	c-KIT, VEGFR2, FLT3	5/6	Tumor growth delay, inhibition in most solid tumor xenografts	[32]
Raf1	Sorafenib	VEGFR, PDGFR, RET, FLT3, c-KIT	2/6	Tumor growth inhibition in various tumor xenografts	[33]
SRC	Dasatinib	ABL, c-KIT, EPHA2, PDGFR	1/6	Limited effectiveness in solid tumor xenografts	[34]
VEGFR1-3	AZD2171	PDGFR, c-KIT	5/5	Tumor growth inhibition in most solid tumor xenografts	[35]

*Xenografts with "intermediate" or "high" response activity as defined by Maris et al. [50].

HGF is also known as "scatter factor," referring to its ability to induce cell motility. Accordingly, MET has been implicated in cytoskeletal reorganization and migration in cancer cells. In RMS cells, HGF promotes chemotaxis and invasion [33–35]. Because of these migratory effects of HGF/MET signaling, the role of this signaling pathway in tumor cell metastasis has been examined. Cells derived from bone marrow secrete HGF, and RMS cells have been shown to home to bone marrow, due in part to MET expression [33, 35, 36]. Furthermore, bone marrow aspirates from RMS patients with metastatic disease have elevated MET expression [37]. Therefore, a major role for MET is to confer migratory and metastatic properties.

MET-null and *Splotch* (*PAX3* mutant) mice both exhibit loss of muscle precursor colonization in the limb bud [30, 38], which revealed an association of PAX3 with MET expression [39]. MET is a transcriptional target of PAX3 and PAX3-FOXO1, and RMS cell lines and tumors express elevated levels of MET compared to normal muscle [34, 40, 41]. Targeted knockdown of MET in human RMS cell lines decreases RMS cell proliferation *in vitro* and tumor burden in mouse xenograft models [35, 42]. Therefore, in addition to regulating migration and metastasis, MET also appears to regulate proliferative properties in RMS.

Several genetically engineered mouse models of RMS either exploit MET signaling or demonstrate deregulated MET expression. The most robust murine model of RMS was generated through manipulation of the HGF/MET signaling axis. While transgenic *HGF* mice were predisposed to a low incidence of many types of cancers, including skeletal muscle-derived tumors [43], transgenic *HGF* mice with a targeted deletion of the *INK4A/ARF* locus had a near complete penetrance of eRMS in young animals [44]. Mouse models utilizing the *PAX3-FOXO1* fusion gene have also defined roles for MET. Conditional replacement of *PAX3* with *PAX3-FOXO1* results in abnormal delamination of myogenic progenitors from the somite that can be reversed with expression of a kinase-inactive MET [45]. aRMS has been modeled by conditional *PAX3-FOXO1* at the *PAX3* locus in either an *INK4A/ARF* or *p53*-null background. MET upregulation was observed in all tumors derived, regardless of the genetic background [46].

Although there is clear evidence for the involvement of MET in RMS initiation, progression, and metastasis, to date there have been no clinical trials evaluating MET inhibition in the context of RMS. Since MET is implicated in many adult malignancies, and phase I clinical trials for monoclonal antibodies and small molecule inhibitors with anti-MET activity have recently begun, we should expect to see trials recruiting pediatric RMS patients.

2.3. EGFR, ErbB2. The ErbB family of RTKs is comprised of four members: EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). Members of this family are similar in size, at 190 kDa, and each are necessary for embryonic development. Each has been implicated in cancer initiation and progression but in different tissue types. Notably, ErbB3 is a noncatalytic receptor but exerts an oncogenic function through heterodimerization with other ErbB family members [47]. ErbB receptors regulate multiple levels of cell physiology in different tissues, including cytoskeletal rearrangement, proliferation, and evasion of apoptosis. In mouse myoblasts, EGFR is expressed and is active in both undifferentiated and differentiated cells. EGFR blockade in murine myotubes induced cell death, suggesting that EGFR regulates prosurvival signaling in myogenic cells [48]. EGFR has been associated with many adult malignancies, including breast, non-small cell lung cancer, glioblastoma, head and neck, gastric, genitourinary, and colorectal carcinomas [49]. Prognosis in these cancers can often be estimated by the presence or absence of EGFR mutations, deletions, or overexpression.

Table 2: Clinical trials evaluating drugs that target RTKs or their ligands, with strata that include rhabdomyosarcoma.

RMS tumor eligibility	Patient age (years)	Drug	Intended RMS Target	Additional Targets	Phase	Start date	Sponsor/ collaborator
Small molecule inhibitors							
Relapsed/refractory	≥15	Imatinib	PDGFR	ABL, c-Kit	I/II	Aug 2000	EORTC
Resistant	≥15 but ≤70	Imatinib	PDGFR	ABL, c-Kit	II	Feb 2001	Novartis
Advanced	≥10	Imatinib	PDGFR	ABL, c-Kit	II	Jun 2002	NCI
Refractory	≤21	Erlotinib	EGFR		I	Feb 2004	COG/NCI
Refractory	≤21	Gefitinib	EGFR		I	Sep 2005	St. Jude's/Astra Zeneca
Metastatic/advanced/ recurrent	≥18	Sunitinib	PDGFR	c-KIT, VEGFR2, FLT3	II	Apr 2007	MSKCC/NCI
Advanced	≥13	Dasatinib	SRC	ABL, c-KIT, EPHA2, PDGFR	II	May 2007	SARC/ Bristol-Myers Squibb
Metastatic/recurrent	≥1 but ≤25	Dasatinib	SRC	ABL, c-KIT, EPHA2, PDGFR	I/II	Sep 2008	Beckman Research Institute/ NCI
Metastatic/relapsed/ refractory	≥18	Pazopanib	VEGFR1-3	PDGFR, c-KIT	III	Oct 2008	EORTC
Refractory/recurrent	≥2 but ≤18	Cediranib	VEGFR1-3		I	Dec 2008	NCI
Monoclonal antibodies against RTK ligands							
Metastatic	≥0.5 but ≤18	Bevacizumab	VEGF	N/A	II	Jul 2008	Hoffman-La Roche
Monoclonal antibodies against RTKs							
Recurrent/refractory	≥2	R1507	IGF-1R	N/A	II	Nov2007	Hoffmann-La Roche/SARC
Unresectable/locally advanced/ metastatic	≥16	IMC-A12	IGF-1R	N/A	I/II	Jun 2008	U. Chicago/NCI
Metastatic/advanced	≥12	IMC-A12	IGF-1R	N/A	II	Jun 2008	ImClone LLC
Relapsed/refractory	≤30	Cixutumumab	IGF-1R	N/A	II	Jan 2009	COG/NCI
Metastatic	≤49	Cixutumumab	IGF-1R	N/A	Pilot	Jan 2010	COG/NCI

Obtained from clinicaltrials.gov website September 2010.

ErbB family proteins were found to be expressed in RMS cells during screening for growth factor signaling pathway members [51–53]. While EGFR is more highly expressed in eRMS tumor tissue [54–56] ErbB2 expression is more prevalent in aRMS tumor tissue, and found in the majority of RMS tumors in the head and neck [55, 57]. ErbB3 is also expressed in RMS cells and may play a role in regulating differentiation, but ErbB4 has not been found to be expressed in RMS cells [52]. Notably, to date no mutations have been identified in the ErbB genes in RMS. Blocking EGFR expression by antisense methods decreases RMS cell proliferation *in vitro* [58]. Unfortunately, follow-up preclinical testing of EGFR inhibitors *in vivo* has not shown efficacy. As an example, the small molecule inhibitor lapatinib was tested in a PPTP screen but had little effect in solid tumors, suggesting that EGFR inhibition alone is not sufficient to inhibit tumorigenesis.

Expression of an activating ErbB2 mutation in combination with loss of p53 is sufficient to induce rhabdomyosarcoma in mouse models. The resulting tumors appear histologically similar to eRMS and express IGF-2 and IGF-1R [59]. This model was used to test a cancer vaccine developed against the ErbB2 receptor, which was successful in preventing spontaneous RMS formation in 50% of mice examined [60]. Even though preclinical studies have not shown promise as monotherapy, ErbB2 may play a supportive role in RMS initiation.

Although inhibition of a single RTK may be beneficial in some circumstances, studies have suggested that this approach will likely not be sufficient treatment for RMS, and this appears to be true of EGFR. This has been appreciated in preclinical models, and therefore human clinical trial design has been modified to evaluate RTK inhibition in combination with a cytotoxic agent. Gefitinib, a small molecule inhibitor for EGFR, is being tested in phase I clinical trials in pediatric solid tumors, in combination with irinotecan [61]. Phase I clinical trials have been completed for erlotinib, which also targets EGFR, done in combination with temozolomide with few adverse effects [62].

2.4. PDGFR. The PDGFR family of RTKs includes PDGFRα and PDGFRβ, both 200 kDa in size, which homo- or heterodimerize to perform their signaling functions. While PDGFRα is believed to be critical in the development of

neural, epithelial, and skeletal tissues, PDGFRβ is important for blood vessel formation and hematopoiesis [63]. In normal myogenesis, PDGFR activation is downregulated, implying that loss of PDGFR signaling is involved in the cell cycle exit that accompanies differentiation [64]. There are four ligands for PDGFR, PDGF-A through PDGF-D. In myoblasts, PDGF-B promotes cell migration and proliferation and reduces differentiation *in vitro* [65, 66]. Therefore, PDGFR signaling is important for embryogenesis, and specifically myogenesis by regulating proliferation, migration, and differentiation in myogenic precursors.

In RMS, the two PDGF receptors show increased expression [67–69], and PAX3-FOXO1 has been shown to activate transcription of PDGFRα [70]. Imatinib, a small molecule inhibitor of PDGFRs, has shown promise as an RMS therapy in preclinical models. In a mouse genetic model of RMS, high expression of PDGFRα was observed in advanced tumors. Loss of function of PDGFRα through siRNA or imatinib induced tumor cell apoptosis. When imatinib or PDGFRα blocking antibodies were used to treat RMS tumors in these mice, 50% of mice had at least a partial reduction of tumor growth [71]. PDGFR inhibition with sunitinib or sorafenib showed promise in PPTP screening, promoting tumor growth delay or inhibition, respectively (Table 1). However, both sunitinib and sorafenib are known to inhibit numerous other kinases, so these effects may not be due to PDGFR inhibition alone. Although targeting PDGFR alone has shown some promise in preclinical models, combination treatment with other chemotherapies may be more beneficial. In a mouse xenograft model of RMS, significant reduction of tumor burden was observed when imatinib was used in combination with the topoisomerase inhibitor topotecan [68].

In RMS patients, high expression of PDGFRs is associated with decreases in failure-free and overall survival, implicating PDGFR signaling in advanced stages of the disease [53, 72]. The PDGFR inhibitor imatinib was tested in advanced sarcomas of various types in a phase II trial. Overall, the results did not support the use of imatinib as a monotherapy, as only 2% of participants saw an effect resulting in partial or complete remission [73]. However, combination treatment of imatinib with other targeted or cytotoxic agents may be more beneficial, as was seen in preclinical models [68].

2.5. VEGFR. The VEGFR family of RTKs is comprised of three members: VEGFR1 (FLT1), VEGFR2 (FLK1/KDR), and VEGFR3 (FLT4). VEGFRs on endothelial cells regulate multiple levels of angiogenesis by promoting endothelial cell proliferation, migration, sprouting, and survival. These receptors are activated by the five members of the VEGF ligand family, VEGF-A through VEGF-D, and placental growth factor (PlGF). VEGFs are produced in a wide variety of tissues in response to hypoxia, in order to recruit vasculature to the hypoxic area [74]. Interestingly, VEGFRs are also expressed in myoblasts, and VEGF promotes myoblast migration and survival. VEGFR expression is downregulated upon myogenic differentiation, suggesting that prolonged VEGFR signaling negatively regulates differentiation [75].

Similar to other cancer cells, when exposed to hypoxia, RMS cells increase their secretion of VEGF [28]. RMS cells express multiple isoforms of VEGF and VEGFRs, implying that RMS tumors may utilize an autocrine loop to not only promote tumor vascularity but induce tumor growth as well. This is supported by evidence that treatment of RMS cells in culture promotes proliferation, while treatment with VEGFR antibody blocked this effect [76]. Furthermore, inhibition of signaling downstream of VEGFR prevents expression of VEGF by RMS cells, suggesting a feed-forward loop promoting proliferation [77].

VEGFR inhibitors have shown promising results in preclinical studies. Monoclonal antibodies to VEGF and VEGFRs and small molecule inhibitors to VEGFRs have been tested in mouse xenografts of RMS reduced tumor volume and vascularity [50, 78–81]. Notably, cisplatin-resistant RMS cells have increased expression of VEGF and VEGFRs, implicating this autocrine signaling in RMS cell survival. Cisplatin-resistant cells were sensitive to VEGFR inhibition, which also blocked VEGF expression [82]. In this way, highly aggressive tumor cells could be targeted with anti-VEGFR therapy. In PPTP screening, VEGFR inhibitors have also shown potential. The inhibitors AZD2171, sorafenib, and sunitinib have each inhibited tumor growth in RMS xenograft models. Currently, there are multiple clinical trials testing VEGFR inhibition in RMS patients. These include the small molecule inhibitors sunitinib, pazopanib, and cediranib as well as the VEGF monoclonal antibody bevacizumab. These studies are ongoing, but whether the strong preclinical data for VEGFR inhibition will translate to positive outcomes in clinical trials remains to be seen.

2.6. FGFR. The FGF receptor (FGFR) family consists of four members, FGFR1 through 4, and vary in size (120–160 kDa), tissue distribution, and ligand affinity. FGFRs affect many aspects of cell and organism physiology including proliferation, migration, and differentiation through activation by FGF ligands, of which there are at least 23 [83]. FGFR4 is considered to be the predominant FGFR in skeletal muscle, regulating skeletal muscle differentiation in chick models and muscle regeneration in mice [84, 85]. As is true for most of the RTKs reviewed here, FGFR4 expression in myoblasts decreases during differentiation, implying that FGFR4 is important in myogenic precursors [86].

While FGFR1 and FGFR3 have been observed to have increased expression in isolated RMS cases [87, 88], and FGF ligands are expressed in RMS cells and tissues [51, 89], signaling through FGFR4 has been the best characterized in RMS tumorigenesis. FGFR4 expression is upregulated in RMS cell lines and tumors [90, 91], and PAX3-FOXO1 promotes FGFR4 expression through 3′ enhancer regions [15]. Recently, FGFR4 was characterized as a regulator of RMS tumor growth and metastasis. Activating mutations within the kinase domain of FGFR4 were identified in 7% (7 of 94) of RMS cases, demonstrating overactive FGFR4 signaling in RMS. These activating mutations were sufficient to transform cells, increase RMS lung metastasis, and decrease survival in mouse xenograft models. Blocking FGFR4 expression decreased RMS tumor size, cell migration,

and metastasis, therefore characterizing FGFR4 as a possible molecular target for RMS [92]. FGFR4 is the most recent RTK implicated in RMS, and as such more research will be needed to verify that FGFR4 is a rational therapeutic target to pursue in preclinical and clinical settings.

3. Regulation of RTK Expression in Rhabdomyosarcoma

Since a signature mutation of aRMS is a chromosomal translocation resulting in the PAX3/7-FOXO1 or PAX3-NCOA1/2 transcription factors, much attention has been focused on the genes regulated by these fusion proteins. In fact, IGF-1R, MET, PDGFR, VEGFR1, and FGFR4 have all been shown to be regulated by PAX3-FOXO1 either by activation of the RTK gene promoter or through 5′ or 3′ enhancing elements [15, 39, 70, 93]. Since transcription factors are generally difficult to target due to chemical intractability, druggable PAX3-FOXO1 transcriptional targets such as RTKs could be more promising than inhibiting PAX3-FOXO1 itself.

Another regulator of RTKs in RMS is the tumor suppressor p53. The importance of p53 function in RMS has been underscored by its role in promoting RMS in mouse models when it is absent [46, 59]. Mutations in p53 have been documented in both histological subtypes of RMS [94], and IGF-1R and PDGFR have been definitively shown to be upregulated in p53 loss-of-function experiments and downregulated when nonmutated p53 is added back to these systems [71, 95]. Although PAX3-FOXO1 and p53 regulation of RTK transcription in specific cases has been informative, there is a need for a better understanding of when and how RTK transcription is activated in RMS tumorigenesis.

Most recently, posttranscriptional regulation of genes has been shown to play a role in RMS tumorigenesis. The most focus has been on microRNAs mir-1 and mir-206, so-called "myo-mirs." Upon myogenic pathway induction, mir-1 and mir-206 expression is upregulated, leading to post-transcriptional downregulation of mir-1 and mir-206 targets. Mir-1 and mir-206 have been found to be sufficient to induce myogenic differentiation in myoblasts [96]. RMS cell lines and tumors do not express mir-1 and mir-206 and therefore are not able to posttranscriptionally regulate mir-1 and mir-206 targets. Surprisingly, MET was found to be implicated in the mir-206 pathway. MET contains two putative binding sites for mir-206 in the MET 3′ untranslated region. Ectopic expression of mir-206 caused loss of MET expression, induction of skeletal muscle differentiation markers, loss of cell proliferation, and decreased tumor burden in mouse RMS xenografts [97, 98]. The mechanism behind the loss of mir-1 and mir-206 in RMS remains to be determined, but its potential to downregulate therapeutic targets like MET may hold promise for RMS treatments in the future.

4. Therapeutic Potential for RTK Inhibition in Rhabdomyosarcoma

As druggable receptors at the plasma membrane, RTKs have been the focus of intense basic and pharmacologic research. Small molecule inhibitors, ligand-neutralizing agents, and monoclonal receptor-blocking antibodies have been generated for many of the RTKs expressed in RMS. However, it is not likely that all of the RTKs in RMS will survive the tests of robust preclinical testing and be evaluated in clinical trials. Therefore, determining which target(s) are the most promising and worthy of clinical trial assignment will be critical. As described below, understanding their mechanisms of upregulation, acquired resistance, and pathway crosstalk will be key to determining how to pharmacologically exploit RTK signaling in RMS.

RTKs are only one of numerous and diverse signaling pathways upregulated in cancer, so identifying the RTKs upregulated in RMS is only a starting point to determine their potential as therapeutic targets. In many cases, blockade of an upregulated RTK will cause cytostatic growth inhibition but not eliminate the cancer cells completely, leading to emergence of resistant clones and refractory disease. However, if the cells have become dependent on a particular RTK signaling pathway for survival, so-called "oncogene addiction" [99], blockade of these pathways should be more effective in disease eradication. The challenge then becomes determining which RTKs confer oncogene addiction.

Another possibility is the presence of activating mutations in RTKs. RTKs containing an activating mutation are much more sensitive to inhibitors targeting that RTK than cells or tumors with a wild-type RTK. An example of this phenomenon was observed in non-small cell lung carcinoma. In clinical trials of EGFR inhibitors, only 10% of patients responded to treatment. Upon further investigation, it was found that the responding patients harbored somatic, activating EGFR mutations [100]. Similarly, in PPTP screening, Kasumi-1 cells (which contain an activating c-KIT mutation) were found to be particularly sensitive to sorafenib [101]. To date, FGFR4 is the only RTK known to have an activating mutation in RMS [92]. Deep sequencing of RTKs implicated in RMS will need to be done to address the possibility that other RTKs are mutated in RMS. A second possibility is genomic amplification or deletion or sheer upregulation of RTKs or their signaling components. This has already proven important in our understanding of IGF-1R in RMS, as the loss of imprinting of the *IGF-2* gene, or higher expression levels of IGF-1R, lead to oncogene dependence even in the absence of an activating mutation and confer sensitivity to RTK blockade [13, 16]. Similarly, wild-type EGFR expression is upregulated in RMS cells. Although inhibition of EGFR does not appear to be a promising candidate for monotherapy, recent studies have suggested that EGFR could be used in targeted immunotherapy applications [102]. In sum, understanding the underlying genetic changes as well as utilizing upregulation of RTKs through novel treatments will guide future RMS therapies.

A drawback of targeted therapies is the ability of tumor cells to adapt and acquire resistance. Through further upregulation of the therapeutic target, mutation of the therapeutic target, or upregulation of a compensating RTK or signaling pathway, cancer cells can rapidly adjust to promote tumor cell survival [103]. For example, in the case of IGF-1R blockade, RMS cells resistant to IGF-1R small

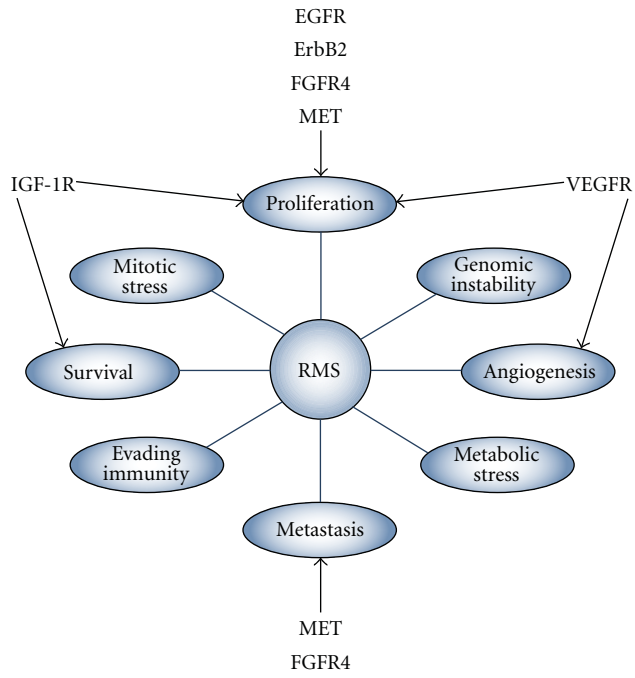

Figure 2: RTKs associated with RMS and their known roles in RMS tumorigenesis or progression.

molecule inhibitors were found to have increased expression of EGFR when compared to those cells that were sensitive. To this end, dual treatment with IGF-1R and EGFR inhibition increased the antitumor effect in RMS mouse xenograft models [24]. Understanding how and if RMS cells adapt to targeted therapies will be critical for successful treatment options.

Although induction of resistance may pose a problem for targeted therapy in RMS, crosstalk within signaling pathways could provide a way to exploit RTK inhibition. For example, the IGF-1R and VEGFR pathways exhibit crosstalk in RMS, and by experimentally inhibiting IGF-1R signaling, VEGF secretion is reduced [28]. Thus, IGF-1R blockade has the potential to thwart both IGF-1R and VEGFR pathways. In addition, many RTKs utilize redundant downstream signaling components. Targeting more than one RTK through multiple individual inhibitors, or using a less specific inhibitor to block several RTKs simultaneously, may prove beneficial by strong inhibition of signaling at a common node. Through a systematic and comprehensive analysis of other as yet undescribed crosstalk mechanisms in RMS, these dependencies can be identified and provide the basis for further preclinical testing.

In addition to the theoretical attraction of combination therapy on blocking signaling pathways, dual or multiple inhibition of RTKs may offer beneficial effects in inhibiting the unique tumorigenic properties of cancer cells. The "hallmarks of cancer" as defined by Hanahan and Weinberg [104], and further classified by Negrini et al. [105], represent specific tumorigenic properties of the cancer cell, as shown in Figure 2. The RTKs we have described impact various cancer cell characteristics. Through targeting of individual RTKs, therapeutic intervention could inhibit distinct malignant properties. When blocked in combination, inhibition of multiple RTKs could have a profound effect on tumor growth and progression. Realistically, these positive outcomes on tumor inhibition may be offset by increased incidence of toxicity and side effects. Therefore, until both preclinical and clinical studies address these issues, combination targeted therapy will pose a sizeable challenge for researchers.

5. Prioritization of Therapeutic Targets

One of the most daunting challenges for pediatric oncology clinical trial design is how to identify the strongest therapeutic candidates to pursue. Because of the limited number of pediatric patients, only the most promising agents under development as cancer treatment should be evaluated in a clinical trial setting. As mentioned above, the PPTP provides a preclinical platform to screen an experimental compound against many types of pediatric cancers. To be considered for a PPTP screen, there must be significant rationale for the proposed agent in pediatric cancers, including the mechanism of action and *in vitro* and *in vivo* efficacy. In some cases, evidence from pediatric preclinical models or adult clinical trials is available, providing pharmacokinetic and dosing data. These cases may receive priority, as they expedite some of the issues addressed in early clinical trials. In terms of prioritization of RTK targets, there is a need to understand the genetic foundation behind activation of specific RTK signaling pathways in RMS cells. Genetic screening of RTKs for mutations or analysis of downstream signaling pathways may provide insight into which therapeutic candidates could have the most profound effects on RMS cells. Clearly, both large scale screens and mechanistic validation will be necessary to prioritize the many candidates.

There are still numerous RTK targets that could be utilized for RMS therapy and warrant further study to provide additional treatment opportunities. Additional study of these targets in preclinical models will be necessary to advance their use to clinical trials, either as single targeting agents, multiple targeting agents, or single targeting agents in combination with cytotoxic therapy. As we expand our knowledge of how RTKs function individually or together, the potential for utilizing RTK inhibition could be a turning point in a new era of targeted therapy for RMS. The future of RMS therapies holds promise and will provide improved options for RMS patients, including those in high risk groups.

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Review Article

The Role of Mirk Kinase in Sarcomas

Eileen Friedman

Department of Pathology, Upstate Medical University, 750 East Adams Street, 2305 Weiskotten Hall, Syracuse, NY 13210, USA

Correspondence should be addressed to Eileen Friedman, friedmae@upstate.edu

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Targeting the tyrosine kinase KIT in gastrointestinal stromal tumors has led to improved treatment. Other kinases might serve as therapeutic targets in the more common forms of sarcoma. The kinase Mirk/dyrk1B is highly expressed in the vast majority of osteosarcomas and rhabdomyosarcomas and mediates their growth, as depletion of Mirk led to tumor cell apoptosis. Mirk is known to increase the expression of a series of antioxidant genes, which scavenge reactive oxygen species (ROS) within various tumor cells, mediating their survival. As a result, depleting Mirk led to increased levels of damaging ROS. Tumor cells depleted of Mirk were also sensitized to low levels of chemotherapeutic drugs that increase ROS levels. In contrast, Mirk expression is quite low in most normal cells, and Mirk depletion or embryonic knockout of Mirk did not detectably affect cell survival. Thus targeting Mirk for intervention in sarcomas might spare most normal tissues.

1. Introduction

Targeting of cellular kinases has proved efficacious for the treatment of various cancers. Kinases are a good target for therapy because they are readily inhibited by small, cell permeable molecules that block their ATP-binding site and because they act catalytically, and so they are in relatively low abundance compared to structural elements within a cell. In gastrointestinal stromal tumors (GISTs), the use of inhibitors of the stem cell factor receptor kinase, KIT has dramatically impacted treatment (reviewed in [1, 2]. The tyrosine kinase KIT is expressed in more than 95% of GISTs, with many exhibiting mutations that increase kinase activity. The Kit inhibitors imatinib and sunitinib have induced stable disease or partial responses in many patients, increasing their length of survival. While GISTs represent only about 5% of all sarcomas, the efficacy of treatment with KIT kinase small-molecule inhibitors suggests that other kinases may represent targets in more prevalent sarcomas.

Mirk/Dyrk1B is a member of the Minibrain/dyrk family of serine-threonine kinases [3–5]. Mirk is expressed at very low levels in most normal tissues [6]. However, Mirk is highly expressed in normal skeletal muscle and in C2C12 myoblasts where it mediates differentiation and survival. Mirk aids in the differentiation of skeletal muscle [7] and maintains the survival of differentiating myoblasts [8]. Mirk is not an essential gene because embryonic knockout of Mirk/dyrk1B caused no evident phenotype in mice [9]. Likewise, normal diploid fibroblasts exhibited no alteration in survival after 20-fold depletion of Mirk [10], suggesting that targeting Mirk for intervention might induce a selective killing of tumor cells.

2. Mirk in Osteosarcomas

Osteosarcoma is the most common malignant bone tumor and is highly metastatic. After chemotherapy, the tumor recurs in about one-third of patients and the life expectancy after recurrence is less than one year [11, 12]. Cytoplasmic kinases and growth factor receptor kinases have been implicated in sarcoma survival including mTOR [13, 14], PDGFR-A [15], and the IGFR1 [16, 17]. Recently an RNA interference screen of the osteosarcoma cell line KHOS was performed using a lentiviral short hairpin RNA library targeting 673 human kinase genes [18]. The Mirk gene was found by this screen to mediate sarcoma cell proliferation and apoptosis, while a Mirk cDNA rescue assay confirmed that the identification of Mirk was not due to off-target effects. Mirk knockdown by shRNA or by synthetic RNAi

duplexes induced apoptosis in each of 3 osteosarcoma lines tested as well as an osteosarcoma in primary culture. No effect was seen on a benign osteoblast cell line. Mirk protein was widely expressed in this cancer, being found in each of 58 osteosarcomas in a tissue microarray. Most significantly, Kaplan-Meier analysis showed that patients whose tumors expressed the highest amount of Mirk protein had significantly worse prognosis than those with low Mirk expression. For this analysis, patients were stratified into two groups, those alive up to 60 months after followup and those deceased. The nonsurvivors had more Mirk in their osteosarcomas, with $P = .0012$. This report [18] indicated that the kinase Mirk was essential for the growth and survival of osteosarcoma cells and that high Mirk protein levels in the cancer were a biomarker for tumor progression.

3. Mirk in Rhabdomyosarcomas

Rhabdomyosarcoma is the most common soft tissue sarcoma in children and is difficult to treat if the primary tumor is nonresectable or if the disease presents with metastases [19, 20]. There are two major histological types, embryonal and alveolar. Alveolar histology is associated with a significantly worse prognosis with a five-year survival rate of less than 30%. The precise etiology of rhabdomyosarcoma is unknown, but it has been suggested to arise in "satellite" cells, the committed skeletal muscle precursor cells [19]. Mirk/Dyrk1B was expressed to some extent in each of 16 clinical cases of human rhabdomyosarcoma examined [21] and in myoblast satellite cells [7]. Furthermore, Mirk was found to be an active kinase in each of 3 rhabdomyosarcoma cell lines tested [21]. In addition, Mirk depletion by synthetic RNAi duplexes induced apoptosis in each of two rhabdomyosarcoma cell lines assayed as shown by increase in both the apoptotic marker Annexin V and DNA breaks revealed by TUNEL staining. Increased apoptosis led to a 3-4-fold decrease in clonogenicity. Thus depleting Mirk led to death of the most aggressive rhabdomyosarcoma cells.

4. Mirk in Skeletal Muscle Myoblasts

Some insight into the possible role of Mirk in rhabdomyosarcoma can be derived from studies of Mirk in skeletal muscle myoblasts. Mirk was expressed in skeletal muscle satellite cells in primary culture and was upregulated about 10-fold when the satellite cells were induced to differentiate, while knockdown of endogenous Mirk by RNA interference blocked myoblast differentiation [7]. Mirk is activated by the stress-activated MAP kinase kinase MKK3 [22]. These results together imply a role for Mirk in the response to cellular injury. Skeletal muscle is regenerated after injury by activation of quiescent satellite cells that enter the cell cycle and then differentiate and fuse with uninjured muscle fibers to repair the damage. Mirk may play some role in muscle regeneration because Mirk is a stress-activated kinase that modulates the activation of the myogenic regulatory factors MEF2 and myogenin, which subsequently mediate myoblast differentiation [8]. Mirk is less likely to play a significant role in embryonic muscle development because a Mirk/Dyrk1B knockout mouse survived to 18 days after conception during which time skeletal muscles were developed [9]. Thus Mirk/Dyrk1B may be a survival factor in skeletal myoblasts undergoing repair.

5. Inactivation of ROS May Be the Mirk Survival Function in Sarcomas

The Mirk kinase gene has been localized to the 19q13 amplicon [6] and is amplified in a subset of pancreatic cancers and ovarian cancers, and less frequently in colon cancers [23–25]. Mirk mediates survival of these cancers at least in part by reducing reactive oxygen species (ROS). ROS are oxygen-containing chemical species with reactive chemical properties, such as hydroxyl radicals, which contain an unpaired electron and the free radical superoxide. Cancer cells often exhibit higher levels of ROS than normal cells because of increased metabolism and oncogenic stimulation, and so they are under increased oxidative stress. Genes which detoxify superoxide (superoxide dismutases 2 and 3) and which prevent the generation of hydroxyl radical (ferroxidase/ceruloplasmin) were found to be upregulated in SU86.86 pancreatic cancer cells [26] and in each of four ovarian cancer cell lines [27] through Mirk. These genes work together to reduce ROS. Superoxide dismutases detoxify superoxide resulting in hydrogen peroxide, which in turn can be metabolized either to water or to hydroxyl radical through the Fenton reaction if Fe++ is available. Conversion to hydroxyl radical is blocked by ferroxidase that converts Fe++ to Fe+++. Mirk is a coactivator for several transcription factors and increases the expression of these antioxidant genes [26]. Thus these Mirk-upregulated genes working together increase antioxidant potential while minimizing hydroxyl production.

6. ROS in Skeletal Muscle

ROS are toxic to cells, decreasing their viability; so ROS levels and cell viability fell following depletion of Mirk from C2C12 myoblasts and from cancer cells. Using immunofluorescence techniques, we have found that Mirk is localized in fast twitch skeletal muscles (Mercer and Friedman, manuscript in preparation). Such muscle endogenously produces ROS in response to repeated contractions. Hydrogen peroxide is produced in contracting muscle, breaking down to ROS species, which can have diverse effects on myoblasts, such as inducing mitochondrial fragmentation [28]. ROS generation within single intact muscle fibers was cytosolic, with a role for NADPH oxidase-derived ROS during contractile activity [29]. Depletion of Mirk from C2C12 myoblasts also led to an increase in ROS (Deng and Friedman, manuscript in preparation), consistent with ROS control being a major role of Mirk in muscle development and function. This protective ROS-decreasing role is likely to have provided a selective pressure to maintain elevated Mirk levels in skeletal muscle and to further upregulate Mirk expression in sarcoma cells. Thus we hypothesize that Mirk mediates sarcoma cell

survival through an increase of its original function in skeletal muscle cells, depletion of ROS.

7. Mirk Depletion/Inactivation Potentiates Certain Chemotherapeutic Drugs

The chemotherapeutic drug cisplatin is one of many known to increase intracellular levels of toxic reactive oxygen species. Thus, an increase in cisplatin toxicity selectively in cancer cells could result from further increasing the cisplatin-elevated ROS levels by targeting antioxidant genes upregulated in cancers such as those mediated by the kinase Mirk/dyrk1B. This possibility was tested, and depletion of Mirk increased cellular ROS levels in each of 4 ovarian cancer cell lines. Mirk depletion averaged only about 4-fold, yet combined with cisplatin treatment enabled low levels of drug to increase ROS to toxic levels in both SKOV3 and TOV21G ovarian cancer cells [27]. Lowering ROS levels by treatment with N-acetyl cysteine limited cisplatin toxicity, resulting in higher cell numbers and decreased cleavage of the apoptotic proteins PARP and caspase 3. Targeting Mirk in sarcomas could increase their response to lower levels of chemotherapeutic drugs, potentially reducing side effects, which often limit therapeutic options in these cancers.

8. Hedgehog Signaling in Sarcomas

Mirk/dyrk1B and Dyrk1A are about 94% identical/homologous within their conserved kinase domains, but unlike within their unique N and C termini. The kinase domain similarity has led many to suspect some common functions between Dyrk1A and Mirk/dyrk1B. Dyrk1A, as one of the Down Syndrome conserved genes, has been intensively investigated. The essential embryonic signaling pathway, Hedgehog, has been implicated in many cancers such as pancreas, lung, and prostate, and Gli1 is a target of this pathway. In initial studies Dyrk1A enhanced Gli1-dependent gene transcription and acted synergistically with Sonic hedgehog to induce transcription [30]. However, the involvement of Mirk in Hedgehog signaling is complex. Mirk is activated by oncogenic K-ras and H-ras [10] and is an active kinase in pancreatic cancers [31], which exhibit a very high rate of K-ras mutation, almost 100% in advanced lesions. Mutant K-ras signaling through Mirk/dyrk1B blocked autocrine Hedgehog signaling to Gli1 within pancreatic cancer cells, only allowing Hedgehog signaling to Gli1 in stromal cells, which do not have mutant K-ras [32]. This is important clinically because most drugs do not reach pancreatic cancer cells because of their dense stroma [33], so the paracrine hedgehog signaling in stromal cells can be targeted [34] to enhance conventional chemotherapy. Activation of the hedgehog pathway confers a poor prognosis in embryonal and fusion gene-negative alveolar rhabdomyosarcoma [35], and the transcription factor Gl1 is a central mediator of EWS/FLI1 signaling in Ewing sarcoma tumors [36]. Since Mirk was found to be an active kinase in each of 3 rhabdomyosarcoma cell lines tested [21], it may also alter Hedgehog signaling to a paracrine mode and thus mediate control of the stromal microenvironment of these tumors. The WD40 repeat protein Han11 can inhibit Dyrk1A-dependent transcription of Gli1 when Han11 also binds the cytoskeletal regulator mDia [37]. Mirk/dyrk1B is found in a 670 kDa complex with unknown proteins [38]. One may be Han11, which binds to Dyrk1A, Dyrk1B/Mirk, the related kinase HIPK2, and the mitogen-activated protein kinase kinase kinase1 (MEKK1) [39]. When downregulated, or conversely when overexpressed, Han11 alters the threshold and amplitude of kinase signaling by HIPK2 and MEKK1, demonstrating a scaffolding function for Han11 in controlling these kinases in a multiprotein complex.

9. Additional Mirk/Dyrk1B Substrates

Several other intriguing Dyrk1A substrates have been identified (CREB, STAT3, and NFAT) [40–43], and have yet to be examined as potential Mirk substrates in sarcomas. The STAT3 signaling pathway is constitutively activated in each of three rhabdomyosarcoma cell lines tested, and two small-molecule compounds inhibited both STAT3 activity and cell proliferation and viability [44]. Mirk and Dyrk1A are coactivators of FOXO1a-dependent glucose-6-phosphatase gene expression [45], and Dyrk1A phosphorylates this transcription factor [46]. Mirk also slightly increased the activity of FOXO3a on a promoter-reporter construct of the CDK inhibitor p27 [47]. The functional relevance of these interactions is unclear. However, Mirk stabilizes p27 by phosphorylation [48], and so it might augment this activity by increasing p27 expression. Increased p27 levels mediate a G0 arrest where damaged cells can repair [49]. A small-molecule Mirk kinase inhibitor would be very useful in confirming the role of putative Mirk substrates in sarcomas.

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Research Article

miRNA Profiling: How to Bypass the Current Difficulties in the Diagnosis and Treatment of Sarcomas

Angélique Gougelet,[1,2] Jennifer Perez,[1,2] Daniel Pissaloux,[3] Anthony Besse,[1] Adeline Duc,[1] Anne-Valérie Decouvelaere,[3] Dominique Ranchere-Vince,[3] Jean-Yves Blay,[1,2,4] and Laurent Alberti[1,2]

[1] *Unité INSERM U590 équipe Cytokines et Cancer, Centre Léon Bérard, 28 rue Laennec, 69373 Lyon cedex 08, France*
[2] *Conticanet (FP6-06188), France*
[3] *Service d'Anatomie et Cytologie Pathologiques, Centre Léon Bérard, 69373 Lyon cedex 08, France*
[4] *EORTC, 83/11 avenue Mounierlaan, 1200 Brussels, Belgium*

Correspondence should be addressed to Angélique Gougelet, angeliquegougelet@gmail.com

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Sarcomas are divided into a group with specific alterations and a second presenting a complex karyotype, sometimes difficult to diagnose or with few therapeutic options available. We assessed if miRNA profiling by TaqMan low density arrays could predict the response of undifferentiated rhabdomyosarcoma (RMS) and osteosarcoma to treatment. We showed that miRNA signatures in response to a therapeutic agent (chemotherapy or the mTOR inhibitor RAD-001) were cell and drug specific on cell lines and a rat osteosarcoma model. This miRNA signature was related to cell or tumour sensitivity to this treatment and might be not due to chromosomal aberrations, as revealed by a CGH array analysis of rat tumours. Strikingly, miRNA profiling gave promising results for patient rhabdomyosarcoma, discriminating all types of RMS: (Pax+) or undifferentiated alveolar RMS as well as embryonal RMS. As highlighted by these results, miRNA profiling emerges as a potent molecular diagnostic tool for complex karyotype sarcomas.

1. Introduction

Sarcomas are rare malignant tumours arising in connective tissues like fat, muscle, bones, and cartilage. According to molecular cytogenetic alterations, sarcomas could be divided into two classes: (1) sarcomas with specific alterations (translocation, oncogenic mutation) including Ewing sarcoma, gastrointestinal stromal tumours, and alveolar rhabdomyosarcoma (2) sarcomas with complex karyotype like leiomyosarcoma, pleomorphic liposarcoma, or osteosarcoma. Osteosarcoma is the most frequent primary malignant bone tumours, characterized by its metastatic potent particularly in lung sites and its resistance to conventional treatments like chemotherapy and radiotherapy [1]. Even if the median survival of osteosarcoma patients has been improved through preoperative administration of chemotherapeutic agents, there are nowadays around 40% poor-responder patients [2]. In fact, osteosarcoma tumours often resist or relapse to presurgical chemotherapeutic treatment, and only few therapeutic options are possible and generally noncurative [3]. A second intensive cure of chemotherapy is currently administered in this case. Thus, it seems essential to develop a diagnosis tool to predict tumour response to chemotherapy to avoid the administration of inefficient drugs. There is also a need for efficient therapeutic alternatives based on the discovery of new targets involved in osteosarcoma tumourigenesis.

Rhabdomyosarcoma (RMS) is one of the most common soft-tissue sarcoma. Three types of RMS are observed: alveolar RMS (20%), embryonal RMS (eRMS, 60%), and pleomorphic RMS (20%). 70% aRMS present a specific translocation of the transcription factor Pax3 at the 3′end of FOXO1, creating a potent transcription factor able to induce myogenesis and survival [4]. 10% aRMS present

a translocation of Pax7 with FOXO1 [5]. aRMS are of bad prognosis as compared to eRMS, particularly those with Pax3 fusion gene [6]. Thus, it appears primordial to obtain a diagnosis tool identifying precisely the RMS subtypes, and particularly discriminating Pax-aRMS from eRMS, difficult to separate according to patient survival characteristics, gene expression profiles, and CGH arrays [7].

Micro-RNAs (miRNAs) are promising diagnosis biomarkers with their tissue specificities and their involve-ment in oncogenic process [8]. miRNAs are non-coding small RNA molecules synthesized from intronic regions with a size range from 16 to 35 nucleotides. They are processed by specific complexes of proteins containing Drosha and Dicer to be matured and finally integrated in RISC complexes [9, 10]. Mature miRNAs match with complementary sequences in messenger RNAs resulting in translation inhibition and accelerated mRNA degradation [11]. miRNA expression levels are characteristic for one tissue to regulate gene expression during growth and development, as it was shown for skeletal tissue and muscle development [12–14]. Their expression is also deregulated in many cancers [15, 16], resulting in a tumour miRNA signature, which could be useful for their classification in line with their tissue origin and molecular alterations [17–19]. Thus, they currently constitute potent biomarkers for cancer diagnosis [18, 20] with their abilities to be detected in patient serum. A noninvasive diagnostic tool based on miRNAs for osteosarcoma could be very useful to adapt chemotherapy protocols to tumour biological specificities.

In this study, we performed the miRNA profiling of sarcoma cell lines, human or rat tumours, to assess if miRNAs could constitute potent biomarkers to surpass the current limitations for rhabdomyosarcoma diagnosis and osteosarcoma treatment. miRNA expression levels were determined using microfluidic cards performing high-throughput TaqMan Low Density Arrays (TLDA), a real-time quantitative PCR (RT-qPCR) assays based on TaqMan technology. We firstly studied the effects of different chemotherapeutic agents on osteosarcoma cell miRNA profiles; we observed that these miRNA signatures were cell specific and drug specific. A CGH array of osteosarcoma tumours obtained from a rat model revealed that this miRNA signature, conserved in rat and human cells, was independent of chromosomal rearrangements, suggesting that miRNA profiles were linked to tumour phenotypes rather than to their genetic background. Of great interest, a miRNA signature was identified in rhabdomyosarcoma tumours from patients in accordance with the molecular translocation Pax3 or Pax7. This signature was in fact a potent tool to discriminate alveolar RMS (Pax-) from embryonal RMS, indistinguishable by the molecular techniques currently used. In conclusion, miRNA profiling constitutes a promising technology as an alternative or a partner of usual molecular techniques to overcome the present difficulties in diagnosis and treatment of sarcomas.

2. Experimental Procedures

2.1. Human Rhabdomyosarcoma Tumours. Seventeen patients treated for rhabdomyosarcoma in the Centre Léon Bérard were included in this study. Four frozen tumours and thirteen formalin-fixed paraffin-embedded tumours were obtained from biopsies realized at the diagnosis. Tumour diagnoses were realized by a referent anatomopathologist specialist for this pathology by immunohistochemistry, FISH, and qPCR.

2.2. Cancer Cell Lines. Five cancer cell lines were obtained from ATCC (Manassas, VA, USA): the two human osteosarcoma MNNG/HOS Cl #5 [R-1059-D] (reference CRL-15-47) and Saos-2 (HTB-85) cells, the chondrosarcoma cell line SW1353 (HTB-94) and the two Burkitt lymphoma Daudi (CCL-213) and Namalwa (CRL-1432) cells. Osteosarcoma and chondrosarcoma cells were grown in DMEM (Gibco, Carlsbad, CA, USA), supplemented with 10% decomplemented fetal calf serum (Lonza, Basel, Switzerland), 10 mL penicillin streptomycin (10 U/mL/10 μg/mL, Gibco, Carlsbad, CA, USA), and 5 mL L-glutamin (200 mM; Gibco) at 37°C humidified atmosphere containing 5% CO_2. Lymphoma cells were grown in RPMI (Gibco, Carlsbad, CA, USA). Cells were exposed to 100 nM RAD-001 (Novartis), 50 μM ifosfamide (ifos, Baxter) or 1 μM cisplatin (CDDP, TEVA) or 100 μM methotrexate (MTX, TEVA) for 24, 48, and 72 h.

2.3. Rat Osteosarcoma Model. Procedures for animal care were performed according to institutional and national guidelines. Animals were anesthetized throughout all surgical and imaging procedures with isoflurane/oxygen (2.5%/2.5%, v/v) (Minerve, Esternay, France). The transplantable orthotopic and metastatic rat osteosarcoma model has been previously described [21–23]. This model mimics its human counterpart in terms of aggressiveness, metastatic spreading and chemoresistance phenotype [21–23]. All the tumours obtained were classified as osteoblastic following histological analyses. Briefly, small tumour fragments (100 mm^3) taken from a hyperproliferative osteogenic tumour area were grafted on 3-weeks old immunocompetents Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA). Using a lateral approach, a tumour fragment was placed contiguous to tibial diaphysis after periosteal abrasion; then, the cutaneous and muscular wounds were sutured. Fourteen days after tumour transplantation, animals underwent a first $^{18}F^{-}$FDG PET Scan and were randomly assigned to a control group treated with saline solution or a treated group exposed to a subcutaneous dose of 10 mg/kg ifosfamide (ifos, Baxter, Deerfield, IL, USA), 7 days apart (at days 15 and 22 after tumour transplantation). A second $^{18}F^{-}$FDG PET Scan was performed 7 days after the second ifos administration. Animals were sacrificed one week after the end of the treatment. Tumour and normal tissue fragments (muscle, bone, and lung) were collected for RNA extractions.

2.4. RNA Extraction and Quantitative Real-Time PCR. FFPE tumours were lysed for 24 h in ATL buffer (Qiagen, France) supplemented with proteinase K (Qiagen) at 60°C in rotative agitation after different washes with toluene, ethanol, and

tris/EDTA in this order. Total RNA was extracted from tumour or cell pellets using a single phenol/chloroform extraction protocol with Trizol, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Five hundred nanograms of total RNA were subjected to the microfluidic PCR technology performed by Applied Biosystems (Foster City, CA, USA). In brief, RNA was reversed transcribed, using multiplexed specific looped miRNA primers from the Taqman MicroRNA Reverse Transcription kit. The second step consists in a real-time quantitative PCR on TLDA: RT products are introduced through microchannels into miniature wells that are preloaded with dehydrated specific primers and probes. Recently, Applied biosystems released the second version of TLDA, consisting of two cards A and B. Analyses were performed for 377 miRNAs on card A and 290 on card B.

2.5. PCR Data Normalization. For each miRNA, the threshold cycle (Ct) was calculated by the ABI 7900 Sequence Detection System software (plate by plate manual Ct analysis with a threshold at 0.25 and automatic baseline). All further data manipulations were done using R scripts. A cutoff of 32 was applied to discard the late Ct values, except for RMS analysis. Around 60% of miRNAs passed the filtering criteria and were used for further analysis. For each TLDA, quality controls were performed on the raw data by checking internal controls and using box plot and scatter plot diagrams. Samples with any kind of problems were discarded so they would not introduce bias during the following normalization procedures. We tested different methods of normalization since the recommended "pseudo" normalization factor mammU6 plotted in each card was not stably expressed in our different samples. Normalization with the two most stable miRNAs identified by GeNorm was not optimal too. Finally, a global normalization by the median was chosen for its reliability over experiments. Tissues included in a given analysis were treated altogether, the normalization procedure being applied separately for the two types of card, A and B. Distribution of normalized data was checked with box plots and correlation plots. The following formula was used to correct Ct values of every card:

$$(\text{Normalized Ct}) = \frac{\text{Ct} \times (\text{mean of medians})}{(\text{median of the card})}. \quad (1)$$

Through this approach, the new median value shared by all samples can be considered as a sort of perfect "virtual housekeeping gene". Therefore, the standard ΔΔCt method can be used to determine the relative quantities (RQ) as follows:

$$\Delta\text{Ct} = (\text{Normalized Ct}) - (\text{New shared median}). \quad (2)$$

For the ΔΔCt calculation, it was more relevant for the statistical analyses to use the mean of all ΔCt obtained across samples for each miRNA, instead of using the ΔCt of a reference sample

$$\Delta\Delta\text{Ct} = \Delta\text{Ct} - (\text{Mean of } \Delta\text{Ct across samples for each miRNA}), \quad (3)$$

$$\text{RQ} = 2^{-(\Delta\Delta\text{Ct})} \quad (4)$$

2.6. miRNA Target Predictions. We compiled 4 databases to determine miRNA targets: TargetScan 5.1, MiRanda, PICTAR, and the miRbase databases. These databases search the presence of conserved 8mer and 7mer sites on the 3′UTR parts of messenger RNA that match the seed region of each miRNA. It also predicts the efficacy of targeting for each matching site. We created our own database which regrouped each miRNA with the geneID of all their protein targets, for rat and human. We only conserved couples miRNA/geneID present in two databases at least.

2.7. miRNA-Regulated Cell Signalling Pathways Predictions. We used the "G-language microarray" web application, which allows the mapping of molecular dataset onto "Kyoto Encyclopedia of Genes and Genomes" (KEGG) pathway maps [24]. We first input miRNA-targeted proteins of interest and the sum of RQ values for all miRNAs that regulate these proteins, contained between 1 and 50; the software then generates KEGG data to create FLASH graphics of cell signalling pathways in which proteins are involved. The colour intensity of a highlighted protein varies with the strength of its regulation by miRNAs.

2.8. Proliferation Assay. Cells were plated in 96 well plates at 5000 cells/well and exposed to 100 nM RAD-001, 50 μM ifosfamide, 100 μM methotrexate, or 1 μM cisplatin or not (NT). Cell growth was measured 24, 48, and 72 h later with 20 μL Cell Titer Glo luminescent reagent (Promega, Madison, WI, USA) for 10 min. Luminescence was recorded using a Microbeta reader (PerkinElmer, Fremont, CA, USA).

2.9. Western Blot. Pelleted cells were resuspended in lysis buffer (Tris 50 mM pH 7.4, NaCl 250 mM, EDTA 5 mM, NaF 50 mM, Triton X-100 0.1%, orthovanadate 1 μM) plus protease inhibitors for 30 min on ice. After a centrifugation at 14000 rpm for 10 min, supernatants were boiled for 5 min in Laemmli sample buffer (Biorad, Hercules, CA, USA). Analysis of protein content was performed on 4%–12% gradient gel. After electrophoretic separation, 30 μg proteins were electrotransferred on a polyvinylidene difluoride membrane (Immobilon P, Millipore corp., Bedford, MA, USA). The membrane was then blocked for 1 h at room temperature with blocking agent 0.2% in PBS/Tween 0.1%, probed overnight with a primary rabbit antibody against the protein of interest, and finally revealed with a secondary antirabbit antibody HRP conjugated (Upstate Biotechnology, Lake Placid, NY, USA) and ECL Advance system (GEhealthcare, Chicago, IL, USA). Primary antibody used was obtained from Cell Signaling (New England Biolabs, Beverly, MA,

USA) used at 1/1000. The β actin was used as a reference (Sigma).

2.10. CGH Array. Oligonucleotide-based microarray analysis was performed using a custom-designed, 244K-feature whole-rat genome microarray manufactured by Agilent Technologies (Santa Clara, CA). Genomic DNA labeling, array hybridization, and washing were performed as specified by the manufacturer (Agilent Technologies). Results of aberration calls consisting of three or more consecutive oligos were then displayed using custom oligonucleotide CGH analysis software (Genespring).

2.11. Statistical Analysis. Normalized RQ data were directly input into the TIBCO Spotfire DecisionSite for Functional Genomics analysis software. We performed unsupervised hierarchical clustering to classify samples by groups. The selection of miRNAs useful to predict tumour response to treatment was statistically realized using ANOVA tests with P values of .05 at least. Results were verified through supervised hierarchical clustering.

Data from miRNA lists of interest were then used as variables in a three-dimensional principal component analysis (PCA) performed with R 2.9.0 package to demonstrate their capabilities to distinguish types of tumours. PCA supplies a simplified three-dimensional picture to our multivariate dataset of miRNA RQ values. By mathematical combination of values according to their strength, three principal components are created that represent as much as possible the variability of the data. Thus, tumours possess three new coordinates in a three-dimensional space. According to their localization in this space, tumours form groups, and their subtypes can be predicted.

3. Results

3.1. miRNA Signatures of Osteosarcoma Cell Lines. In our recent study published in International Journal of Cancer, we showed that the two osteosarcoma Saos-2 and CRL-15-47 (15-47) cells mimic the biological response of human osteosarcoma and tumours obtained from a rat model. In fact, we identified in an osteosarcoma rat model a panel of 61 miRNAs discriminating tumours with a good response to ifosfamide from those with a bad response [25]. On the basis of this signature, we realized a principal component analysis allowing predicting tumour response. In this PCA diagram, we could notice that the Saos-2 cells were predicted as sensitive to ifosfamide contrary to 15-47 cells (Figure 3(b) [25]), according the results obtained by a proliferation assay (Figure 6(a) [25] and Figure S1). This was confirmed by a PCA analysis realized with the miRNA signature identified in human tumours (Figure S2). We so considered that these two cell lines were an interesting model to study the importance of miRNAs in cell response to treatment and to identify new therapeutic strategies.

3.2. miRNA Signatures of Human Cancer Cell Lines. We firstly performed a preliminary miRNA profiling on different cell models to compare the miRNA profiles of osteosarcoma cells used in our laboratory to perform *in vitro* experiments, Saos-2 and 15-47 cells, with the chondrosarcoma cells SW-1353 (chondro) and the Burkitt lymphoma Daudi and Namalwa cells. In a previous study, we identified 61 miRNAs involved in osteosarcoma cell response to treatment [25]. We only conserved these miRNAs to realize an unsupervised hierarchical clustering with the five cancer cell lines. As shown in Figure 1(a), this miRNA signature was representative of the two human osteosarcoma cell lines, since these two cells clustered together independently but closely to the chondrosarcoma cells. These three cell lines were classed in a distinct group from the two lymphoma cells Daudi and Namalwa. This confirmed that each cancer cell line presents a miRNA signature in accordance with their origin, as shown by others [15, 16].

3.3. miRNA Profiles in Response to Chemotherapeutic Agents Were Cell Specific. Then, we assessed if miRNA profiles were specifically modified in response to chemotherapy. We chose to expose osteosarcoma and lymphoma cells to ifosfamide, an alkylating chemotherapeutic agent currently used for paediatric osteosarcoma. A proliferation assay based on ATP measurement showed that the only Saos-2 cell line was moderately sensitive to 50 μM ifosfamide after 48 h exposure (proliferation inhibition around 30%) (Figure S1). Based on this observation, we decided to expose these cells to 50 μM ifosfamide for 24 h to realize miRNA profiling. On the basis of the panel of 61 miRNAs identified in our previous study [25], osteosarcoma cells were markedly different from lymphoma cells, confirming that miRNA profiles were cell specific as shown by the unsupervised hierarchical clustering in Figure 1(b). We could notice that Saos-2 cells present a unique miRNA signature in which the majority of miRNAs were overexpressed (in red in Figure 1(b)). A supervised hierarchical clustering realized following an ANOVA $P < .03$ between the Saos-2 sensitive cells versus the resistant cells revealed that they effectively clustered according to their sensitivity to ifos: Saos-2 in one hand, independently to 15-47 cells and both lymphoma cells (Figure 1(c)). We confirmed this observation with the other chemotherapeutic agent ciplatin. As previously, cells were classified according to their susceptibility to CDDP on the supervised hierarchical clustering in Figure S3A (ANOVA $P < .03$): the 15-47 and Namalwa cells, sensitive to CDDP based on the proliferation assay in Figure S3B, clustered together, independently to Daudi and Saos-2 cells refractory to this treatment.

3.4. Osteosarcoma Cell miRNA Profiles Were Specific of Each Chemotherapeutic Agent. Thus, since miRNA signatures of untreated as well as treated cells were cancer specific, we assessed if each chemotherapeutic drug induced a different miRNA profile in a same cell. As suggested previously for osteosarcoma cells, cisplatin and ifosfamide exposure resulted in quite different miRNA profiles. After a statistical analysis with an ANOVA $P < .03$, we only found two dicriminating miRNAs common to both miRNA signatures induced by ifos and CDDP in the two cell lines (Figure S3). In this context, we test a third cytotoxic agent currently administered in osteosarcoma pathology, the methotrexate.

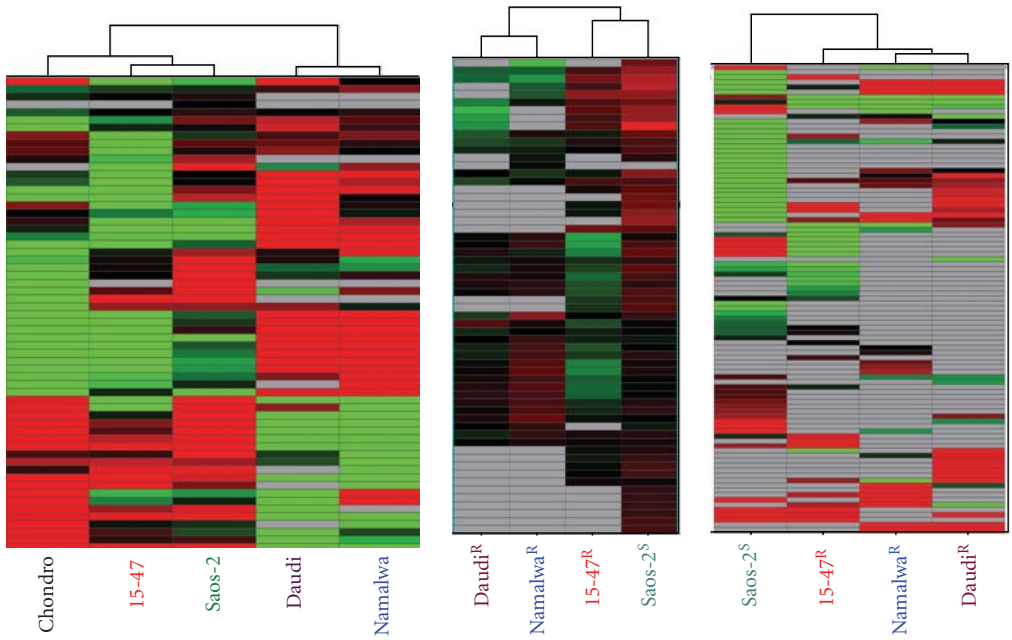


Figure 1: Cancer cell miRNA signatures were consistent with their tissue origin and with their sensitivity to ifosfamide. (a) This unsupervised hierarchical clustering only conserved the 61 miRNAs which discrimated osteosarcoma cells according to their response to treatment. Osteosarcoma cell lines clustered together near chondrosarcoma cells and independently to the lymphoma Daudi and Namalwa cells. Each row represents the relative levels of expression for each miRNA, and each column shows the expression levels for each sample. The red or green colour indicates relatively high or low expression, respectively, while grey squares indicate no expressed miRNA. (b) and (c) miRNA profiles after exposure to 50 μM ifosfamide for 24 h. (b) This unsupervised hierarchical clustering only conserved the 61 miRNA differently expressed in osteosarcoma cells according to their sensitivity to ifos following an ANOVA ($P < .03$). (c) This supervised hierarchical clustering conserved miRNAs differently expressed in cells according to their response to treatment following an ANOVA ($P < .05$) after removing the miRNAs whose expression depends on the cell types. The red or green colour indicates relatively high or low expression, respectively, while grey squares indicate no expressed miRNA.

As shown in the unsupervised hierarchical clustering in Figure 2, only conserving the 61 miRNAs of interest for osteosarcoma response, as explained above, the miRNA signature in the two osteosarcoma cells Saos-2 and 15-47 strongly differed from those observed for ifsofamide and cisplatin. It is important to note that a majority of these miRNAs were overexpressed in both cell lines in response to MTX. This was relevant with their sensitivity to MTX as shown in the proliferation assay in Figure 2(b). In brief, it seems that discriminating miRNAs were generally overexpressed in the cells after exposure to a cytotoxic agent, to which they were sensitive, as it was also shown for ifosfamide in the Saos-2 cells (Figure 1(b)). This also confirmed that miRNAs predicting cell response to a treatment differed according to the drug.

On the basis of these preliminary *in vitro* results, we could suggest that miRNA profiles, due to their drug specificity, could be a potent tool to predict a cancer cell response to a treatment. Since osteosarcoma is currently resistant to conventional treatments, the prediction of its response to one agent could be a progress for this pathology.

3.5. Osteo- and Chondrosarcoma Cell Response to the mTOR Inhibitor RAD-001. As highlighted by these previous data, we were able to classify and predict osteosarcoma cell response to chemotherapy. Our algorithms were not only interesting for chemotherapeutic agents but also promising to identify new targeted therapies to encounter osteosarcoma resistance. Thus, we tested a potent drug for skeletal sarcoma treatment, which inhibits the pro-oncogenic protein mTOR, called RAD-001 (Everolimus, Novartis). mTOR is often aberrantly activated in cancers and, in particular in chondrosarcoma [26] and osteosarcoma [27]. mTOR signalling has been described as implicated in tumour development, metastasis, and drug resistance [28, 29]; thus, mTOR targeting successfully inhibits tumour growth and renders them sensitive to conventional treatments [30, 31]. RAD-001, acting in a similar manner than rapamycin through the inhibition of mTORC1 complexes, is currently tested in various clinical trials for renal cell carcinoma (RECORD program), advanced papillary tumours (RAPTOR), metastatic neuroendocrine tumours (RAMSETE), or breast cancers (BOLERO).

Thus, we performed *in vitro* experiments on chondrosarcoma and osteosarcoma cells with 100 nM RAD-001. The Saos-2 and chondrosarcoma cell proliferation was reduced of 40% following exposure to RAD-001 during 72 h contrary to 15-47 cell growth (Figure 3(a)). In parallel, we

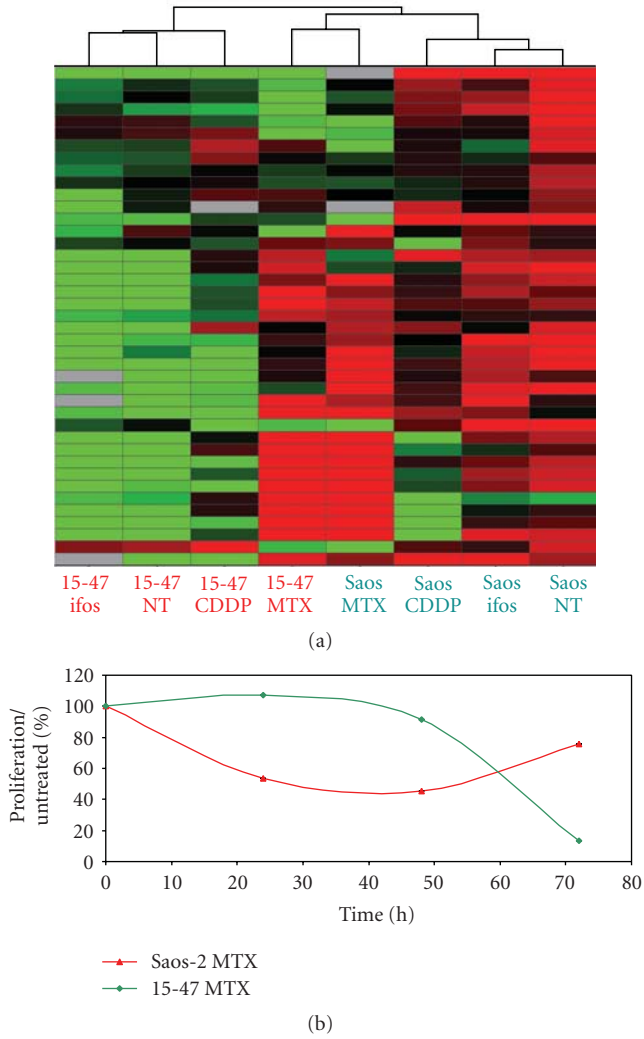


Figure 2: miRNA expression profiles osteosarcoma cells were specific for each chemotherapeutic agent. (a) This unsupervised hierarchical clustering conserved the miRNAs identified as discriminating for ifosfamide response after removing the miRNAs whose expression depends on the cell types. Each row represents the relative levels of expression for each miRNA, and each column shows the expression levels for each sample. The red or green colour indicates relatively high or low expression, respectively, while grey squares indicate no expressed miRNA. (b) Cell growth was measured by the Cell Titer GloLuminescent assay as described in Section 2.6 24, 48, and 72 h after exposure to 100 μM methotrexate. Results were represented as the mean % of proliferation normalized to untreated cells of two independent experiments realized in duplicate.

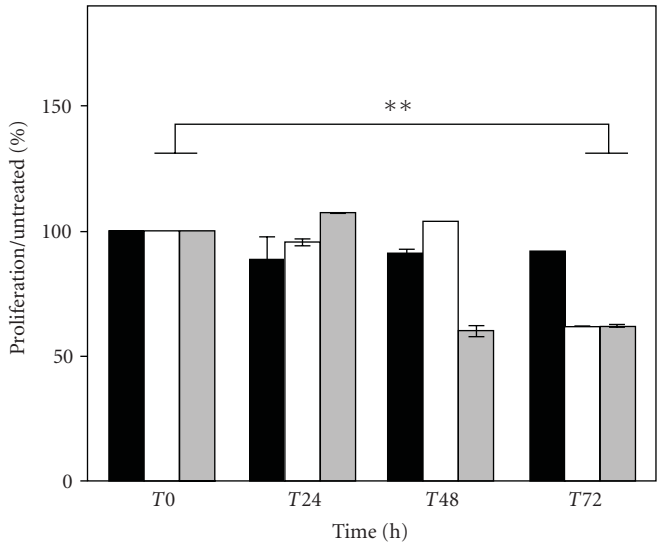
realized Western blot with RAD-001 on chondrosarcoma and osteosarcoma cells concerning the major actors of the mTOR cell signalling pathways. This revealed that the mTOR pathway was inhibited by RAD-001 in chondrosarcoma cells contrary to 15-47 cells, in particular eIF4G and p70 S6 kinase whose phosphorylation level was decreased (Figure 3(b)).

Thus, we analysed if the miRNA signatures of these cells were different and could explain their differential response to RAD-001. We performed a supervised hierarchical clustering between untreated Saos-2, chondrosarcoma and 15-47 cells following an ANOVA with $P < .05$. This clustering revealed that 16 miRNAs discriminated the chondrosarcoma and Saos-2 cells in one hand and the 15-47 cells in the other hand (Figure 4). Except miR-146, Saos-2 and chondrosarcoma overexpressed these contributory miRNAs.

Thereafter, as we have explained in our previous study on osteosarcoma [25], miRNA profiling constitutes a potent tool to identify miRNA-targeted cell signaling pathways through an *in silico* approach. In our case, we searched if these miRNAs shown as differently expressed in cells according to their response to RAD-001 potentially target the mTOR signalling pathway. We created a database, as described in Section 2.6 which determine the predicted targets for these miRNAs described in the miRbase. Then, we summed up the RQ values for each miRNA in the Saos-2 and chondrosarcoma cells sensitive to RAD-001 and concatenated with the geneID of their protein targets. We finally inserted these data in the G-language microarray web application, which connects miRNA targets according to their involvement in similar KEGG pathways, the mTOR pathway in this case. As shown in Figure 5, the mTOR pathway is targeted by these miRNAs and particularly its downstream proteins implicated in VEGF signaling and autophagy processes, in particular RICTOR, ATG1, and HIF-1a. Thus, the Saos-2 and chondrosarcoma cells overexpressed miRNAs that potentially inhibit mTOR signalling. Inhibition of these miRNAs through the use of Locked Nucleic Acid (LNA) and qPCR measurement of RICTOR, ATG1, and HIF1a could confirm this concept.

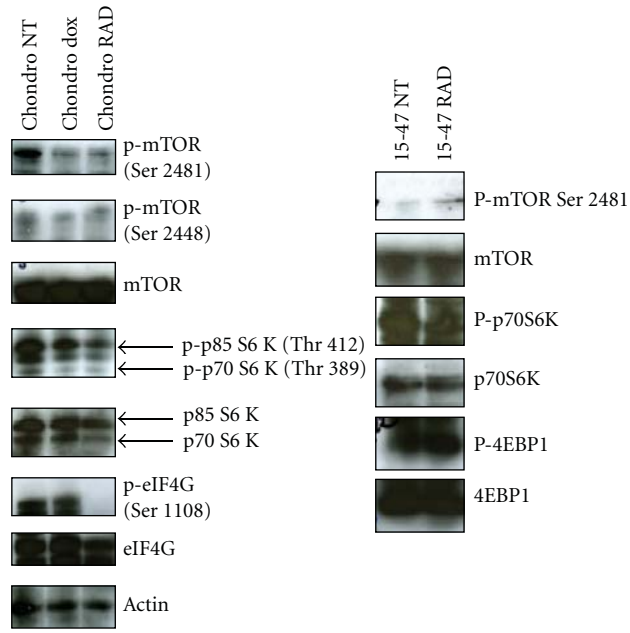
To resume, miRNAs constitute potent biomarkers to determine the susceptibility to a treatment and could be very useful to identify new therapeutic targets as an alternative of chemotherapy for chondrosarcoma and osteosarcoma often refractory to this treatment. In the next steps, we assessed if these observations were relevant *in vivo*, with a model of rat osteosarcoma and with patient samples.

3.6. Predictive miRNA Signature of a Rat Osteosarcoma Model Was Probably Not Related to DNA Aberrations. As described in other studies realized by members of our team [21, 22, 25], we possess a rat osteosarcoma model mimicking the human pathology concerning aggressiveness, chemoresistance and the apparition of lung metastases (see Section 2.6). The treatment of animals with ifosfamide results in two groups, the good versus the bad or moderate responders, in a proportion closer to that observed for patients. By miRNA profiling, we were able to distinguish tumours sensitive to ifosfamide from those refractory to this drug and above all to predict the response of untreated tumours with ten miRNAs through the use of statistical algorithms created in our lab [25]. Following these interesting data, we would like to confirm that this miRNA signature was specific of tumour response to treatment and not related to different tumour genetic backgrounds. We thus realized an analysis in CGH array with the same tumours used for miRNA profiling. We analysed two tumours of each type, untreated, treated with ifosfamide and good responder, or treated with ifosfamide and bad responder, as compared to the



P value	*T*24 versus *T*0	*T*48 versus *T*0	*T*72 versus *T*0	*T*72 versus *T*48
15–47	NS	NS	NS	NS
Saos	NS	NS	0.026	0.019
Chondro	NS	0.019	0.026	NS

(a)



(b)

Figure 3: mTOR inhibition by RAD-001 in chondrosarcoma resulted in cell proliferation inhibition. (a) Cell growth was measured by the Cell Titer GloLuminescent assay as described in Section 2.6 24, 48, and 72 h after exposure to 100 nM RAD-001. Results were represented as the mean % of proliferation normalized to untreated cells of two independent experiments realized in duplicate. A Fisher test was realized, NS corresponds to "nonsignificant". (b) Western blot analysis of RAD-001 effects on the mTOR pathway in the chondrosarcoma and 15-47 osteosarcoma cells exposed or not (NT) to doxorubicin (dox). 30 μg protein extracts were analysed by Western blot with antibodies 1/1000 against actors of the mTOR pathway (phosphorylated form or not) (Cell signalling, Beverly MA).

same untreated bone sample, the reference tissue in CGH analysis. The majority of chromosomal aberrations observed in CGH array was common to untreated tumours and treated tumours, regardless of their response to treatment (Figure 6). The few different abnormalities were essentially linked to individual tumour biological specificities.

We compiled all abnormalities and verified in our "home-made" database if any miRNA, identified as discriminating of tumour response, was located in these DNA regions. Interestingly, this *in silico* analysis also revealed that neither miRNA nor gene were present in the few differential aberrations observed in these tumours, in particular in the chromosome 4 (Figure S4), suggesting that the different miRNA profiles were rather linked to tumour response to treatment and not due to upstream chromosomal rearrangements. Although we could not rule out that some trans-acting proteins could be deregulated consequently to these aberrations, this suggested that all tumours were homogeneous and that an increase in some miRNAs in sensitive tumours were due to their upregulation and not to a genetic amplification.

It seems that molecular diagnosis based on miRNA profiling highlights the tumour behaviour, that is, in response to a treatment, and thus a phenotype rather than a genotype contrary to CGH array. These two molecular techniques could be a couple of choice to improve the care of patients with pathologies currently hardly to diagnose.

3.7. Rhabdomyosarcoma miRNA Profiles Were Correlated to their Histological Subtypes. Finally, to corroborate the previous idea considering that miRNA profiling could be very helpful for uncertain diagnoses, we performed the miRNA profiling of rhabdomyosarcoma samples. In fact, we recently showed that miRNA profiling was reliable for osteosarcoma diagnosis on 29 formalin-fixed paraffin embedded (FFPE) biopsies of patients [25]. Based on the expression level of a panel of five miRNAs, we successfully separated good responders from bad responders to treatment. So, we assessed if our TLDA platform was also competitive for RMS diagnosis. We obtained seventeen tumours including alveolar RMS patients, (Pax3+) (3 patients) or Pax7+ (2), embryonal RMS patients (6) and negative fusion aRMS (6).

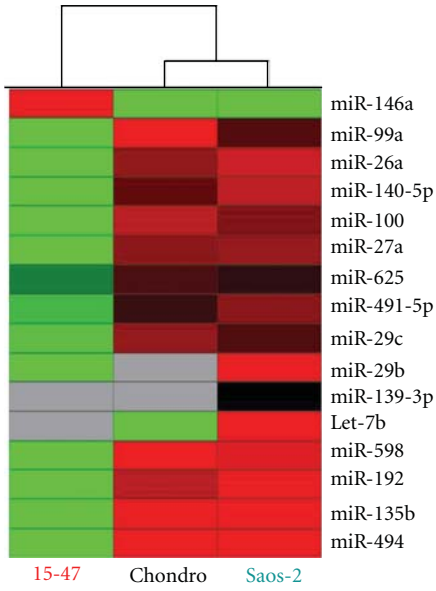


Figure 4: miRNA expression profiles of osteosarcoma and chondrosarcoma cells were consistent with their sensitivity to the mTOR inhibitor RAD-001. This hierarchical clustering only conserved miRNA differently expressed in tumours according to their response to treatment following an ANOVA ($P < .05$). Each row represents the relative levels of expression for each miRNA and each column shows the expression levels for each sample. The red or green colour indicates relatively high or low expression, respectively, while grey squares indicate no expressed miRNA.

All these tumours were diagnosed through the use of immunohistochemistry, FISH and qPCR, which were validated by a referent anatomopathologist (Table S5). A supervised hierarchical clustering on rhabdomyosarcoma tumours following an ANOVA with a P value $< .03$ between the four types of RMS, revealed that tumours clustered according to their molecular alterations Pax3/FOXO1, Pax7/FOXO1 or no translocation, on the basis of the expression level of 10 miRNAs (Figure 7(a)). (Pax+) tumours, particularly those (Pax3+) overexpressed all these miRNAs. Then, we performed a statistical analysis with these ten miRNAs based on Principal Component Analysis, a method which allows studying the variability between a set of variables. This consists of assigning a new system of three coordinates to each contributory miRNA by a mathematical procedure. Then, RQ values of each miRNA are adjusted for each tumour by the new coefficients obtained previously and summed up. Thus, a 3-dimension PCA diagram was realized with the three new coordinates for each tumour (Figure 7(b)). Through this mathematical representation, we could distinguish (Pax+) from fusion negative aRMS and eRMS. eRMS also constitutes an independent group with a high value of component 2 (represented in the y-axis on Figure 7(b)). The fusion negative aRMS constitute a separate group even if some samples were difficult to classify in accordance with their uncertain diagnosis. Even if the number of samples was low for each subset, a statistical analysis showed a significant P value between (Pax3+) and (Pax7+) and between (Pax+) and (Pax−) tumours, 0.05 and 0.0005 respectively (Figure S6).

We showed that miRNA profiling was a potent tool to discriminate fusion negative aRMS from embryonal RMS. miRNAs could be useful biomarkers to improve the diagnosis of this type of RMS, since fusion negative aRMS are currently molecularly indistinguishable from eRMS [7].

4. Discussion

miRNA signatures are observed for many types of cancers, that is, sarcoma [19], breast and prostate cancers [18, 32]. These signatures constitute potent diagnosis and prognosis tools for chronic lymphocytic leukemia [33], colon adenocarcinoma [34], or lung cancers [35]. Here, we showed that osteosarcoma cell lines also expressed miRNA patterns different from those of chondrosarcoma and lymphoma cells (Figure 1(a)) and which allow us to discriminate cell response to chemotherapeutic treatment (Figures 1(b), 2, and S3). In addition, osteosarcoma miRNA signatures were cell and drug specific (Figure 2(a)). This drug specificity of osteosarcoma has also been observed by Song et al. with U2-OS osteosarcoma tumour xenografts, in which different miRNAs were deregulated in response to the chemotherapeutic agents doxorubicin, cisplatin, and ifosfamide; only 3 miRNAs were commonly found deregulated in response to all drugs [36]. With their specificity, miRNAs constitute promising biomarkers to anticipate the tumour response to a treatment of interest. As we have recently shown, through miRNA profiling, we were able to predict osteosarcoma tumour response to chemotherapy for rat tumours as well as for patient FFPE biopsies [25]. Here, we showed that miRNA profiles of osteosarcoma cells were in accordance with their response to the mTOR inhibitor, RAD-001 (Figures 3 and 4). The miRNAs deregulated in response to this drug in sensitive cells, effectively targeted the mTOR pathway, in particular the downstream proteins eIF4G and p70 S6 kinase (Figure 3(b)), and potentially RICTOR, ATG1 and HIF1a, which might be validated by qPCR analysis (Figure 5).

In brief, miRNAs appeared very useful for the identification of new exciting therapeutic approaches through the targeting of some miRNA protein targets or some miRNAs involved in tumour development themselves. In future, we would like to confirm the implication of these miRNAs in treatment response *in vitro* through the use of miRNA mimics or inversely of Locked Nucleic Acid (LNA) against these miRNAs. As mentioned in this study, we possess an interesting *in vitro* osteosarcoma model, on which we could test the miRNA functionality in the presence of the different drugs used in this work. Following the validation of miRNA involvement *in vitro*, we would also test these mimics or LNAs *in vivo* in the model of rat osteosarcoma. This approach has been successfully employed in rhabdomyosarcoma through the conditional expression of miR-206 in mice [37] and could become a potent therapeutic strategies [38].

In addition to the identification of new targets, miRNA also constitute an interesting alternative to the conventional

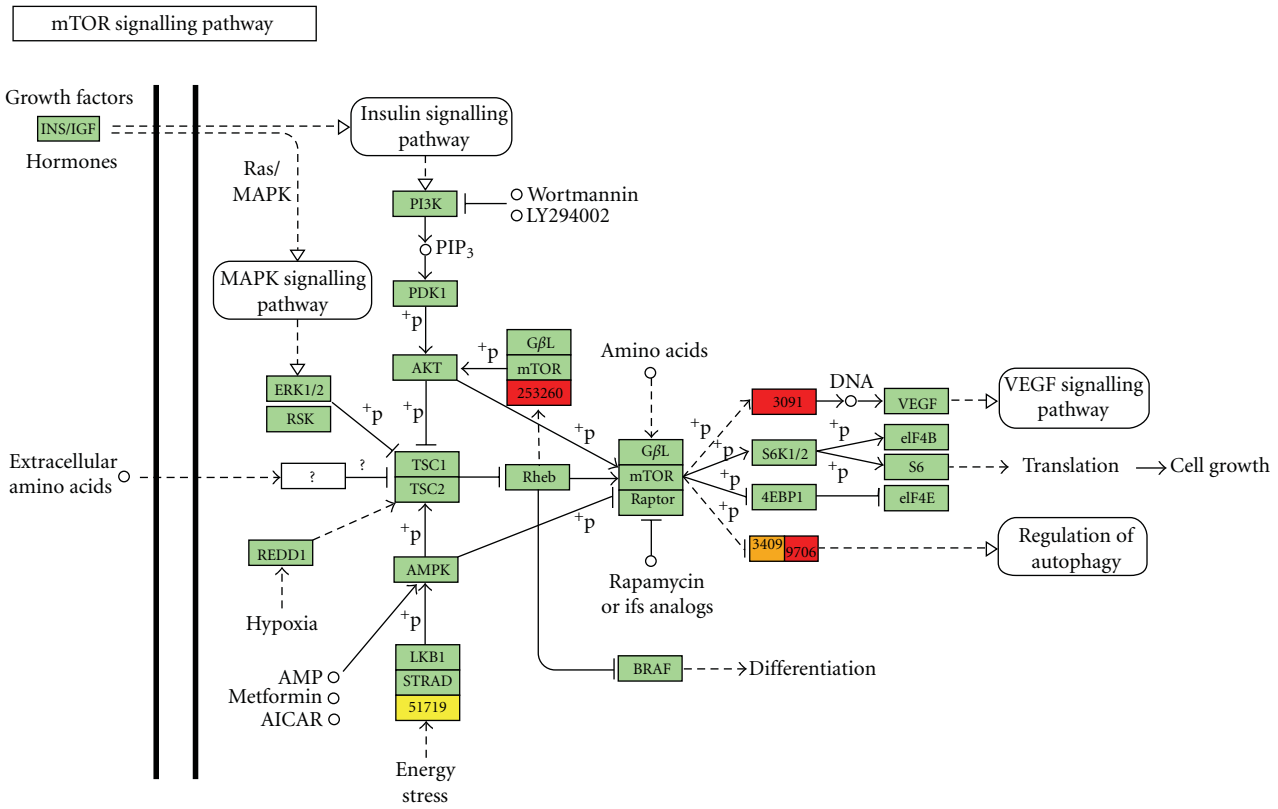


Figure 5: The discriminating miRNAs interfered with the mTOR pathway. Proteins in yellow, orange, and red colours represent targets of miRNAs; a yellow square represents a weak repression, while red represents the maximal repression; green squares were not targeted.

molecular technologies routinely used for cancer diagnosis. In fact, osteosarcoma present complex karyotypic alterations rendered them difficult to diagnose with current diagnostic methods, like CGH array [39]. With the rat osteosarcoma model, we confirmed that tumours presented numerous long chromosomal aberrations (Figure 6). These abnormalities were generally common to all tumours, regardless to their susceptibility to treatment and neither miRNAs of interest nor genes were located in these regions (Figure S4). Even if some proteins involved in the regulation of miRNA expression (trans-acting factors or epigenetic regulating factors) could be deregulated following these mutations, the miRNA profiles observed in rat tumours might be correlated to the effects of the cytotoxic drugs on the miRNA machinery and no to upstream DNA rearrangements. Even if miRNAs could be submitted to epigenetic regulation like methylation or acetylation, this only concerns 5% to 10% miRNAs, and we could consider that this process is minor for the miRNA signature of osteosarcoma tumours and cells based on 61miRNAs [40, 41]. Enthusiastically, our work is the first suggesting that miRNA signatures were not correlated to DNA amplifications, as it was observed for neuroblastoma [42] or in mixed lineage leukemia [43]. Although our cohort was not fully satisfying, it appeared that miRNA profiling could predict tumour response to treatment by reflecting tumour biological specificities and not genotypic characteristics. This work also highlights miRNA measurement as an interesting partner to CGH array in the case of pathologies with unstable karyotypes. In the same way, Selvarajah et al. was the first to suggest a combination of CGH array and interphase FISH to better understanding osteosarcoma pathogenesis [44].

miRNA patterns were not only related with osteosarcoma phenotypic properties but also with rhabdomyosarcoma histological subtypes. By miRNA profiling, we were able to discriminate the different subtypes of rhabdomyosarcoma: Pax3+ or Pax7+ or fusion negative, classically difficult to diagnose by histological analysis (Figure 7(a)). This miRNA pattern was unique since all miRNAS identified as discriminating are no or weakly described in the literature. Very interestingly, on the basis of their miRNA profiles, our algorithms allow us to discriminate embryonal RMS from fusion negative aRMS (Figure 7(b)). It was in agreement with the work of Wachtel et al. identifying different expression profiles linked to aRMS (Pax+), fusion negative aRMS, and eRMS [45].

Altogether, it seems that miRNA measurement is advantageous for sarcoma with complex karyotype, since fusion negative RMS, similarly to osteosarcoma, are characterized by a complex karyotype linked to allelic imbalance, loss of heterozygoty and heterogeneous gene expression profiles. Although the molecular classification of fusion negative RMS is always controversial, our work corroborates the study of Davicioni et al. suggesting that Pax/FOXO1 dictates a specific

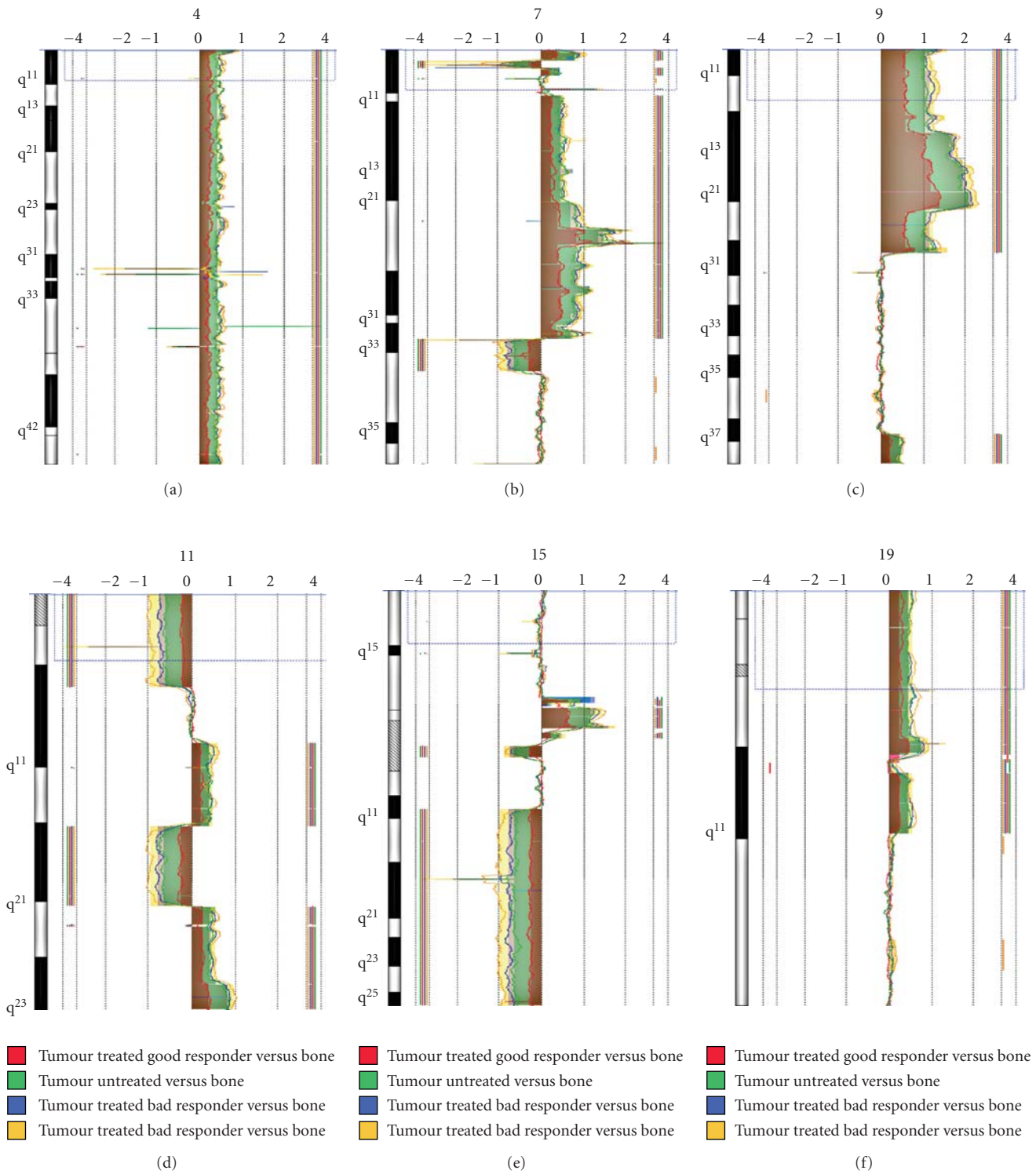
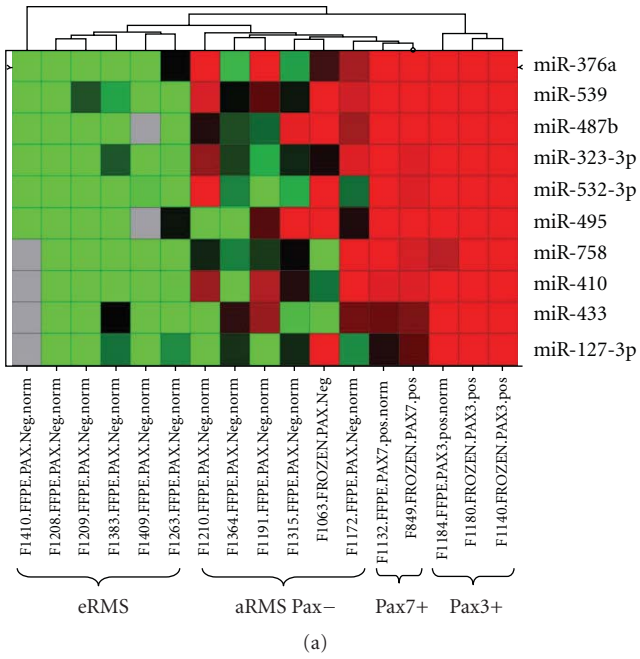
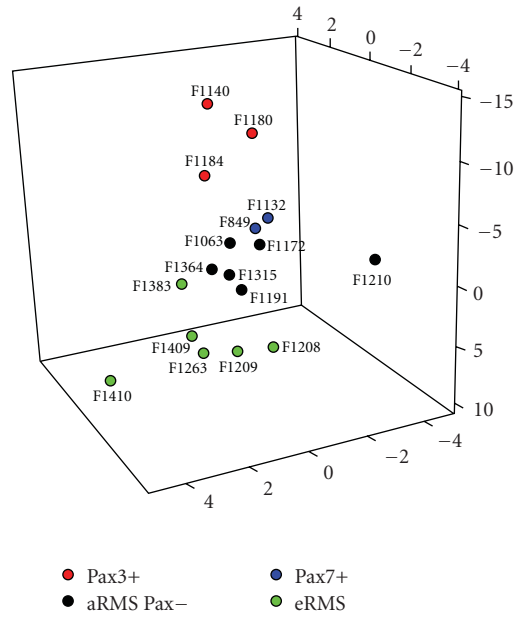


Figure 6: CGH analysis of six tumours from an osteosarcoma rat model. Here, we represent six chromosomes among the twenty + X chromosomes present in rat genome. All the analyses were performed with the same untreated bone sample as a reference.

expression signature in RMS by oligonucleotide microarray expression profiling [46]. Inversely, this differs from the recent work of Williamson suggesting that fusion negative alveolar rhabdomyosarcoma is difficult to distinguish from embryonal rhabdomyosarcoma concerning patient survival characteristics, gene expression profiles, and CGH arrays [7]. In fact, our work and theirs were not totally contradictory, since they only focused on genomic analysis. As suggested for osteosarcoma, miRNA patterns reflect the phenotypic tumour properties rather than its genetic and could be



(a)



(b)

Figure 7: Rhabdomyosarcoma miRNA signatures were consistent with their molecular alterations. (a) This supervised hierarchical clustering only conserved miRNA differently expressed in the different subtypes of RMS following an ANOVA ($P < .03$). Each row represents the relative levels of expression for each miRNA, and each column shows the expression levels for each sample. The red or green colour indicates relatively high or low expression, respectively, while grey squares indicate no expressed miRNA. (b) Principal component analysis of RMS tumours as a tool to determine the potential tumour response to treatment. RQ values of the ten selected miRNAs for each tumour were corrected by the coefficients determined in (a), as described in Section 2.6.

a promising alternative for RMS diagnosis to surpass the current limitations of molecular analysis combined to traditional histopathology.

Thus, it seems that miRNA profiling could be very useful for osteosarcoma and rhabdomyosarcoma diagnosis. Here, we showed that on the basis of ten miRNAs, we were able to separate the different subtypes of RMS. We have previously suggested that a panel of five miRNAs was statistically sufficient to distinguish the potent response of osteosarcoma patients to treatment [25]. The TLDA technology presents numerous advantages including its need for few amount of total RNA and the possible analysis of FFPE samples, as it was previously shown by others [47–49]. This method is especially useful to detect circulating miRNAs in patient serum, an emerging field these two past years [50, 51]. A blood-based molecular diagnosis tool through miRNA profiling from patient serum could be a major advance for osteosarcoma, requiring a biopsy for its diagnosis, which could result in a secondary amputation.

Altogether, these promising results open up the way to a new diagnosis tool based on miRNA for osteosarcoma as well as rhabdomyosarcoma, which could improve patient survival in both cases through the prediction of patient response to chemotherapy and the precise identification of RMS subtypes, respectively.

Abbreviations

CDDP:	Cisplatin
Chondro:	Chondrosarcoma cells
Ct:	Threshold cycle
Dox:	Doxorubicin
FFPE:	Formalin-fixed paraffin embedded
ifos:	Ifosfamide
KEGG:	Kyoto encyclopedia of genes and genomes
LNA:	Locked nucleic acid
NT:	Non treated
PCA:	Principal component analysis
RAD:	RAD-001
RMS:	Rhabdomyosarcoma
RQ:	Relative quantity
RT-qPCR:	Real-time quantitative PCR
TLDA:	Taqman low density array
15-47:	Human osteosarcoma cells CRL-15-47.

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Research Article

Delineation of Chondroid Lipoma: An Immunohistochemical and Molecular Biological Analysis

Ronald S. A. de Vreeze,[1] Frits van Coevorden,[1] Lucie Boerrigter,[2] Petra M. Nederlof,[2] Rick L. Haas,[3] Johannes Bras,[4] Andreas Rosenwald,[5] Thomas Mentzel,[6] and Daphne de Jong[2]

[1] *Department of Surgical Oncology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, 1066 CX, Amsterdam, The Netherlands*
[2] *Department of Pathology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, 1066 CX, Amsterdam, The Netherlands*
[3] *Department of Radiation Oncology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, 1066 CX, Amsterdam, The Netherlands*
[4] *Department of Pathology, Academic Medical Centre, 1105 AZ, Amsterdam, The Netherlands*
[5] *Department of Pathology, University of Wuerzburg, 97074 Wuerzburg, Germany*
[6] *Department of Dermatopathology, Bodensee, D-88048 Friedrichshafen, Germany*

Correspondence should be addressed to Ronald S. A. de Vreeze, rdevreeze@gmail.com

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Aims. Chondroid lipoma (CL) is a benign tumor that mimics a variety of soft tissue tumors and is characterized by translocation $t(11;16)$. Here, we analyze CL and its histological mimics. *Methods*. CL ($n = 4$) was compared to a variety of histological mimics ($n = 83$) for morphological aspects and immunohistochemical features including cyclinD1(*CCND1*). Using FISH analysis, *CCND1* and *FUS* were investigated as potential translocation partners. *Results*. All CLs were strongly positive for *CCND1*. One of 4 myoepitheliomas, *CCND1*, was positive. In well-differentiated lipomatous tumors and in chondrosarcomas, *CCND1* was frequently expressed, but all myxoid liposarcomas were negative. FISH analysis did not give support for direct involvement of *CCND1* and *FUS* as translocation partners. *Conclusions*. Chondroid lipoma is extremely rare and has several and more prevalent histological mimics. The differential diagnosis of chondroid lipomas can be unraveled using immunohistochemical and molecular support.

1. Introduction

Lipomatous lesions show a broad morphological spectrum and clinically range from benign to highly malignant diseases. Over the last few years, studies focusing on lipomatous tumors have led to the delineation of new variants of lipomatous proliferations as well as to the introduction of new concepts, mainly as a result of the fruitful interactions between molecular genetics and pathology [1–4]. As a result, chondroid lipoma has been described and considered a benign tumor of soft tissue that may mimic a variety of soft tissue tumors [1, 5–7].

At gross examination, chondroid lipoma resembles lipoma, presenting as a solitary, slowly growing mass that is located either within skeletal muscle, muscle fascia, or in the deep subcutis. The main cytological features consist of clustered, variably mature, multivacuolated hibernoma-like cells enmeshed in a capillary plexus, in a background of chondromyxoid material. This tumor may show histologic features resembling myoepithelioma, myxoid liposarcoma, extraskeletal myxoid chondrosarcoma, hibernoma, and other lipomatous or chondroid neoplasms, resulting in diagnostic and consequently therapeutic dilemmas [8].

Cytogenetic data on a few cases of chondroid lipoma are available and show a balanced translocation $t(11;16)$ (q13-p12) [9–11]. The typical and recurrent involvement of 11q13 has also been described in other classes of lipomatous tumors such as ordinary lipoma and hibernoma, but not in

association with 16p12-13. Several genes at the breakpoint regions may be relevant candidate genes, and recently MKL/myocardin-like 2 (MKL2) has been implicated. Recent findings show that cyclinD1 (*CCND1*) is not only involved in cell cycle regulation, but also in the regulation of cellular metabolism, cellular migration, and especially fat cell differentiation, making this a relevant candidate gene [12]. Another candidate fusion gene is the *FUS* gene located on chromosome 16p11. This gene is involved in one of the typical mimics of chondroid lipomas: myxoid liposarcoma. Although *FUS* is located at a different chromosomal location (16p11 versus 16p12) and therefore involvement in chondroid lipomas is not highly likely, it has not been properly investigated.

Here, we describe a histopathological, immunohistochemical and fluorescence in situ hybridization analysis in a series of chondroid lipomas and histological mimics.

2. Methods

To retrieve all cases diagnosed as chondroid lipomas in the Netherlands between 1997 and 2007, a search was performed in the Dutch nationwide pathology registry database (Pathologic Anatomic National Automated Archive, PALGA). The PALGA database contains all reports of potential cases and anonymous patient characteristics such as age, gender, conclusions, and coded summaries of all pathology reports in the Netherlands since 1992. Potential cases were retrieved and corresponding formalin fixed paraffin embedded tissue blocks were collected from the original pathology laboratories. Eleven cases were reviewed on hematoxylin and eosin-stained slides with additional immunohistochemical and molecular data if needed (RdV, DdJ and JB); consensus was obtained at the multiheaded microscope. Two additional cases of chondroid lipoma were retrieved from the files of the Department of Dermatology, Bodensee, Friedrichshafen (Dr. T. Mentzel) and also included for further study. Classification of all biopsy and resection material was performed according to the WHO classification for soft tissue tumors [13]. Four cases were diagnosed as chondroid lipoma four cases as myoepithelioma and selected for further study. The remaining cases were diagnosed as myxoid liposarcoma ($n = 1$), lipoma ($n = 1$), and hibernoma ($n = 1$), chondrolipoma ($n = 2$).

Specifically, to further investigate the role of *CCND1* in the spectrum of lipomatous tumors, 21 lipomas, 28 well-differentiated liposarcomas, 18 myxoid liposarcomas, and 10 chondrosarcomas, both extraskeletal myxoid chondrosarcomas and primary chondrosarcomas of bone with extension into the soft tissue that were diagnosed in the same period, were randomly selected from the files of the Netherlands Cancer Institute to complete the morphological spectrum.

2.1. Immunohistochemical Analysis. Immunohistochemical staining was performed according to standard methods. In brief, after a pretreatment of citrate-based microwave antigen retrieval, the sections were incubated with the following antibodies overnight at 4°C without pretreatment: *CCND1* antibody (SP-4 AB-5 Labvision, Fremont, USA) dilution 1 : 100, *CD34* antibody (QBEND Labvision, Fremont, USA) dilution 1 : 3000, *CD68* antibody (KP1 DAKO Glostrup Denmark) dilution 1 : 50000, S100 antibody (polyclonal DAKO Glostrup Denmark) dilution 1 : 6000, Pan Keratin antibody (MNF116 + LP34 DAKO Glostrup Denmark) dilution 1 : 1600, *P63* antibody (MNF116 + LP34 LabVission Fremont USA) dilution 1 : 5000, *SMA* antibody (1A4 Zymed Carlsbad USA) dilution 1 : 5, vimentin antibody (3B4 DAKO Glostrup Denmark) dilution 1 : 400 and visualized with diaminobenzidine. The percentage of tumor cells with nuclear staining was assessed semiquantitatively. Staining intensity was ranked in three levels (positive, focal positive, and negative). Immunohistochemical staining of all slides were scored by two observers (DdJ and RdV). Slides could only be scored negative if positive internal controls were present. In cases of discrepancies or equivocal interpretations, consensus was obtained at the multiheaded microscope.

2.2. Fluorescence in Situ Hybridization. After confirming the most histologically typical areas using hematoxylin and eosin stained sections, dual-colour fluorescence in situ hybridization assay was performed according to standard methods on 5-μm-thick tissue sections of formalin fixed paraffin embedded specimens. After dewaxing, hydration, and pretreatment (DAKO Glostrup Denmark) at 95°C for 10 min, a protease digestion was performed in for 15 min at 37°C. Incubation was performed according to the dual-color break-apart principle with two differently labeled probes flanking the gene of interest. For *CCND1*, one Texas Red-labeled DNA probe (*CCND1-Upstream*) covering 163 kb centromeric to the *CCND1* breakpoint cluster region and one fluorescein-labeled DNA probe (*CCND1-Downstream*) covering 644 kb telomeric to the *CCND1* breakpoint cluster region were cohybridized (DAKO Glostrup Denmark). For analysis of *FUS* (16p11), one Spectrum Green labeled probe distal to the *FUS* gene and one Spectrum Orange labeled probe proximally from the *FUS* gene were used. Slides were incubated at 37°C for 48 h in a humidified chamber. After stringent washing at 72°C for 2 min and counterstaining, fluorescent signals were scored using a Nikon Microphot-SA fluorescence microscope with appropriate filters, and the resulting images were captured using a charge-coupled-device camera. Fifty to 60 evaluable nuclei were counted by two different individuals (PN and RdV), and the percentages of single and fused signals were calculated. A positive result was defined as the presence of split signals in more than 10% of the cells when the distance between the flanking signals was three times the estimated signal diameter. In case of two single color pairs in more than 90% of the cells, cells were regarded negative for translocation.

3. Results

3.1. Patient Selection. In the initial PALGA and the Bodensee, Friedrichshafen (Dr. T. Mentzel) selection, 15 patients were retrieved. Of these, 2 cases were excluded because representative material could not be obtained. At review, four lesions were considered true chondroid lipomas. Further,

Table 1: Patient and tumor characteristics.

Patient characteristics	Chondroid lipoma	Myo epithelioma	lipoma	Well-differentiated liposarcoma	Myxoid liposarcoma	Chondrosarcoma
Male/female	1/3	3/1	11/10	13/15	10/8	8/2
Median age at Diagnose, yrs (range)	36 (32–75)	56 (48–64)	49 (21–69)	62 (28–80)	43 (26–70)	54 (29–90)
Mean tumor circumference (range)	3 (2–10)	8 (2–20)	5 (1–18)	13,5 (2–30)	14 (4–30)	6 (2–13)

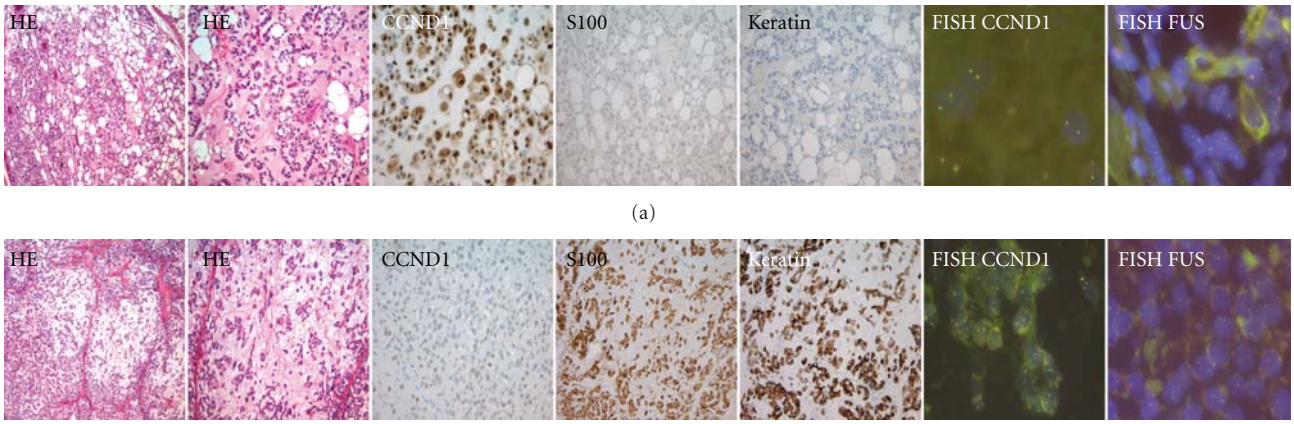

Figure 1: (a) Chondroid lipoma and (b) myoepithelioma.

four myoepitheliomas: one myxoid liposarcoma, one lipoma, one hibernoma, and two chondro lipomas were diagnosed (Table 1). The four chondroid lipomas and four myoepitheliomas were further analyzed and compared to 83 mimics randomly selected cases collected at the Netherlands Cancer Institute as described above.

3.2. Histopathological Analysis. Chondroid lipomas ($n = 4$) showed fibrous capsule and were dominated by a mature lipomatous proliferation with sheets, clusters, and nests of cells with eosinophilic, vacuolated cytoplasm in an eosinophilic cartilagenous matrix. The extracellular myxohyaline matrix showed a cartilagenous appearance, and the vascularisation was rich (Figure 1). The eosinophilic and vacuolated tumor cells were arranged in sheets, clusters, and cords and contain irregular, hyperchromatic nuclei with inconspicuous nucleoli; some of the vacuolated cells are indistinguishable from lipoblasts. Mitotic activity was absent.

3.3. Immunohistochemical Analysis. All (4/4) myoepitheliomas stained positive for keratin, whereas 1/4 chondroid lipoma stained focally positive and 3/4 stained negative. Immunohistochemical staining with S100 showed 1/4 positive and 2/4 focal positive lesions in chondroid lipoma and 3/4 positive and 1/4 focal positive myoepithelioma. Immunohistochemical staining of chondroid lipomas and myoepithelioma for vimentin, *SMA, CD34, CD68*, and *P53* was not distinctive.

Detailed immunohistochemical results are listed in Table 2 and Figure 1.

3.4. CCND1 and FUS as Candidate Genes for Translocation in Chondroid Lipoma. In all chondroid lipomas, both the obvious lipogenic cells and the eosinophilic tumor cells were immunohistochemically uniformly positive for *CCND1*, whereas 1/4 myoepitheloma lesion stained positive for *CCND1*, and 3/10 chondrosarcomas stained positive while 3/10 were focal positive and 4/10 were negative. The lipomas and well-differentiated liposarcomas that were immunohistochemically analyzed for *CCND1* showed scattered positivity in a majority of cases 32/49 (65%), whereas 16/49 (33%) stained negative for *CCND1* and 1/49 stained positive. None of the 18 myxoid liposarcomas showed immunohistochemically *CCND1* expression. Therefore, *CCND1* could be used for distinction between myxoid liposarcoma and chondroid lipoma: 4/4 (100%) positivity in chondroid lipoma and 0/18 (0%) positivity in myxoid liposarcoma.

By using fluorescence in situ hybridization for *CCND1* and *FUS*, respectively, no breaks in these genes could be detected in chondroid lipoma or in myoepithelioma.

4. Discussion

This study shows that true chondroid lipomas are extremely rare soft tissue tumors. The fact that in a Dutch nationwide search in a 10-year period by PALGA only two unequivocal cases were diagnosed that were retrieved within a spectrum of mimics underlines the rarity of the diagnosis and shows that awareness of the characteristics of chondroid lipoma is particularly important in reaching a chondroid lipoma diagnosis.

Table 2: Immunohistochemical and fluorescence in situ hybridization results.

Histology	IHC *CCND1* (%)	IHC CD34	IHC CD68	IHC S100	IHC keratin	IHC P63	IHC SMA	IHC vimentin	FISH Split apart *CCND1*	FISH Split apart *FUS*
Chondroid lipoma ($n = 4$)										
Positive	4	0	0	1	0	0	0	3	0	0
Focal positive	0	0	1	2	2	0	0	0	n.a.	n.a.
Negative	0	4	3	1	2	4	4	1	4/4*	4/4*
Myoepithelioma ($n = 4$)										
Positive	1	0	0	3	3	0	0	4	0	0
Focal positive	1	0	1	1	1	1	0	0	n.a.	n.a.
Negative	2	4	3	0	0	3	4	0	4/4*	3/3*
Lipoma ($n = 21$)										
Positive	0	—	—	—	—	—	—	—	—	—
Focal positive	16 (76)	—	—	—	—	—	—	—	—	—
Negative	5 (24)	—	—	—	—	—	—	—	—	—
Well differentiated liposarcoma ($n = 28$)										
Positive	1 (4)	—	—	—	—	—	—	—	—	—
Focal positive	16 (57)	—	—	—	—	—	—	—	—	—
Negative	11 (39)	—	—	—	—	—	—	—	—	—
Myxoid liposarcoma ($n = 18$)										
Positive	0	—	—	—	—	—	—	—	—	—
Focal positive	0	—	—	—	—	—	—	—	—	—
Negative	18 (100)	—	—	—	—	—	—	—	—	—
Chondrosarcoma ($n = 10$)										
Positive	3 (30)	—	—	—	—	—	—	—	—	—
Focal positive	3 (30)	—	—	—	—	—	—	—	—	—
Negative	4 (40)	—	—	—	—	—	—	—	—	—

* There were no translocations observed.
n.a.: not applicable.
IHC: immunohistochemical.

This study furthermore shows that the histological mimics of chondroid lipomas as described in the literature can be distinguished by means of immunohistochemical analysis. Especially immunohistochemistry for *CCND1* and FISH analysis for the specific translocations may be supportive to discriminate between myxoid liposarcoma and chondroid lipoma. Although some apparent histologic hallmarks of chondroid lipomas can be readily recognized such as nests and cords of uni- and multivacuolated cells within a prominent myxohyaline to chondroid matrix, the immunohistochemical marker pattern is very helpful. Myoepithelioma of the soft tissues is an important mimic that expresses, in contrast to chondroid lipoma, the epithelial markers keratin as well as *S100* protein. Well-differentiated liposarcoma with myxoid changes may be a mimic that may be recognized on the basis of clinical setting, morphology as well as by the specific genetic changes. Also chondroid lipoma may be characterized by a specific translocation $t(11;16)$. Recently, MKL/myocardin-like 2 (MKL2) and C11orf95 (chromosome 11 open reading frame 95) were identified as translocation partners in 3 cases of chondroid lipoma. Although the extent of possible variant translocations may not be clear yet, this finding provides an important addition for further support in differential diagnostic problems [11, 14]. In the present study, all cases of chondroid lipomas showed high immunohistochemical expression of *CCND1*. Since the *CCND1* gene is located on 11q13, this makes it an attractive candidate gene for the $t(11;16)$ translocation in chondroid lipoma. Based on a spilt apart FISH assay, *CCND1* did not show rearrangement, however. This indicates that the breakpoint is most probably not located in the *CCND1* region and does not give support for involvement of this gene in the oncogenesis of chondroid lipoma, despite expression of the protein. Indeed, *CCND1* is expressed broadly in several types of well-differentiated lipomatous tumors, such as lipoma and well-differentiated liposarcoma and also in tumors with chondroid differentiation, including extraskeletal myxoid chondrosarcoma and primary chondrosarcoma of bone in support of this notion. As expected, *FUS* indeed was shown not be involved in the translocation.

5. Conclusion

Chondroid lipoma is extremely rare and has several and more prevalent histological mimics. The differential diagnosis of chondroid lipomas can be unraveled using immunohistochemical and molecular support. Although chondroid lipoma shows high expression of *CCND1*, this expression

should not be regarded as deregulated and there is no support that *CCND1* is directly involved as a translocation partner in the characteristic *t*(11;16).

Conflict of Interests

The authors declared that there is no conflict of interests.

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Research Article

Human Chondrosarcoma Cells Acquire an Epithelial-Like Gene Expression Pattern via an Epigenetic Switch: Evidence for Mesenchymal-Epithelial Transition During Sarcomagenesis

Matthew P. Fitzgerald,[1] Francoise Gourronc,[2] Melissa L. T. Teoh,[1] Matthew J. Provenzano,[3] Adam J. Case,[1] James A. Martin,[2] and Frederick E. Domann[1]

[1] *Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa, Iowa City, IA 52242, USA*
[2] *Department of Orthopaedics and Rehabilitation, University of Iowa, Iowa City, IA 52242, USA*
[3] *Department of Otolaryngology-Head and Neck Surgery, University of Iowa, Iowa City, IA 52242, USA*

Correspondence should be addressed to Frederick E. Domann, frederick-domann@uiowa.edu

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Chondrocytes are mesenchymally derived cells that reportedly acquire some epithelial characteristics; however, whether this is a progression through a mesenchymal to epithelial transition (MET) during chondrosarcoma development is still a matter of investigation. We observed that chondrosarcoma cells acquired the expression of four epithelial markers, *E-cadherin, desmocollin 3, maspin*, and *14-3-3σ*, all of which are governed epigenetically through cytosine methylation. Indeed, loss of cytosine methylation was tightly associated with acquired expression of both *maspin* and *14-3-3σ* in chondrosarcomas. In contrast, chondrocyte cells were negative for *maspin* and *14-3-3σ* and displayed nearly complete DNA methylation. Robust activation of these genes was also observed in chondrocyte cells following 5-aza-dC treatment. We also examined the transcription factor *snail* which has been reported to be an important mediator of epithelial to mesenchymal transitions (EMTs). In chondrosarcoma cells *snail* is downregulated suggesting a role for loss of *snail* expression in lineage maintenance. Taken together, these results document an epigenetic switch associated with an MET-like phenomenon that accompanies chondrosarcoma progression.

1. Introduction

Chondrosarcoma is a rare but deadly form of bone cancer and is the second most common type of bone cancer accounting for nearly 26% of all bone cancers [1]. These tumors are stubbornly resistant to both chemotherapy and radiation therapy, therefore surgical ablation is still the most effective treatment [2, 3]. However since surgical resection is often difficult and not practical for metastatic disease, more effective treatments are needed.

Chondrosarcomas have been presumed to arise from the chondrocyte lineage of mesenchymal cells; of mesodermal origin because they are the most similar cells however, the exact origin or subtype of cells is still an area of active investigation. Numerous studies have shown the occurrence of genetic alterations in chondrosarcomas including loss of heterozygosity (LOH) on multiple chromosomes, wide variation in ploidy status, and mutations in the tumor suppressors p53, p16ink4a, pRB, among others [4–6]. In contrast, relatively little is known about the epigenetic alterations that occur during chondrosarcoma progression [7, 8].

The malignant progression to chondrosarcoma has been suggested to involve some degree of a mesenchymal to epithelial transition (MET), and it has been shown in an *in vitro* model that chondrosarcoma cells can transition to a more epithelial-like phenotype under certain conditions [9]. MET is a fundamental developmental process which is important to vertebrate embryogenesis in vascular, urinary, and genital tissues [10, 11]. Although much has been learned about the more commonly known and well-studied reciprocal process, the epithelial to mesenchymal transition

(EMT) during carcinoma progression, the mesenchymal to epithelial transition (MET) in sarcoma progression is considerably less well understood. These lineage transitions have important consequences to cell morphology, cell to cell adhesion, cell motility, and in the extracellular matrix of cells. However, the phenotypic plasticity conferred to cells as a result of these transitions which are so critical to development may also become coopted by cells during the process of carcinogenesis.

MET during carcinogenesis has been shown to be induced by the *c-met* proto-oncogene [9, 12, 13]. *P140 c-met* is a receptor tyrosine kinase for HGF/SF and increased expression leads to epithelial differentiation [14, 15]. In addition to epithelial specification by *c-met*, 5-azacytidine, a DNA methyltransferase inhibitor with broad spectrum epigenetic effects, has been used to induce the conversion of mesenchymal cells into epithelial cells *in vitro* [16]. More recently research on the transcription factor *snail* has been linked to aberrant DNA methylation of the epithelial specific E-cadherin promoter in association with EMT, and stable RNA interference of *snail* expression in carcinoma cell lines induced a complete MET [17, 18]. Finally, as corneal stromal keratinocytes differentiate into corneal fibroblasts they undergo an epigenetic switch with respect to *maspin* expression [19]. Such results highlight the possible role played by epigenetic changes through DNA methylation in a cell's ability to transdifferentiate from a mesenchymal to a more epithelial phenotype.

To investigate whether chondrosarcoma cells are displaying some characteristics of MET we examined four epithelial markers to confirm the acquisition of more epithelial-like expression. These epithelial markers included *E-cadherin, desmocollin 3, maspin, and 14-3-3σ*. Next to investigate whether epigenetic changes are occurring in chondrosarcomas we examined protein and RNA expression along with the DNA methylation at two distinct and separate loci, *maspin* and *14-3-3σ*. Both have been identified as specific epithelial markers and have separately been shown in lung, pancreas, prostate, and other cancers to be epigenetically controlled through DNA methylation [20–24]. Finally we measured expression of the *snail* transcription factor which has been reported to be an important mediator of EMT in part through epigenetic mechanisms [25].

Maspin is a member of the serpin family of protease inhibitors (*SERPINB5*) and has been described as an epithelial marker and a type II tumor suppressor gene based upon its ability to inhibit invasion and motility of mammary tumors [26–29]. Zhang and colleagues also found *maspin* to function as an inhibitor of angiogenesis [28, 30, 31]. *Maspin* gene expression is regulated in part through methylation of its promoter in human normal cells [29]. In addition, silencing of the *maspin* gene in association with aberrant DNA methylation has been reported in cancer cells from breast, melanoma, and thyroid [20, 32, 33]. Nevertheless, loss of *maspin* expression in cancer is not a universal phenomenon. In other malignancies such as pancreatic, lung, ovarian, and gastric cancers, *maspin* expression is paradoxically increased in malignant cells compared to their normal cells of origin [21, 34–36].

14-3-3σ, also known as stratifin or HME1, was originally identified as an epithelial-specific marker downregulated in breast cancer cell lines [37]. *14-3-3σ* has been shown to be involved in a wide variety of cellular processes, including its response to DNA damaging agents and gamma radiation through activation by p53, which then contributes to G2 cell cycle arrest [38, 39]. Studies have shown that, similar to *maspin*, downregulation of *14-3-3σ* was associated with aberrant hypermethylation of the *14-3-3σ* CpG island [23, 40, 41]. Since the original report, hypermethylation of *14-3-3σ* leading to silencing has been reported in prostate, hepatocellular carcinomas, and others [23, 41]. However, just as with *maspin*, *14-3-3σ* is not always downregulated and in fact is upregulated in pancreas and squamous cell carcinomas [42, 43].

Two members of the cadherin family of cell adhesion molecules *E-cadherin* and *desmocollin 3*, have been shown to be downregulated in several types of cancers through DNA methylation [44, 45]. This decrease in expression has been correlated with the epithelial to mesenchymal transition. The *snail* transcription factor has been shown to repress E-cadherin expression and has been reported to be an important mediator of epithelial to mesenchymal transitions. Recently, it has been shown that *snail* binds to the E-boxes of the E-cadherin promoter and can recruit the histone deacetylase HDAC1 and DNA methyltransferase DNMT1 to help in the epigenetic silencing of *E-cadherin* [46].

In this study we show an upregulation of four distinct epithelial markers and the downregulation of snail, all consistent with cells that have undergone to some extent an MET transition. Next we show that epigenetic alterations in two of these genes, *maspin* and *14-3-3σ*, are consistent with their gain of expression in chondrosarcomas. We demonstrate that loss of DNA methylation at both the *maspin* and *14-3-3σ* loci led to increased expression of these two epithelial specific genes during chondrosarcoma carcinogenesis. These results link the mesenchymal to epithelial transition in chondrosarcoma to an epigenetic switch in lineage-specific gene expression.

2. Materials and Methods

2.1. Cell Culture. Chondrosarcoma cells and normal chondrocytes were isolated by overnight digestion of chopped tissues with 0.5 mg/mL type IA collagenase and pronase E (Sigma) in Dulbecco Modified Eagle's medium with 10% fetal calf serum (Life Technologies). All cells were cultured as monolayers in growth medium containing 40% Dulbecco Modified Eagle's medium, 40% Minimum Essential medium, 20% Ham's F12, 10% fetal calf serum supplemented with 1.0 units/ml insulin, 20 μg/mL hydrocortizone (Sigma), and 40 μg/mL gentamycin or 100 units/ml penicillin/streptomycin and grown at 37°C with 5% CO_2 in a humidified cell culture incubator. The SNM83 are the normal chondrocyte cell strain used in this study. The cell line JJ was a generous gift from Dr. Joel Block. The *in vitro* morphologies of several of these cell lines

have been previously reported [47]. Briefly, we found that the *in situ* morphology of chondrocytes and low-grade chondrosarcoma cells changed in monolayer culture, where both transitioned from a spindle cell shape to a more polygonal cell shape after a few passages. High-grade (2-3) chondrosarcoma cells in monolayer culture retained their spindle shaped in culture.

2.2. Real-Time RT-PCR Assays for Gene Expression. Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and quantified using a biophotometer (Eppendorf, Westbury, NY, USA). For real-time RT-PCR analysis of *maspin* and *14-3-3σ* mRNA expression, a reverse transcription step was performed using a High Capacity cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA). The reverse transcription reaction of 2 μg of RNA was primed with random primers and incubated at 25°C for 10 min followed by 37°C for 120 min. The primer/probe PCR reactions consisted of 100 ng of cDNA added to 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems Inc.), 1.25 μL of gene-specific *maspin* and *14-3-3σ* primer/probe mix (Assays-on-Demand, Applied Biosystems Inc.), and 6.25 μL PCR grade water, for a 25 μL total reaction. For *E-cadherin*, *desmocollin 3,* and *snail* the primers were designed using ABI primer express software. Primer sequences are available upon request. The PCR reactions consisted of 100 ng cDNA with 0.6 μM primers in Power SYBR green PCR MasterMix (Applied Biosystems Inc.) with a total reaction volume of 25 μL. The genes were not multiplexed but rather amplified in separate tubes. The PCR conditions for all reactions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, with annealing at 60°C for 1 min. Real-Time PCR was performed on an ABI 7000 real-time sequence detection system. Both the *maspin* and *14-3-3σ* gene-specific TaqMan probes were labeled with a 5′ reporter dye, 6-FAM, and a 3′ end containing a nonfluorescent quencher and a minor groove binder. Fold differences in mRNA expression were calculated using their respective mRNA expression calibrated to 18-s ribosomal RNA expression and computed using ABI relative quantitation software (Applied Biosystems Inc.).

2.3. Western Blot Analysis. Proteins were isolated from SNM83 and NH69 cells using RIPA buffer and quantified using a Bradford assay. Twenty μg of protein were size fractionated by electrophoresis on a 12% SDS-PAGE gel and then transferred to nitrocellulose membranes. The membranes were then probed with a *maspin* antibody (Pharmingen) and *14-3-3σ* antibody (Chemicon).

2.4. Sodium Bisulfite Genomic DNA (gDNA) Sequencing. Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and quantified using a biophotometer (Eppendorf). Five micrograms of genomic DNA was modified under conditions previously described [32]. The *maspin* and *14-3-3σ* CpG islands were amplified from the bisulfite modified DNA by two rounds of PCR utilizing nested PCR primers specific to the bisulfite modified sequence of the *maspin* promoter and the *14-3-3σ* CpG island as described previously [32, 48]. The final PCR product was cloned into a TOPO TA vector according to the manufacturer's instructions (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). Five positive recombinants were isolated using Qiaprep Spin Plasmid Miniprep Kit (Qiagen) according to manufacturer's instructions and sequenced on an ABI automated DNA sequencer. The methylation status of individual CpG sites was determined by comparison of the sequence obtained with the known *maspin* and *14-3-3σ* sequences. The number of methylated CpGs at a specific site was divided by the number of clones analyzed ($n = 5$) to yield the percent methylation for each site. For total promoter methylation calculation, the total of all the 19 CpG sites for *maspin* and the 27 CpG sites in *14-3-3σ* that were methylated from the 5 clones was counted and divided by the total CpG sites.

2.5. Gene Reactivation Using 5-Aza-2′-Deoxycytidine. For 5-aza-2′-deoxycytidine (5-aza-dC) reactivation studies in the CS8E chondrosarcoma cell line, cells were plated at 5×104 cells in 6-well plates and were treated with 10 μM 5-aza-dC in complete media on days 0, 2, and 4 then harvested for total RNA on day 5 using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was then analyzed by real-time RT-PCR.

3. Results

3.1. Gain of mRNA Expression of Epithelial-Specific Genes in Chondrosarcomas. Compared to normal chondrocytes, chondrosarcoma cell lines displayed gain of expression of several epithelial-specific markers. *E-cadherin* mRNA expression was significantly upregulated in 4 of the 5 chondrosarcoma cell lines ranging from a 6- to 189-fold induction over the normal SNM83 normal chondrocyte cell strain as shown in Figure 1(a). Similarly, *desmocollin 3* mRNA expression was acquired in 4 of the 5 cell lines examined, ranging from a 2- to a 12-fold increase as compared to the normal counterpart SNM83, as shown in Figure 1(b). *Maspin* mRNA levels were also similarly affected. *Maspin* mRNA expression was virtually undetectable in the normal SNM83 chondrocyte cell strain and in stage I early CS8E chondrosarcoma cell line. In contrast, the chondrosarcoma cells CSPG, JJ, NH69, and CS13H displayed abundant *maspin* expression as shown in Figure 2(a) and Table 1. The chondrosarcoma cell lines displayed approximately 10^2- to 10^5-fold higher levels of *maspin* mRNA expression when compared to the normal SNM83 chondrocyte cell line. Finally, we determined that *14-3-3σ* mRNA expression was also virtually undetectable in the SNM83 normal chondrocyte cell strain and in CS8E cancer cell line and low in the CSPG cell line. In the other chondrosarcoma cells JJ, NH69, and CS13H there was approximately 100-fold higher levels of *14-3-3σ* mRNA expression as shown in Figure 2(b) and Table 1. To our knowledge this is the first report showing the upregulation of either *maspin* or *14-3-3σ* in chondrosarcoma cell lines.

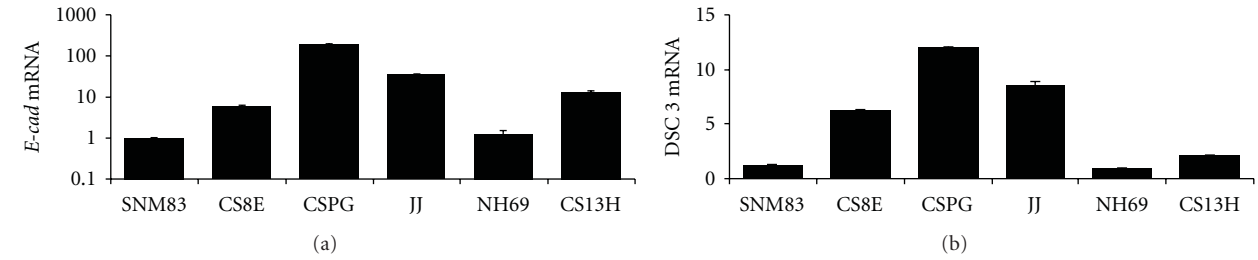

Figure 1: Chondrosarcomas acquire aberrant expression of epithelial-specific genes *E*-cadherin and desmocollin-3. (a) *E-cadherin* mRNA expression was measured by real-time PCR. Four of the five chondrosarcoma cell lines (CS8E, CSPG, JJ, and CS13H) showed a significant increase in *E-cadherin* mRNA expression from approximately 6- to 189-fold increase when compared to the normal SNM83 chondrocyte cell line. SNM83 had minimal expression but was set at 1 on graph as calculated using ΔΔCT relative quantification method. (b) *Desmocollin* 3 mRNA expression was measured by real-time PCR. Four of the five chondrosarcoma cell lines (CS8E, CSPG, JJ, and CS13H) showed a significant increase from approximately 1.5- to 12-fold more *Desmocollin* 3 mRNA when compared to normal line. Again SNM83 had minimal expression but was arbitrarily set at 1 for calculation purposes.

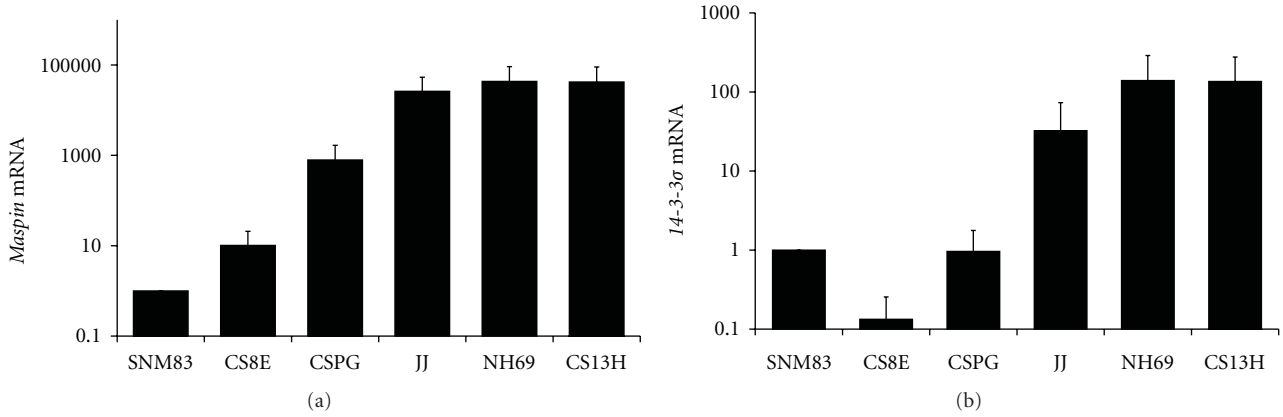

Figure 2: Chondrosarcomas acquire aberrant expression of epithelial-specific genes *maspin* and *14-3-3σ*. (a) *Maspin* mRNA expression was measured by real-time PCR. Four of the five chondrosarcoma cell lines (CSPG, JJ, NH69, and CS13H) showed a significant increase in *maspin* mRNA expression when compared to the normal SNM83 chondrocyte cell line. SNM83 had minimal expression but was set at 1 on graph as calculated using ΔΔCT relative quantification method. (b) *14-3-3σ* mRNA expression was measured by real-time PCR. Three of the five chondrosarcoma cell lines (JJ, NH69 and CS13H) showed a significant increase from approximately 50- to 150-fold more *14-3-3σ* mRNA when compared to normal SNM83 and the CS8E and CSPG chondrosarcoma cell lines. Again SNM83 had minimal expression but was arbitrarily set at 1 for calculation purposes.

Table 1: Summary of *maspin* and 14-3-3σ mRNA expression and cytosine methylation states in human chondrosarcoma cell lines.

Cell Line	Expression	Fold change mRNA	Methylation	% Methylation
Maspin				
SNM83	−	1	+	93
CS8E	−	10	+	86
CSPG	+	1000	−	7
JJ	+	32,000	−	3
NH69	+	63,000	−	1
CS13H	+	63,000	+/−	65
14-3-3σ				
SNM83	−	1	+	85
CS8E	−	.15	+	90
CSPG	−	1	+	95
JJ	+	45	−	27
NH69	+	150	−	6
CS13H	+	145	−	4.4

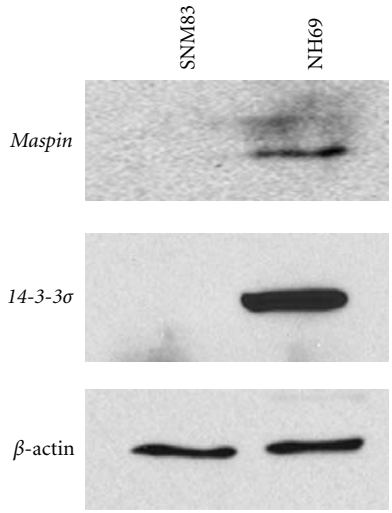

Figure 3: Chondrosarcoma cells expression of *maspin* and *14-3-3σ* proteins. Western blot analysis for maspin and *14-3-3σ*. Lane 1 is the SNM83 chondrocyte cell line which shows no protein expression for either maspin or *14-3-3σ*, and in lane 2 the NH69 chondrosarcoma cell line shows a robust induction of both proteins. These are consistent with real-time PCR results of mRNA expression. Beta-actin was used as the loading control.

3.2. Gain of Epithelial-Specific Protein Expression. To determine whether the increases in mRNA observed were translated into functional proteins we performed western blotting on the epithelial-specific markers *maspin* and *14-3-3σ* in two representative cell lines as shown in Figure 3. The normal SNM83 cells showed no detectable expression of *maspin* or *14-3-3σ* while the chondrosarcoma cell line NH69 showed robust expression of both proteins consistent with the previously observed upregulation in the *maspin* and *14-3-3σ*'s mRNA levels (Figure 2).

3.3. Maspin and 14-3-3σ Gene Methylation in Chondrocytes. Figures 4(a) and 4(b) represent the normal methylated state of the *maspin* promoter in the normal chondrocyte cell line SNM83. Figure 4(a) is a histogram representing the percent methylation at each of the 19 CpG locations and their distribution across the *maspin* promoter' whereas Figure 4(b) shows the five analyzed clones individually and the methylation at each CpG site. The overall methylation for the *maspin* promoter was 93% in the SNM83 cells (Table 1). This high degree of promoter methylation taken along with the undetectable SNM83 *maspin* mRNA and protein levels represented in Figures 2(a) and 3 is consistent with reported studies linking high *maspin* promoter methylation with silenced gene expression.

Figures 4(c) and 4(d) represent the methylation status of 27 CpG's in the *14-3-3σ* gene in the SNM83 cells. Similar to the *maspin* promoter, *14-3-3σ* shows a highly methylated state of the CpG's in normal *14-3-3σ* negative chondrocytes. Figure 4(c) is a histogram representing the overall percent methylation from the five picked clones at each CpG site and its distribution across the gene; whereas Figure 4(d) shows the individual clones and methylation at each CpG site. The overall methylation of SNM83 from the sequenced clones is 85% methylated. This methylation pattern of *14-3-3σ*, similar to *maspin*, is associated with silenced *14-3-3σ* expression.

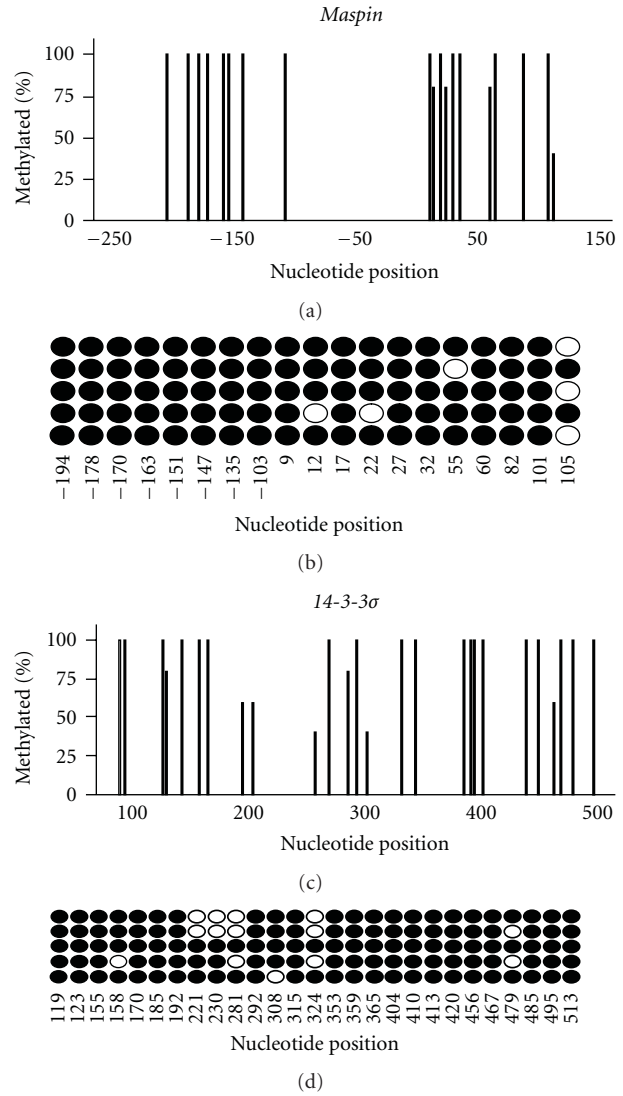

Figure 4: Normal chondrocyte cells have a highly methylated *maspin* promoter and *14-3-3σ* CpG island. (a) and (c) Histograms representing the percent methylation at each of the CpG's spanning the *maspin* promoter and *14-3-3σ* CpG island in SNM83 chondrocyte cells. (b) and (d) Bubble charts of Maspin and *14-3-3σ* in SNM83. Each of the five rows represents a sequenced amplicon, while each of the columns represents the position of the nucleotide relative to the transcription start site. Darkened circles represent methylated cytosines while open circles represent unmethylated cytosines. Nucleotide positions relative to start site were based on UCSC genome browser build 17.

3.4. Loss of Maspin Promoter Methylation in Human Chondrosarcomas. Figure 5(a) illustrates the methylation frequency and distribution at each of the 19 CpG's we measured

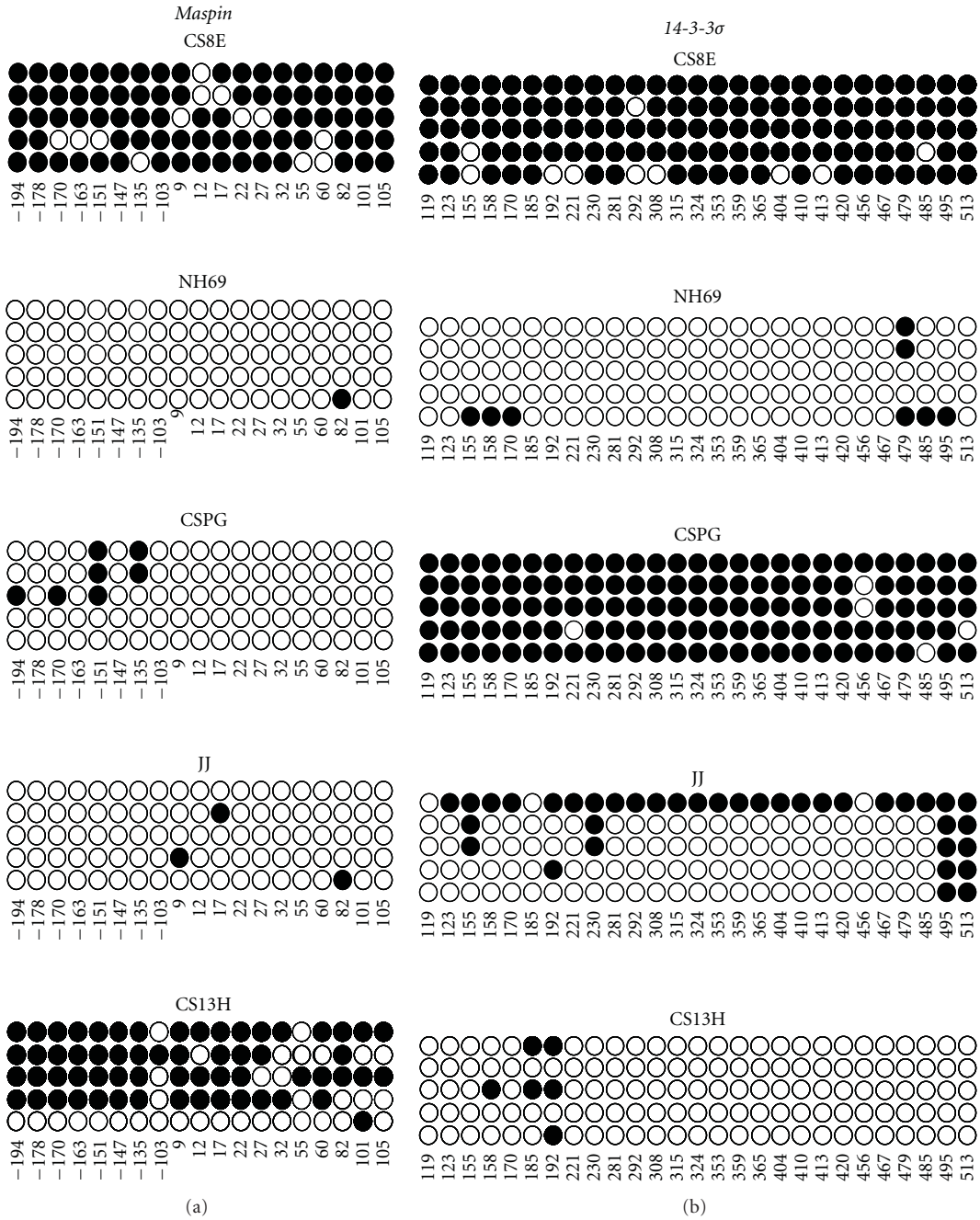

Figure 5: The *maspin* promoter and *14-3-3σ* gene are hypomethylated in mRNA expressing chondrosarcoma cell lines. Bubble charts of the maspin promoter (a) and *14-3-3σ* gene (b) for the five chondrosarcoma cell lines analyzed. Each of the five rows represents a sequenced amplicon while each column represents the nucleotide position of the CpG measured relative to the transcription start site. Darkened circles represent methylated cytosines, and clear circles represent unmethylated cytosines. Nucleotide positions relative to start site were based on UCSC genome browser build 17.

in the *maspin* promoter in the five human chondrosarcoma cell lines analyzed. SNM83 (Figure 4) and CS8E displayed overall *maspin* promoter methylation percentages of 93% and 86%, respectively, while CS13H showed an intermediate methylation percentage of 65%. In sharp contrast, the remaining chondrosarcoma cell lines CSPG, JJ, and NH69 displayed significantly lower percentages of overall promoter methylation of 7%, 3%, and 1% respectively (Table 1). The intermediate promoter methylation of CS13H may be due to the phenotypic heterogeneity displayed in the tumor tissue from which the DNA was extracted (data not shown), or could be due to a reversion of this late stage chondrosarcoma back to a more "normal" methylation profile similar to chondrocytes. Nonetheless, these data are consistent with our

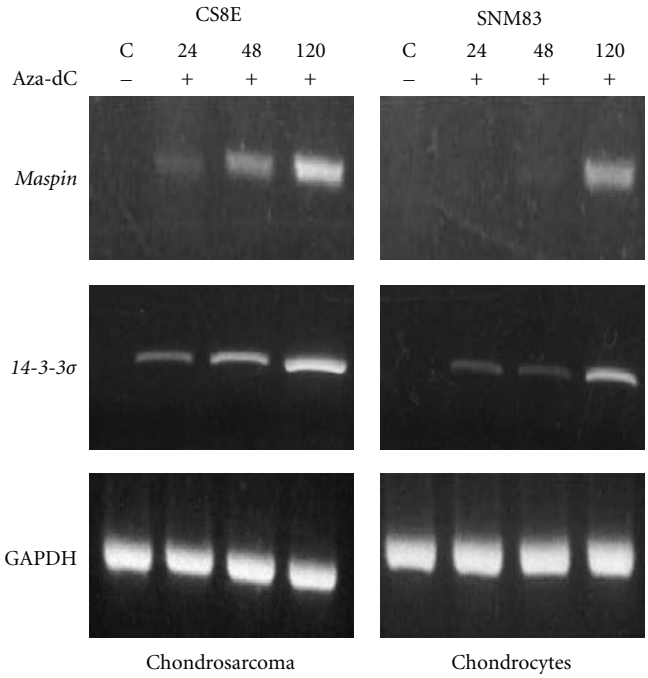

Figure 6: The methyltransferase inhibitor 5-Aza-dC reactivated both *maspin* and *14-3-3σ* mRNA expression in chondrocytes and chondrosarcomas cell lines. The CS8E chondrosarcoma cell line and SNM83 normal chondrocyte cell line were exposed to 10 uM 5-Aza-dC. RNA was harvested after 48, 72, and 120 hours and subjected to RT-PCR for *maspin* and *14-3-3σ*. GAPDH was used as loading control.

previous report in human pancreatic carcinoma cell lines as well as reports from human breast, lung, ovarian, and thyroid cancers that tightly associate *maspin* expression to promoter methylation.

3.5. 14-3-3σ Gene Methylation in Chondrosarcomas. Figure 5(b) also illustrates the methylation frequency and distribution at each of the 27 CpG sites of the *14-3-3σ* CpG island among the sequenced amplicons from the same five chondrosarcoma cell lines. CS8E and CSPG displayed a percentage of overall DNA methylation of 90% and 95%, respectively. In contrast, the remaining chondrosarcomas cell lines display relatively little DNA methylation (Table 1). This supports previous work with *14-3-3σ* that shows the tight association of DNA methylation with low expression and hypomethylation with higher levels of *14-3-3σ* expression [40, 42].

3.6. Gene Reactivation with 5-Aza-Deoxycytidine. The mRNA expression and sodium bisulfite DNA sequencing data alone shows a potential association but does not fully establish a cause and effect relationship between cytosine methylation and *maspin* and *14-3-3σ* gene expression. Therefore we investigated whether we could induce *maspin* and *14-3-3σ* expression by treating the *maspin* and *14-3-3σ* negative SNM83 chondrocyte cells and the CS8E chondrosarcoma cells with the DNA methyltransferase

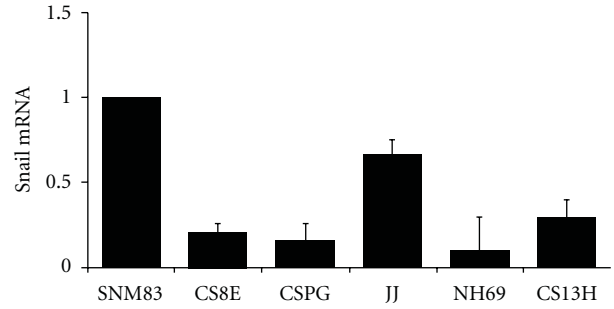

Figure 7: *Snail* mRNA expression is downregulated in human chondrosarcomas. All five chondrosarcoma cell lines showed a significant decrease ranging from 1.5- to a 10-fold decrease in *snail* mRNA expression when compared to the normal SNM83 chondrocyte cell line as determined by real-time PCR using ΔΔCT relative quantification method. Expression is normalized to SNM83 chondrocyte expression.

inhibitor 5-aza-2′-deoxycytidine (5-aza-dC). After 120 hours of 5 μM 5-aza-dC both the SNM83 and CS8E cells showed a significant increase from almost undetectable levels of *maspin* and *14-3-3* μ to crisp mRNA expression when compared to the untreated controls as shown in Figure 6. These data are consistent with previous reports of induced *maspin* and *14-3-3σ* expression following 5-aza-dC treatment of hypermethylated and nonexpressing cell lines [21, 32, 41, 49].

3.7. Loss of Snail mRNA Expression in Chondrosarcomas. To begin to assess the potential underlying molecular mechanism(s) for the apparent epigenetic switch in lineage-specific gene expression we observed and we measured *snail* mRNA expression, since *snail* has been reported to participate in the MET. Interestingly, s*nail* mRNA expression was significantly downregulated in all five chondrosarcoma cell lines when compared to the SNM83 normal chondrocytes, ranging from a 1.5- to 10-fold decrease in mRNA expression as shown in Figure 7. This decrease is supportive of our observation that *E-cadherin* and other epithelial-specific markers mRNAs were induced in the majority of human chondrosarcoma cell lines analyzed and is noteworthy because of recent reports showing that stable RNA interference of *snail* can lead to a MET transition [25].

4. Discussion

In this study we examined whether epigenetic changes are associated with a mesenchymal to epithelial-like transition in chondrosarcomas. To first investigate whether that our chondrosarcoma cells were acquiring more epithelial-like characteristics we queried the expression of four separate epithelial markers: *E-cadherin, desmocollin 3, maspin,* and *14-3-3σ*. All of these genes have been shown to be deregulated in association with cytosine methylation and are involved with the malignant progression of many cancers [20–24, 50–52]. Therefore, two of these genes, *maspin and 14-3-3σ*, were further examined as representatives

for epigenetic alterations. We identified the acquisition of expression of all four epithelial-specific markers in four of the five chondrosarcoma cell lines examined. Moreover, the robust acquisition of *maspin* and *14-3-3σ* expression in chondrosarcomas is associated with a significant loss of DNA methylation at those loci when compared to normal SNM83 chondrocyte cell strain.

The acquisition of the epithelial markers *E-cadherin* and *desmocollin 3* in four of the five chondrosarcoma cell lines is consistent with reports showing that sarcomas can to some degree transition through MET from their parental cell lineage [48, 53]. The acquisition of *E-cadherin* is interesting because of the numerous reports indicating that downregulation of *E-cadherin*, frequently by aberrant methylation, is a hallmark of EMT. An important mediator of EMT and *E-cadherin* downregulation in cancer is the zinc finger transcription factor *snail.* An example of *snail's* important role in EMT and control of *E-cadherin* has been shown in *snail* knock-out mice which show embryonic lethality, and the embryos fail to complete EMT, forming an altered mesodermal layer while still retaining *E-cadherin* expression. Among the chondrosarcoma cell lines assessed here, all five showed a significant decrease in the mRNA expression of the *snail* transcription factor. Although the amount of decrease in *snail* expression was not predictive of the fold mRNA increase of *E-cadherin*, the downregulation of *snail* is suggestive of a less repressed *E-cadherin* and therefore could help to explain the observed increase of *E-cadherin* in our chondrosarcoma cells. In recent reports *snail* has also been investigated as an effector of the epigenetic changes observed in the downregulation of *E-cadherin.* It has been shown in these reports that *snail* binds to the E-boxes of the *E-cadherin* promoter and helps to recruit both histone deacetylase 1 (HDAC1) and DNA methyltransferase 1 (DNMT1). Indeed when *snail* was stably overexpressed in Hep3b cells the *E-cadherin* promoter became hypermethylated and histone H3 and H4 acetylation were decreased [46]. In support of this, a similar study by Cano et al. showed that stable interference of *snail* mRNA in *snail* overexpressing Madin Darby canine kidney (MDCK) cells led to a full MET and re-expression of *E-cadherin*. They extended and confirmed these results when they stably knocked down *snail* in two mouse epidermal carcinoma cell lines with similar results [54].

The acquired expression in our chondrosarcoma cells of four epithelial markers that have been shown in other cancers to be deregulated by promoter methylation, taken together with the downregulation of *snail,* led us to examine whether epigenetic changes could be associated with this MET-like transition in the chondrosarcoma cell lines assessed. To do this we compared the methylation status of *maspin* and *14-3-3σ* in chondrosarcoma cells that acquired the expression of these markers to their nonexpressing normal counterpart, the SNM83 chondrocyte cell line. We report here that acquisition of both *maspin* and *14-3-3σ* in chondrosarcoma cell lines is tightly associated with aberrant hypomethylation of their CpG islands and that expression of both epithelial markers could be induced in a nonexpressing chondrosarcoma cell line by addition of the DNA methyltransferase inhibitor 5-Aza-dC. These findings are consistent with the epigenetic control of these loci documented in recent studies of multiple normal and cancer cell types [21, 22, 34, 55]. The acquisition of *maspin* and *14-3-3σ* in chondrosarcomas and other cancers, while still seemingly paradoxical to its role as a tumor suppressor, may be considered as a loss of epigenetic control. However, this may be better viewed as an epigenetic switch whereby the cancer cell, during progression, coopts the normal epigenetic mechanism(s) to propagate diverse cell types of differing lineage specificities. An example of this epigenetic switch may occur in solid tumors such as in colorectal cancer which uses the EMT transition to acquire a more metastatic phenotype but subsequently undergoes the reciprocal MET at the site of metastasis to reacquire, at least in part, the phenotype of the originating tumor [10]. This phenotypic reversion may confer a selective advantage to its new environment at the site of metastasis and thus allow for more successful colonization. The dynamic interconversions between EMT and MET in malignant progression cannot simply be explained by irreversible genetic alterations. These interconversions are suggestive of epigenetic mechanisms playing a role in these transitions because epigenetic changes, in contrast to irreversible genetic changes, are not permanent and allow a tumor cell more plasticity to alter its gene expression to adapt to different environments that can ultimately lead to phenotypic changes.

One of the ways a tumor cell can accomplish this epigenetic switch is through variable DNA methylation of the CpG sites in the transcriptional control regions of genes. Specific examples of this switch in *maspin* and *14-3-3σ* expression have been documented to occur both in ovarian and breast carcinomas. It is interesting to note that *maspin* expression is silenced during breast cancer progression, but activated during ovarian cancer progression [34, 35]. Ovarian surface epithelial cells being derived from the mesoderm activate *maspin* expression through DNA hypomethylation as they transition through MET [56]. Conversely, breast carcinomas typically silence *maspin* expression through hypermethylation and undergo the EMT to become more mesenchymal [32, 40, 57]. It is also noteworthy to mention that *snail* expression has been shown to be decreased in ovarian cancer cells during MET and to increase in breast cancer cells during EMT [58, 59]. To speculate that *snail* is affecting the epigenetic control of *maspin* expression in these cancers as well as in our chondrosarcoma cells is intriguing but has yet to be examined. However a bioinformatics search of both *maspin* and *14-3-3σ* promoters reveals putative *snail* binding sites but further research is needed to elucidate any direct interaction. These examples may however help to explain the paradoxical gain of expression of *maspin* and *14-3-3σ* in chondrosarcomas as well as in pancreatic and ovarian cancers. While epigenetic changes in the expression of *maspin*, *14-3-3σ*, *desmocollin 3*, and *E-cadherin* in chondrosarcoma cells and in other cancers are associated with the EMT or MET transitions, the extent to which they are a contributing factor to these process remains to be determined. Nevertheless, they may provide new biomarkers for differential diagnosis of cartilaginous

diseases and provide better insights into how cells loss lineage maintenance in cancer.

In addition to showing an epigenetic mechanism for *14-3-3σ* upregulation in chondrosarcoma cells it is interesting to speculate on what affects this acquired expression could have on treatment. The abundant expression of *14-3-3σ* might help to confer the drug and radiation resistance commonly found in the treatment of chondrosarcomas [4, 40, 60]. In a recent study *14-3-3σ* was identified as an important contributor to drug resistance in human breast and pancreas cancer cells, and the exogenous overexpression of *14-3-3σ* led to a greater resistance to chemotherapeutics and radiation [60, 61].

5. Conclusion

In summary we show that chondrosarcoma cells acquire four epithelial-specific markers *maspin, 14-3-3σ, desmocollin 3*, and *E-cadherin,* which when taken together, is suggestive of chondrosarcoma cells undergoing to some degree an MET transition. We also report that all of the chondrosarcomas examined showed a significant downregulation of the *snail* transcription factor which may help to explain the re-acquisition of *E-cadherin* and MET-like transition in our cell lines. The reports that *snail* has been shown to act as an epigenetic repressor of *E-cadherin* by recruitment of histone deacetylase 1 and DNA methyltransferase led us to examine whether additional changes in the epigenetic maintenance of two well-known epithelial markers *maspin* and *14-3-3σ* were occurring during this MET-like transition. We show here for the first time that chondrosarcoma cells acquire both *maspin* and *14-3-3σ* mRNA expression which is associated with vastly decreased DNA methylation of their genes. The acquisition of expression of these genes could be playing a role in malignant progression, or their expression could simply be biomarkers of progression. Expression of these genes, especially 14-3-3σ, could also be contributing to some of the characteristics of chondosarcomas such as resistance to chemotherapy and radiation [40]. We now report that epigenetic changes through loss of DNA methylation occur to activate epithelial specific genes *maspin* and *14-3-3σ* and that they are associated with the upregulation of *E-cadherin, desmocollin3* and the downregulation of *snail* during the transition of chondrocytes to chondrosarcomas. These epigenetic changes have not been extensively studied in MET, and this new knowledge could lead to more insight into the mechanisms underlying this important process, as well as aid in identifying new markers for better staging, diagnosing, and treating chondrosarcomas.

Abbreviations

5-Aza-dC: 5-Aza-2′-deoxycytidine
LOH: Loss of heterozygosity
MET: Mesenchymal to Epithelial transition
EMT: Epithelial to Mesenchymal transition
E-cad: E-Cadherin
DSC3: Desmocollin 3.

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Review Article

Spinal Chondrosarcoma: A Review

Pavlos Katonis,[1] Kalliopi Alpantaki,[1] Konstantinos Michail,[1] Stratos Lianoudakis,[1] Zaharias Christoforakis,[1] George Tzanakakis,[2] and Apostolos Karantanas[3]

[1] *University Hospital, University of Crete, Heraklion 711 10, Greece*
[2] *Department of Histology, Medical School, University of Crete, Heraklion 710 03, Greece*
[3] *Department of Radiology, University Hospital, University of Crete, Heraklion 711 10, Greece*

Correspondence should be addressed to Pavlos Katonis, katonis@hol.gr

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Chondrosarcoma is the third most common primary malignant bone tumor. Yet the spine represents the primary location in only 2% to 12% of these tumors. Almost all patients present with pain and a palpable mass. About 50% of patients present with neurologic symptoms. Chemotherapy and radiotherapy are generally unsuccessful while surgical resection is the treatment of choice. Early diagnosis and careful surgical staging are important to achieve adequate management. This paper provides an overview of the histopathological classification, clinical presentation, and diagnostic procedures regarding spinal chondrosarcoma. We highlight specific treatment modalities and discuss which is truly the most suitable approach for these tumors. Abstracts and original articles in English investigating these tumors were searched and analyzed with the use of the PubMed and Scopus databases with "chondrosarcoma and spine" as keywords.

1. Introduction

According to the World Health Organization, chondrosarcomas represent a heterogenous group of tumors characterized by their ability of cartilage formation [1]. Chondrosarcoma is the third most common primary malignant bone tumor after osteosarcoma and Ewing's sarcoma. However, the incidence of spinal chondrosarcomas is estimated to be from 2% to 12% in various series [2]. The thoracic spine is the most frequent localization, followed by the cervical and lumbar region [3]. Unlike most other malignant spinal tumors, the lesions may arise in the vertebral body (5%), the posterior elements (40%), or both (45%), since there are three growth centers in each vertebra from which the tumor originates [4]. The most common presenting symptom in chondrosarcoma is pain. Other complaints include a palpable mass and neurologic deficits in half of the patients [3].

The radiological features of chondrosarcomas vary significantly depending upon the histologic grade. The spectrum of findings starts with lysis, which is difficult to discriminate form enchondromas. High-grade tumors are demonstrated radiographically with a moth-eaten destruction and interrupted periosteal reaction. Higher grade of differentiation is related to the presence of a "rings and arcs" pattern of calcification into the tumor matrix. The differential diagnosis depends on the presence of calcifications. If present, then the main consideration is enchondroma. If absent, many lesions should be also considered such as metastases, malignant fibrous histiocytoma, and fibrosarcoma. The following criteria favor a diagnosis of chondrosarcoma: deep endosteal scalloping (>2/3 of cortical thickness), cortical disruption, periosteal reaction, soft tissue mass, and intense radionuclide uptake. Associated soft tissue mass is a common finding, and, thus, CT or MRI are important to fully appreciate the extraosseous extension [5].

The histologic grading is just one indicator that can predict the tumor's biological behavior. Prognosis is also related to management. The clinical challenge is to prevent recurrence and to optimize treatment options. Chondrosarcomas are typically resistant to known protocols of radiotherapy and chemotherapy; therefore, surgical removal is essential, and the outcome is based on the margins achieved [6, 7]. This review focuses on the most relevant issues relating to classification, diagnostic work-up, and surgical management of spinal chondrosarcomas. The principles of surgical excision

and reconstruction as well as novel treatment options like radiofrequency ablation and cryosurgery are also discussed.

2. Histopathological Classification of Chondrosarcoma

Chondrosarcoma has been classified into conventional and variant types. The variant types of chondrosarcoma include the least aggressive *clear cell* type and the *high-grade* mesenchymal and dedifferentiated tumors associated with poor prognosis. Conventional chondrosarcoma, which constitutes approximately 85% of all chondrosarcomas, is further classified into *primary* (85%) and *secondary* (15%) [8]. The primary chondrosarcoma arises *de novo* within the bone and can extend through the cortex with a large soft-tissue mass. A secondary chondrosarcoma develops on the surface of the bone mostly as a result of malignant transformation within the cartilage cap of a pre-existing benign tumor such as osteochondroma [1, 9]. It has been reported that secondary chondrosarcomas tend to be of a lower grade exhibiting a better prognosis than primary tumors [10]. In general, primary and secondary chondrosarcomas are histologically similar, and, for both, three different grades are recognized, which is one of the most reliable predictors of clinical behavior [11]. These histological grades are directly connected with prognosis and the risk of metastases. Grade I tumors are characterized by low cellularity and lack of pleomorphism; they contain a rich hyaline cartilage matrix and rarely metastasize [12]. In contrast, grade III chondrosarcomas are extremely cellular with pleomorphism and mitotic figures. Mucomyxoid matrix areas are frequent in grade III tumors and metastases occur in 70% of patients. Grade II chondrosarcoma hold some of the characteristics of both grade I and grade III [11]. In addition, to histological grade of the lesion, the prognosis depends on the possibility of performing *en bloc* excision with proper oncologic margins. Because of the difficulties associated with *en bloc* surgery in the spine, tumors of the vertebral column have had a deprived prognosis independent of the histological grade [9].

It seems that chondrosarcomas may be biologically dynamic, since up to 13% of recurrent tumors display a higher grade of malignancy or even dedifferentiation compared to the initial neoplasm, with a severe adverse prognosis. Alterations in TP53 as well as the *CDKN2A* (p16) tumor suppressor gene are thought to be important for the progression of low-grade towards high-grade chondrosarcoma. [13, 14].

Although primary and secondary chondrosarcomas show similarity in histopathologic features, they differ at the molecular genetic level [14]. The exostosin (*EXT*) genes, which are connected with the development of multiple osteochondromas (MOs), are involved in the origin of osteochondroma and secondary chondrosarcoma. The *EXT* genes participate in heparan sulphate biosynthesis and the resulting heparan sulphate proteoglycans (HSPGs) are fundamental for cell signaling [15]. Although it is quite clear that inactivation of EXT1 and EXT2 encourages osteochondroma development, the exact molecular trigger causing its malignant transformation is unclear [16]. It is evident that several growth-signaling pathways which are normally activated during skeletal growth such as the Indian hedgehog (IHH)/parathyroid hormone-like hormone (PTHLH) factor, wingless type (Wnt) protein, and transforming growth factor (TGF) signaling pathways are deprived in secondary chondrosarcoma. The IHH signaling and the Wnt signaling are downregulated while the TGF signaling and the PTHLH signaling, which is downstream of the IHH and it is responsible for chondrocyte proliferation, are up regulated and increased with increasing histological grade [13, 16].

On the contrary, *EXT* genes are not involved in the development of primary chondrosarcoma, and, in this case, the initiate event remains unidentified [17]. These tumors are usually aneuploid, with complex karyotypes, and 96% of them contain alterations at some level in the pRb pathway [18].

3. Rare Chondrosarcoma Subtypes

In addition to conventional chondrosarcoma, several variant subtypes of chondrosarcoma are recognized which are extremely rare especially when they originate in the spine [19].

Clear cell chondrosarcoma is a rare variant chondrosarcoma with relatively good prognosis. It is described as a "round cell" neoplasm with clear, empty cytoplasms. Benign giant cells may be present, which is the reason that it might erroneously be diagnosed as a chondroblastoma. Vascularity is a common feature in this tumor. Although it has a reasonably benign biological behavior, clear cell chondrosarcoma needs to be treated as a malignancy. Metastases are rare, but may occur up to 20 years following initial diagnosis; consequently, long-term followup is required [20]. On the molecular level, recent studies have shown that there is evidence of extra copies of chromosome 20 and loss or rearrangements of 9p. Also, expression of PTHLH, PDGFIHH, Runt-related transcription factor 2, and matrix metalloproteinase 2 [21, 22] were found.

Mesenchymal chondrosarcoma is another rare variant of chondrosarcoma, which is highly malignant. The prognosis of this tumor is extremely poor. It can involve both the bone and soft tissues. Huvos et al. classified mesenchymal chondrosarcoma into hemangiopericytoma-like and small dark round cell type. The same team reported that this tumor occurs in relatively young patients (mean age of presentation 26 years) [23]. Histopathologically, it is characterized by varying amounts of differentiated cartilage admixed with undifferentiated petite round cells [24]. On the molecular level, more than 60% of the tumors demonstrate p53 overexpression. In addition, expression of the antiapoptotic BCL2, protein kinase C- (PKC-), and platelet derived growth factor receptor- (PDGFR-)pathways were found [25, 26].

Dedifferentiated chondrosarcoma is an extremely aggressive variant type of chondrosarcoma with deprived prognosis. It is defined as a borderline low-grade chondrosarcoma

next to high-grade noncartilaginous anaplastic sarcoma, with a remarkably sharp junction between the two components [27, 28]. These two components hold identical genetic aberrations with additional genetic changes in the anaplastic component, suggesting a common ancestor cell with early diversion of the two components [29].

4. Risk Factors and Epidemiology

Several hypotheses have been proposed regarding the risk factors of spinal chondrosarcomas. Moreover, recurrence of chondrosarcoma of the spine is very common in case of invasion of the epidural space [30, 31]. Hereditary multiple exostoses is a syndrome that seems to be connected with spinal chondrosarcoma and constitute a significant risk factor [23]. Furthermore, there are benign lesions, such as chondromas, that can undergo a malignant transformation to spinal chondrosarcoma [22]. Epidemiological data shows a fairly equal gender representation between men and women, a range of age from 13 to 78 years, and a mean age of 33 years [7, 9, 32]. Location of chondrosarcoma involves the lumbar spine in 68% of the cases, the thoracic spine in 23%, and the cervical spine in 9%, and classification as peripheral and central chondrosarcoma is, almost in 2/3 of the cases, in favor of the peripheral [9]. Other studies show that these tumors have higher frequency in the thoracic than the rest of the spine as a result of the greater number of thoracic segments relative to cervical and lumbar regions [7]. Finally, almost 90% of tumors were classified as low grade (Enneking Stage I) and had a greater incidence among Caucasians [7, 9, 32].

5. Radiologic Features and Imaging

Plain radiographs demonstrate spinal chondrosarcoma as a well-defined mass with internal calcification [33]. In case that the mass projects into the lung fields, a well-defined opacity may be seen (Figures 1(a) and 1(b)). Computed tomography (CT), with its ability to overcome overlying structures, is able to depict the anatomic origin of the lesion and the pattern of calcification, namely, "rings and arcs" (Figure 1(c)). CT may also reveal paravertebral extension of the tumor, the displacement and potential infiltration of the surrounding structures, and involvement of adjacent levels [33–36]. Occasionally, spinal chondrosarcoma may appear as a lytic lesion involving the vertebral body, which may be complicated by a compression fracture of the superior or inferior end-plates [34, 35]. Magnetic resonance imaging (MRI) demonstrates the tumor as a low-signal intensity on T1-w and heterogeneous low and high-signal intensities on T2-w and STIR images, suggesting mineralized and nonmineralized matrices (Figures 2, 3, and 5) [33, 35]. In addition, MRI is better compared to CT in depicting the epidural and intraforaminal extension highlighting possible compression of the neural structures [34]. Fat-suppressed contrast-enhanced T1-w images show peripheral and lobulated rim enhancement (Figure 4) whereas lesions with limited calcification may appear with homogenous enhancement (Figure 5) [33, 35]. Scintigraphy by means of Tc-99m HMDP will show focal accumulation in the tumor site [33].

Table 1: The Enneking system for the surgical staging of bone and soft-tissue tumors is based on grade (G), site (T), and metastasis (M) [40].

Stage	Grade	Site	Metastasis
IA	G1	T1	M0
IB	G1	T2	M0
IIA	G2	T1	M0
IIB	G2	T2	M0
III	G1 or G2	T1 or T2	M1

6. Histological Diagnosis and Staging

The histological examination of the spinal chondrosarcoma shows vacuolated tumor cells with irregular hyperchromatic nuclei and clear cytoplasm, encircled by a network of fine osteoid trabeculae and spicules of nontumoral infiltrated bone [33]. In other cases, the tumor manifests a biphasic pattern with solid and cellular proliferation of small round-short spindle tumor cells and differentiated chondroid islands with endochondral ossification [33]. According to Enneking staging system, the lesions are classified as follows: histologically low-grade intracompartmental (IA), histologically high-grade intracompartmental (IIA), histologically low-grade extracompartmental (IB), and histologically high-grade extracompartmental (IIB) (Table 1) [9, 37, 38]. The second column of Table 1 is explained below.

Grade. In the Enneking system, bone tumors are graded as follows:

(i) G0: benign lesion,

(ii) G1: low-grade malignant lesion,

(iii) G2: high-grade malignant lesion.

The third column of Table 1 is explained below.

Site. In the Enneking system, the site and local extent of bone tumors are classified as follows:

(i) T0: a benign tumor that is confined within a true capsule and the lesion's anatomic compartment of origin (i.e., a benign intracapsular, intracompartmental lesion),

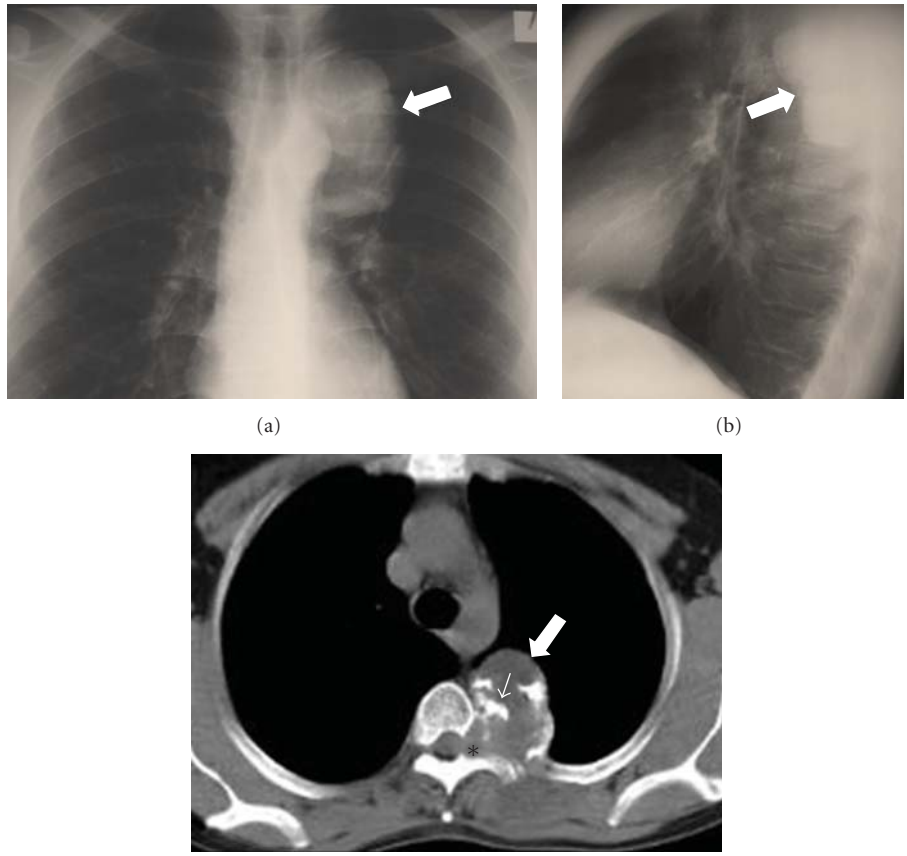
(ii) T1: intracompartmental lesion,

(iii) T2: extracompartmental lesion.

The fourth column of Table 1 is explained: metastatic classification in the Enneking system is as follows.

(i) M0: no regional or distant metastasis,

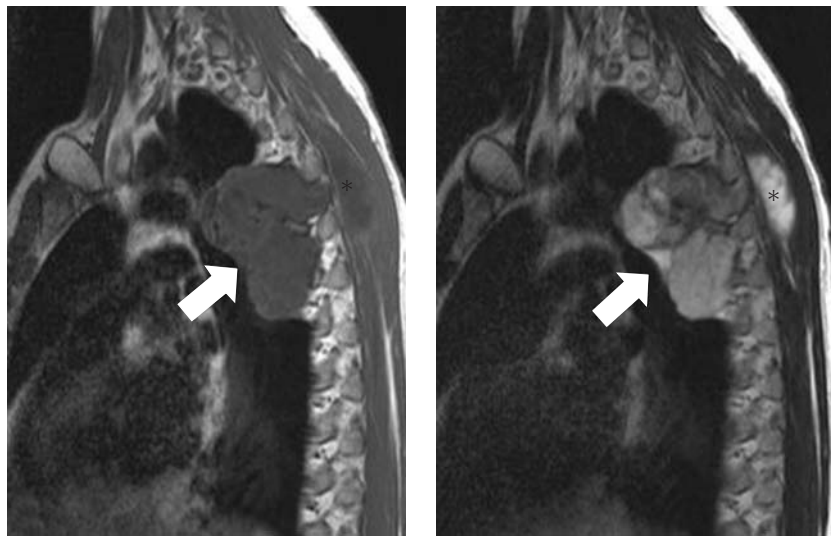
(ii) M1: regional or distant metastasis.



(a) (b)

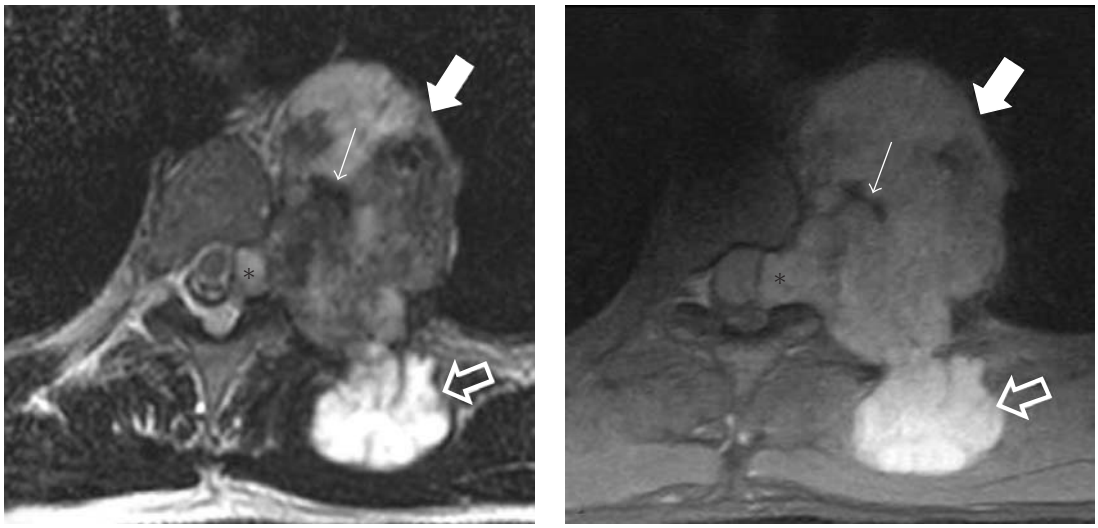
(c)

Figure 1: A 32-year-old man with chondrosarcoma. The posteroanterior (a) and lateral (b) chest radiographs, show a well-defined radiopaque lesion in the left posterior paraspinal location (arrows). (c) The axial MDCT image demonstrates a soft-tissue mass (arrow) with amorphous "rings and arcs" calcified matrix (thin arrow) and adjacent neural foramina widening (asterisk).



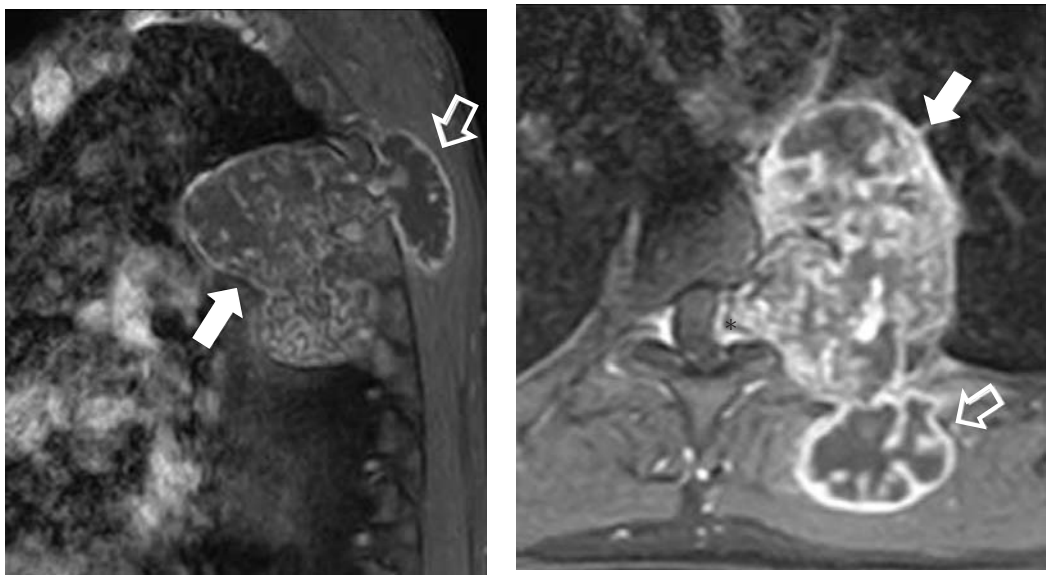
(a) (b)

Figure 2: MR imaging of the same patient. The sagittal T1-w (a) MR image shows a hypointense lobulated lesion (arrow). (b) The sagittal T2-w MR image shows the lesion with heterogeneous but predominantly high signal intensity (arrow). Note the superficial palpable component of the tumor (asterisks).



(a) (b)

Figure 3: MR imaging of the same patient. The axial T2-w (a) and the axial fat-saturated PD-w (b) MR images show a heterogeneous high intensity mass (thick arrows) with mineralized elements that demonstrate low signal intensity (thin arrow). Note the superficial (open arrows) as well as the neural foraminal extension (asterisks) of the tumor.



(a) (b)

Figure 4: MR imaging of the same patient. The sagittal (a) and axial (b) contrast-enhanced fat-saturated T1-w MR images show intense heterogeneous enhancement of both the intrathoracic (arrows) and the superficial (open arrows) tumor components. Enhancement is also observed in the intraforaminal component of the tumor (asterisk).

Staging.

(1) Under the Enneking system, malignant tumors are classified into stages I–III, with further subdivisions into A and B. Grade 1 and grade 2 tumors are stage I and stage II, respectively. T1 and T2 tumors are stage A and stage B, respectively. Tumors with distant metastasis are stage III.

Furthermore, the extent of the lesions has been classified according to the Weinstein-Boriani-Biagini (WBB) staging system with data taken from radiographs, CT and MRI scans, and surgical reports (Figure 6) [9, 39]. The vertebral body is topographically divided in twelve zones similar to the clock hours and five layers beginning from the paravertebral bony compartment until the meningeal layer, and the site of

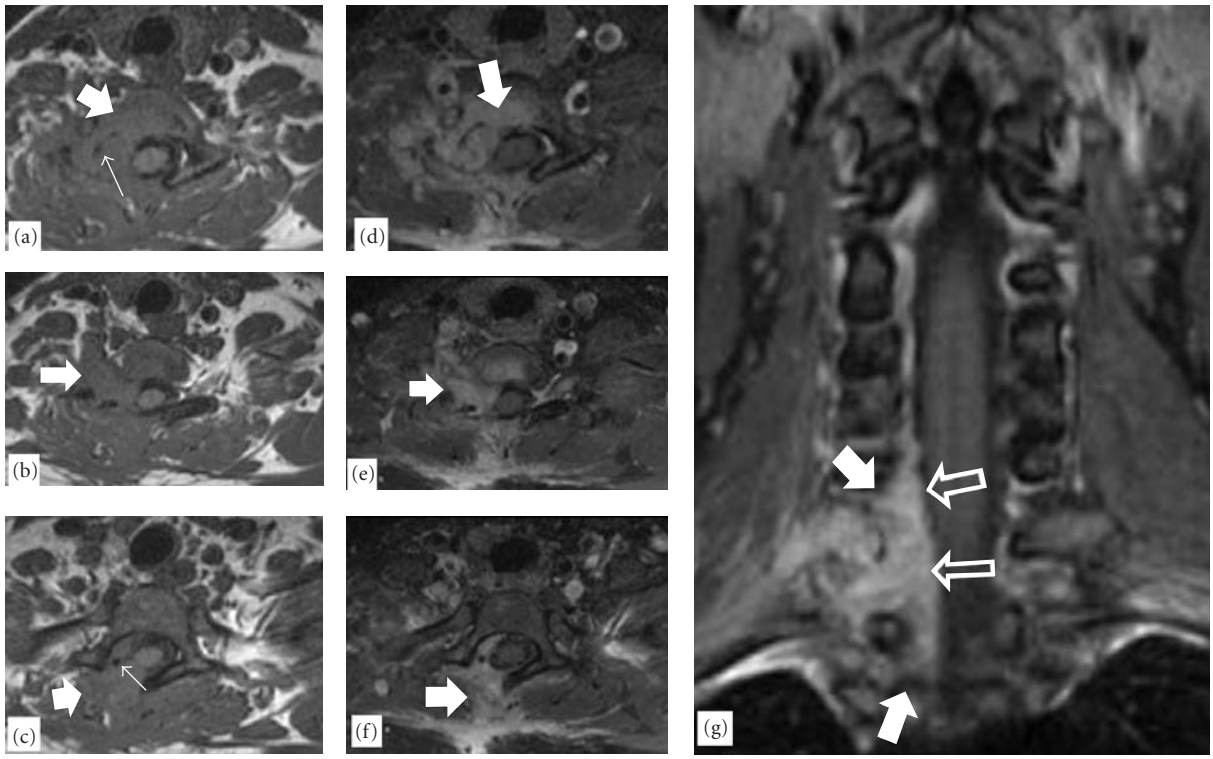


Figure 5: A 41-year-old female with a recurrent chondrosarcoma of the lower cervical spine. The axial T1-w MR images ((a)–(c)), show a soft-tissue mass in the right lower cervical spine (arrows) with foci of calcifications (thin arrows). The fat-suppressed contrast-enhanced MR images ((d)–(f)) show the intense enhancement of the lesion (arrows). (g) The coronal fat-suppressed contrast-enhanced MR image, shows the extension of the lesion within the right epidural space (arrows), with spinal cord displacement (open arrows).

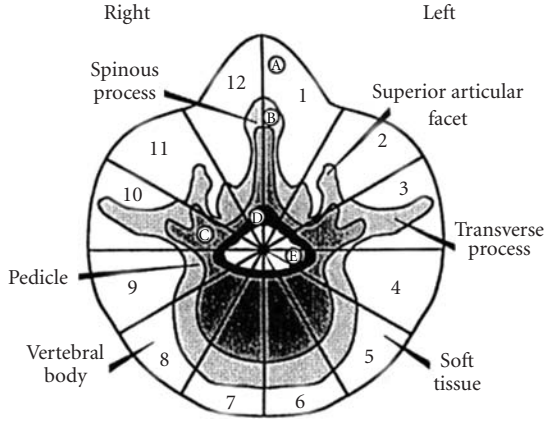


Figure 6: Weinstein-Boriani-Biagini surgical staging for spinal tumors, the transverse plane, and into five layers (A to E, from the paravertebral extraosseous region to the dural involvement). [40]. (A) Extraosseous (soft tissues), (B) intraosseous (superficial), (C) intraosseous (deep), (D) extraosseous (extradural), (E) extraosseous (intradural), and (M) metastasis.

the tumor is recorded. Finally, the Tomita staging is as follows: lesion within the vertebral body (I), the lesion extends to the pedicle (II), lesion extends to the whole vertebra (III), extension to epidural space (IV), extension to paravertebral space (V), extension to paravertebral space and neighboring vertebral levels (VI), and extension to multiple levels (VII) [9, 30, 33].

Even though primary spinal chondrosarcoma is uncommon, it represent an enormous therapeutic challenge. Consequently, it is necessary a reliable, validated, and evidence-based classification system on which to base treatment and conduct future research. A resent study shows that the intraobserver reliability for both Enneking and WBB classifications are substantial to near perfect; however, the interobserver reliability was considered fair to moderate. Therefore further work is needed to investigate the validity of these classification systems [40].

7. Differential Diagnosis

Tumors to be included in the differential diagnosis are the angioblastic meningioma, osteosarcoma, Ewing's sarcoma, and hemangiopericytoma. However, their histological features are distinct [36]. In case of a coexistence of a pathologic fracture, osteoporosis should be excluded from the diagnosis [35].

8. Management and Outcome (Prognosis)

The inherent resistance of chondrosarcoma to conventional radiation and chemotherapy makes the choice of surgical resection an inevitable necessity for patients suffering from

such a tumor [9]. A proper oncologic [37, 38] (Enneking) and surgical [31, 39] (Weinstein- Boriani- Biagini) staging of the tumor by a multidisciplinary oncologic team is a prerequisite for making the right decision on the most appropriate surgical technique, combined or not with any adjuvant medical modalities [31, 41]. Once a biopsy is to be undertaken before the definite procedure, this should be part of the whole treatment plan and carried out under the guidance and supervision of the spine surgeon [19, 41]. A closed CT-guided biopsy using a 16–18G trocar instead of a fine needle is preferred from an open one [19, 41], as being the most correct according to the oncologic rules and principles. It is fundamental that the biopsy path is contained by the excision margins at the definite surgery [19, 41].

8.1. Surgery. Surgery is of critical importance when treating spinal chondrosarcomas and should aim at preserving or even improving functionality, relieving pain, and controlling local tumor recurrence, promising a prolonged survival [31]. The spectrum of oncologically established surgical procedures applicable to the spinal column [39] varies from the most complex *en block* resection (defined as an attempt for surgical tumor removal in a single piece surrounded by healthy tissue) to the simplest one implying a piecemeal removal of the tumor (curettage). *En block* resection should be accompanied by a histological inspection and description of the resected margins [39], defined as "wide" (through the healthy tissue outside the pseudocapsule [37–39].

En block resection for primary treatment of chondrosarcoma successfully performed, wide, with disease-free margins, provides the best results regarding local tumor control with reported rates of recurrence as low as 3–8% [9, 42]. In contrast, an intralesional or curettage procedure is deemed to be unacceptable with regression rates up to 100% [3, 5, 9]. Recurrence usually appears within 3–5 years postoperatively [7, 9, 30] and much closer to the operation when a subtotal excision instead of an *en block* resection had been performed [7, 9]. However, isolated cases of late relapse as far as 10 years have been described making a long-term follow-up period for these patients essential [9].

Similarly, *en block* excision with negative histological margins offers patients the greatest chance for a prolonged survival compared with any other procedure resulting in subtotal resection, and tumor-related death estimated 12% versus 42%, respectively, for the two groups during the follow-up period [7, 9, 19, 31, 42]. Local recurrences after intralesional debulking procedures can be treated with operations of adequate margins and may give satisfactory results whereas a repetition of curettage does not prevent recurring even if accompanied by radiation [9, 19, 30].

Although *en block* resection with tumor-free margins is the optimum surgical treatment for spinal chondrosarcomas that guarantees a long recurrence-free interval and patient survival [3, 5, 7, 9, 19], at the same time, induces a significant surgical challenge [31, 41], quite often requiring a spondylectomy. The proximity of neural, vascular, and visceral structures combined with the complex spinal anatomy makes the goal of wide margins a difficult task, which is not always feasible even if a meticulous preoperative plan has been employed [3, 5, 9, 41, 43]. Complications deriving from *en block* excision are not meaningless, comprising mainly of wound problems and blood loss for the early postoperative period and implant failure and local regression for the late period [3, 41, 43].

In recent years, the innovative work of WBB [39] and Tomita [44] on surgical staging of spine tumors in combination with the evolution of modern surgical techniques [32, 33, 43, 45–47] regarding approach, reconstruction, and stabilization of vertebral column without endangering nerve structures and functional outcome or compromising the oncologic result have demonstrated that *en block* primary spine tumor resection, like chondrosarcoma, may be a feasible and oncologically justified procedure [40], provided that an experienced oncological multidisciplinary team has set the indication and properly planned it [41, 42].

8.2. Radiation and Chemotherapy. Both radiation and chemotherapy have been used as adjuvant therapies after completion of surgery [5, 7, 9], but their positive effect on patient survival and local tumor recurrence seems to be of little importance [3, 5, 7, 9, 43]. Chemotherapeutic agents have not proved to affect the outcome at all in spinal chondrosarcoma and their role is limited [5, 7, 9].

A reasonable explanation of chemotherapy incompetence might be expression of the multidrug-resistance 1 gene, P glycoprotein, resulting in resistance to doxorubicin in vitro. Also, the large amount of extracellular matrices, the poor vascularity, and the low proliferation rate of the tumor cells make chemotherapy agents even more ineffective [13]. Tumors with small cells and low percentage of cartilage matrix show more sensitivity to chemotherapy. Mesenchymal chondrosarcoma, although there is lack of prospective studies, seems to be responsive to doxorubicin-based combination chemotherapy. These patients should be considered for adjuvant chemotherapy, and in the case of metastatic disease, palliative chemotherapy [23]. Yet there is a pressing need for new standard chemotherapy treatment options for the patients with unresectable or metastatic disease. Recently, new chemotherapy agents such as histone deacetylase and aromatase inhibitors as well as angiogenesis inhibitors have been studied in vitro and in vivo, and several studies are currently ongoing [13].

Although radiotherapy is frequently used in patients with inadequate margins [5], survival for these patients remains low compared to those who had a margin-free *en block* excision and no adjuvant radiation [9, 43]. One reason explaining these results, apart from tumor resistance, could be the fact that these modalities are implemented mainly on chondrosarcomas of higher grade or patients who cannot tolerate a major operation [7]. Radiotherapy applied in high doses (65 Gy) [48] or proton beam radiation [49, 50] becomes mostly important when treating chondrosarcoma of the upper cervical spine, due to the technical difficulties that an effort for wide surgical excision in this peculiar anatomical location entails. Local control rates of up to 92% have been reported [50] but the follow-up period is short

(<5 years). Recently, a systematic review [42], including a multicenter cohort, concludes that radiation as an adjunct to surgery, in case that an incomplete excision of the mass has been achieved, may have a small beneficial effect on outcome and mainly on local tumor control. Radiation as a primary treatment for chondrosarcoma of the spine is strongly not indicated [42].

8.3. Cryosurgery and Radiofrequency Ablation. Although latest publications report the effectiveness of cryoablation in combination with curettage, as an alternative to more radical procedures, for the treatment of low grade I chondrosarcoma of the appendicular skeleton [51, 52], this is not documented by the current literature regarding the mobile spine. Radiofrequency ablation is another minimal invasive percutaneous technique used mainly for palliating painful skeletal metastasis [53–55], including the spine region [56], but there is no study, to our knowledge, addressing the application of this technique in primary spinal tumors and more specifically chondrosarcoma.

8.4. Prognosis. Besides, histological grade of the tumor, the prognosis depends on the possibility of performing *en bloc* excision with appropriate oncologic margins. A successful operation, in terms of complete tumor excision with disease-free margins is a major independent prognostic factor for a favorable course of the disease, affecting critically both local tumor control and patient survival [5, 7, 9, 19, 42]. Regarding tumor recurrence, it is reported to rate higher (up to 100%) when inadequate margins (intralesional or contaminated) have been accomplished during the operation [7, 9, 19, 41, 42] and/or a primary treatment (including biopsy) has taken place outside the reference center [19, 41]. Distal metastases are sparsely reported in the literature [7, 41], occurring during the course of the disease and related to a higher tumor grade [41] and a local tumor recurrence [19].

Regarding survival, it is difficult to extract accurate rates due to the lack of large series and standardization of surgical techniques in the existing literature. However, York et al. [7] estimate an overall 5- and 10-year survival rate at 64% and 40%, respectively, for 21 surgically treated patients. Similarly, Bergh et al. [19] in their study of 69 cases of the axial skeleton (including 12 spinal chondrosarcomas) calculate overall 5-, 10-, and 15-year survivals for the whole series at 72%, 67%, and 63%. Factors adversely affecting survival are considered an older patient age and a higher tumor grade [19], inadequate surgical margins [5, 19, 41], and a local recurrence [9, 19, 42]. Failed local control of the disease, as a consequence of insufficient surgery, is deemed to be crucial for survival, with a rate of tumor-related death as high as 61% for patients suffering a local regression [19].

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Review Article

Advances in Molecular Characterization and Targeted Therapy in Dermatofibrosarcoma Protuberans

Piotr Rutkowski,[1] Agnieszka Wozniak,[2] and Tomasz Switaj[1]

[1] *Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Roentgena 5, 02-781 Warsaw, Poland*
[2] *Laboratory of Experimental Oncology, Department of General Medical Oncology, Catholic University Leuven and University Hospitals Leuven, 3000 Leuven, Belgium*

Correspondence should be addressed to Piotr Rutkowski, rutkowskip@coi.waw.pl

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The molecular pathogenesis of dermatofibrosarcoma protuberans (DFSP) involves distinctive rearrangement of chromosomes 17 and 22 leading to formation of the *COL1A1-PDGFB* fusion gene. The knowledge of molecular events underlying development of DFSP resulted in the implementation of targeted therapy with imatinib—a tyrosine kinase inhibitor (TKI), to the clinical practice. The striking efficacy of imatinib in advanced cases of DFSP has been demonstrated in a few clinical trials. Thus, imatinib is currently considered the gold standard in the treatment of inoperable and/or metastatic and/or recurrent cases of DFSP. Therapy with imatinib may potentially facilitate resection or decrease possible disfigurement related to radical surgical procedure. Following partial response on imatinib significant percentage of patients may be rendered free of the disease by surgery of the residual tumor.

1. Introduction

Dermatofibrosarcoma protuberans (DFSP) is a rare cutaneous-origin sarcoma with usually indolent growth (over years) and low metastatic potential. Regional/distant metastases probability is less than 5% [1, 2]. Metastases develop more commonly in DFSP-containing areas of high-grade fibrosarcoma—fibrosarcomatous-DFSP (DFSP-FS) [3–6], which is characterized by more aggressive course. If distant metastases occur they are often restricted to lungs, and less commonly to lymph nodes. The standard treatment of the localized disease is radical, wide local excision. It is recommended that margins of the surgical excision should exceed 2-3 cm [1, 7]. This procedure often requires application of reconstructive techniques and may result in cosmetic disfigurement or functional impairment. Unfortunately, the microscopically infiltrating pattern of tumor growth might lead to high rates of unexpected positive margins. Local recurrences may occur late, and they have been reported within the range of 24–90% [1, 3, 8–14]. Nevertheless, several reports provided data demonstrating lower frequency of recurrence rate [15–17]. Recurrent disease is more challenging surgically, due to tumor fixation to deeper structures. Microscopic infiltrations spreading from the tumor might also lead to high probability of unexpected nonradical resection. There is only limited experience with Mohs micrographic surgery in the treatment of localized DFSP [18–20].

2. Molecular Pathogenesis

DFSP is characterized by the presence of distinctive, reciprocal rearrangement of chromosomes 17 and 22 in the form of translocation t(17;22)(q22;q13) or supernumerary ring chromosomes containing material from chromosomal regions 17q22 and 22q 13 [21–31]. The rearrangement leads to the fusion of alpha chain type a (*COL1A1)* localized on 17q22 to the platelet-derived growth factor beta (*PDGFB)* localized on 22q13 (Figure 1) [32].

The *PDGFB* gene product is a growth factor that serves as a ligand for the transmembrane receptor kinase PDGFRB. The formation of *COL1A1-PDGFB* fusion gene results in the constitutional upregulation of *PDGFB* expression, leading

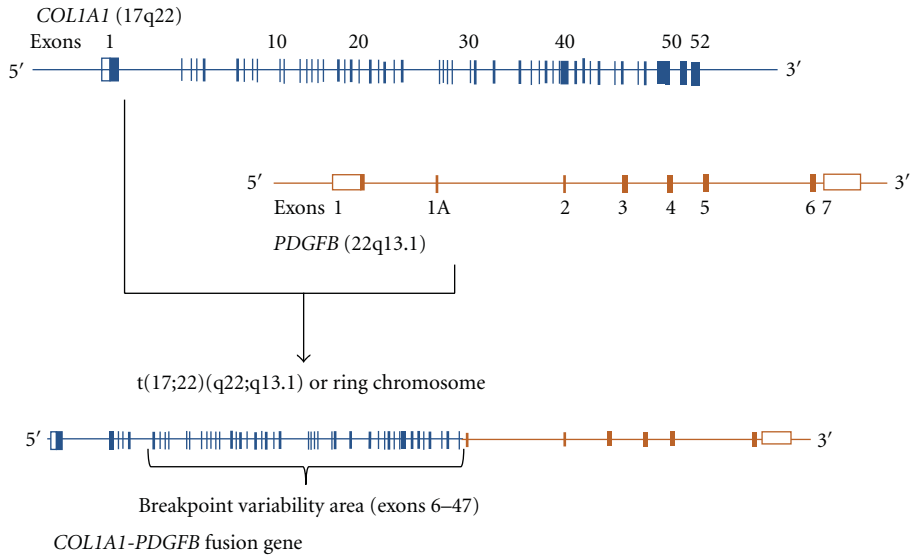


Figure 1: Schematic presentation of the *COL1A1/PDGFB* fusion gene formation.

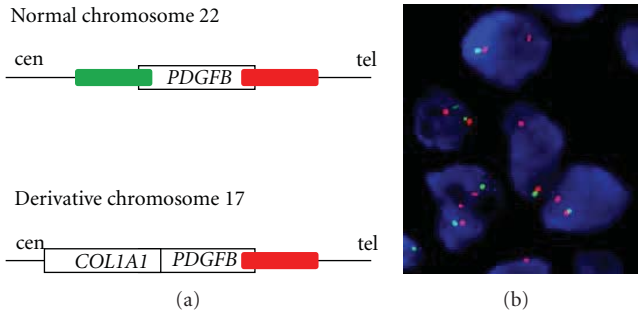


Figure 2: *PDGFB* break-apart FISH in interphase nuclei from DFSP. (a) Schematic localization of FISH probes; (b) *PDGFB* rearrangement detected by FISH, evidenced by one copy (red probe) of the telomeric *PDGFB* signal in tumor cells (courtesy of Professor M. Debiec-Rychter).

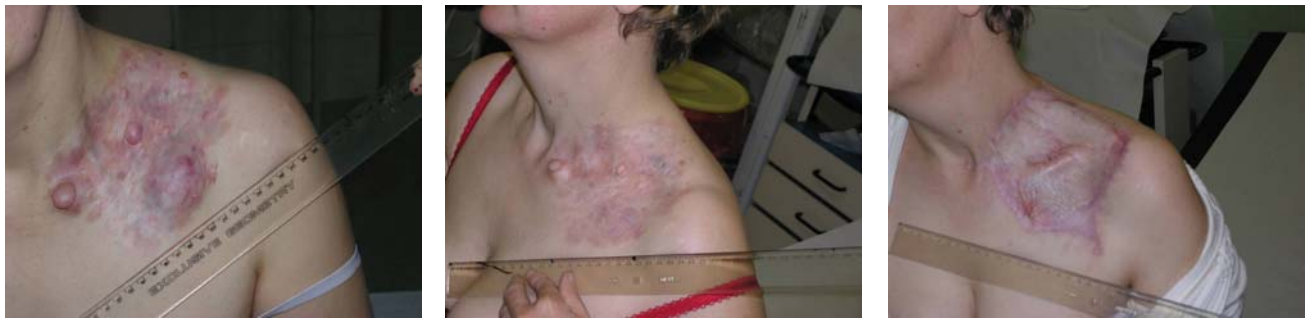
to continuous autocrine activation of PDGF receptor B (PDGFRB) and as a consequence to propagation of the mitotic signal by formation of an autocrine and paracrine loops [33–35]. Greco et al. [36, 37] provided evidence, that transfection with *COL1A1-PDGFB* fusion gene could transform NIH3T3 cells. Furthermore, it was shown that by using suramin, a compound known to interfere with PDGF-PDGFR ligand-receptor interaction, the *COL1A1-PDGFB* transformed phenotype in NIH3T3 cells can be reversed [36].

Interestingly the presence of the specific *COL1A1-PDGFB* fusion transcript was also identified in giant cell fibroblastoma (GCF) that is a histologic variant of DFSP. GCF primarily affects children so it is also called the juvenile form of DFSP [38–41]. In DFSP-FS increased copy numbers of *COL1A1-PDGFB* fusion gene were observed suggesting a possible oncogenic mechanism of the clonal evolution from DFSP into DFSP-FS [42].

Although there is no need for molecular confirmation of the diagnosis in the majority of DFSP cases, the detection of the chromosomal 17;22 rearrangements or the *COL1A1-PDGFB* fusion is a valuable diagnostic tool for differential diagnosis of atypical, metastatic DFSP or DFSP-FS. Currently two main molecular techniques are used: fluorescence *in situ* hybridization (FISH) or multiplex reverse transcription polymerase chain reaction (RT-PCR). FISH can be performed on interphase nuclei from cell suspensions, touch prints, or frozen or fixed paraffin-embedded sections most commonly using break-apart *PDGFB* or *COL1A1-PDGFB* fusion approach (Figure 2). On the other hand, RT-PCR requires RNA extracted from tumor fragments and necessitates the simultaneous use of several *COL1A1* primers (multiplex approach) as the breakpoint can randomly occur between exons 6 and 47 [43–45].

3. Targeted Therapy

Advances in the understanding of molecular mechanisms of DFSP resulted in the implementation of targeted therapy based on PDGFR inhibition to the treatment of this sarcoma. Imatinib mesylate is a tyrosine kinase inhibitor rationally developed and specifically directed against BCR/ABL, KIT, FMS (receptor for Colony Stimulating Factor 1), ARG (ABL-related gene), and PDGFR alpha and beta. It has been also found to be the first effective systemic therapy in DFSP. Imatinib competes with adenosine triphosphate (ATP) molecule, blocking tyrosine kinase receptor ability for autophosphorylation, which in return results in inhibition of the aberrant signal transduction pathway and partial restoration of proper intracellular signaling. The observation that autocrine overproduction of PDGFB caused by gene rearrangement is a key pathogenetic factor [33, 34] forced the *in vitro* research, which showed inhibition of DFSP cells growth *in vitro* after exposure to imatinib [36, 46]. The



(a) (b) (c)

Figure 3: Images of advanced dermatofibrosarcoma protuberans of the supraclavicular region before and after therapy with imatinib, and after resection of residual disease. The patient is now 3 years free of disease.

further demonstration of the imatinib inhibitory effect on six different DFSP cell lines both *in vitro* and *in vivo* [37] has led to the investigation of this new therapeutic approach in the clinic. Early case reports on small series of patients suggested the usefulness of imatinib in metastatic and locally advanced DFSP [47–52]. Next series of 10 patients with locally advanced and/or metastatic DFSP treated within Imatinib Target Exploration Consortium Study B2225 showed responses in all patients, including complete responses in five out of 10 of locally advanced cases and one partial response lasting seven months in metastatic case [53]. As a consequence imatinib was registered as a therapy of choice in advanced (inoperable and/or metastatic) DFSPs (Figure 3). In a phase II trial [54] evaluating the activity of imatinib in life-threatening malignancies expressing imatinib-sensitive tyrosine kinases DFSP was the only one of five tumor types in which a notable activity was shown including extensive regression in 10/20 cases (50% partial remissions, 33,3% complete remissions).

Combined analysis of prematurely closed, two phase II, single arm, open-label trials on efficacy of imatinib in advanced DFSP (European Organisation for Research and Treatment of Cancer no. 62027 and the Southwest Oncology Group no. S0345) has demonstrated the clinical benefit with rate exceeding 70% and median time to progression of 1.7 years on 25 patients with advanced DFSP [55]. Although there were some differences in both trials' design, the observed responses' rates were similar. These results imply that the imatinib dose of 400 mg daily has similar efficacy to 800 mg daily in this entity. Rutkowski et al. [56] have proved striking activity of imatinib mesylate in advanced DFSP in the group of 15 patients treated with imatinib in routine clinical practice outside any trial, with clinical benefit rate approaching 80% as well as median PFS and OS being not reached. In Table 1 the efficacy results of imatinib in advanced DFSPs from pooled analysis of phase II trials [55] and 15 patients treated outside clinical trials is presented [56].

It has also been shown that DFSPs-FS with t(17;22) are still imatinibsensitive although responses seem to last shorter [57] while DFSPs-FS lacking the specific aberration do not respond to the treatment [53]. Therefore the confirmation of the molecular target (*COL1A1-PDGFB* fusion) presence seems to be obligatory in every case prior to the start of imatinib therapy.

Table 1: The best overall responses, progression, and survival status in combined phase II clinical trials [55] and in group of patients treated outside clinical trials [56].

	Group of 24 patients treated in phase II trials [55]	Group of 15 patients treated outside clinical trials [56]
	N (%)	
Progression status		
Progression-free	12 (50)	11 (73)
Progression	12 (50)	4 (27)
Survival status		
Alive	18 (75)	12 (80)
Dead	6 (25)	3 (20)
Best overall response		
Partial response	11 (45.9)	11 (73)
Stable disease	6 (25)	1 (7)
Progressive disease	4 (16.6)	3 (20)
Not evaluable	3 (12.5)	0

Complete, wide surgical excision is the standard treatment in localized, resectable cases, and in advanced cases it may result in cosmetic disfigurement or serious functional impairment. Thus the neoadjuvant imatinib strategy leading to tumor downstaging and decrease of excision morbidity by tissue-sparing appears to be very attractive. Kérob et al. [58] presented report on 25 resectable DFSP (median size: 4.5 cm) treated in phase II trial with preoperative imatinib at the dose of 600 mg daily for two months. The objective partial response according to RECIST was observed in nine cases (36%). The median relative tumor volume decrease was 20% (range: 12.5–100%). Available clinical data indicate that some DFSP patients initially evaluated as

unresectable/metastatic or necessitating mutilating surgery turned out to have resectable tumor after imatinib therapy. This rational approach enabling achievement of complete remission may be potentially curative, although longer followup is needed. Further studies are required for elucidating whether preoperative imatinib therapy reduces the need for wide surgical margins or whether imatinib has activity as adjuvant therapy in cases with positive margins after excision or in other high-risk patients.

Majority of patients treated with imatinib experienced side effects during treatment, but almost all are mild and manageable. The most common were fluid retention/edema, anemia, fatigue, nausea, skin rash, thrombocytopenia, vomiting, neutropenia, and diarrhea, and they are similar to those observed in patients with gastrointestinal stromal tumor (GIST).

There are still several questions regarding imatinib mechanism of action, and possible resistance to this targeted therapy in DFSP. There is also a need to identify novel predictive molecular markers for patients' outcome. It was presumed that imatinib effect resulted from inhibition of PDGFR phosphorylation. Surprisingly, clinical activity of imatinib in DFSP is striking even in DFSP expressing relatively low amounts of activated receptor. It seems that inhibition of low-level receptor tyrosine kinase may be effective clinically if tumor cells are dependent on that signaling mechanism, what has been observed also in pigmented villonodular synovitis/tenosynovial giant-cell tumor [59, 60]. The better understanding of the downstream effects caused by imatinib-PDGFB interaction would allow defining additional treatment strategies for DFSP patients. In case of disease progression after initial response to imatinib the investigation of other multitargeted tyrosine kinase inhibitors seems to be justified.

To summarize, imatinib therapy is currently the gold standard in the treatment of inoperable and/or metastatic and/or recurrent cases of DFSP, and this targeted therapy may potentially facilitate resection or decrease possible disfigurement. Significant percentage of patients may be rendered free of disease by surgery of residual disease following partial imatinib responses. Current therapy of DFSP with t(17;22) translocation should be conducted by multidisciplinary team, including oncological surgeon. The use of imatinib mesylate as initial therapy should be considered to decrease possible extent of surgery and related morbidity.

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Review Article

Molecular Approach to Uterine Leiomyosarcoma: LMP2-Deficient Mice as an Animal Model of Spontaneous Uterine Leiomyosarcoma

Takuma Hayashi,[1,2] Akiko Horiuchi,[3] Kenji Sano,[4] Nobuyoshi Hiraoka,[5] Yae Kanai,[5] Tanri Shiozawa,[3] Susumu Tonegawa,[6] and Ikuo Konishi[7]

[1] *Department of Immunology and Infectious Disease, Shinshu University Graduate School of Medicine, 3-1-1, Asahi Matsumoto, Nagano 390-8621, Japan*
[2] *Promoting Business Using Advanced Technology, Japan Science and Technology Agency (JST), Saitama 332-0012, Japan*
[3] *Department of Obstetrics and Gynecology, Shinshu University School of Medicine, Nagano 390-8621, Japan*
[4] *Department of Laboratory Medicine, Shinshu University Hospital, Nagano 390-8621, Japan*
[5] *Pathology Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[6] *Picower Institute and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139-4307, USA*
[7] *Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan*

Correspondence should be addressed to Takuma Hayashi, takumah@shinshu-u.ac.jp

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Uterine leiomyosarcoma (LMS) develops more often in the muscle tissue layer of the uterine body than in the uterine cervix. The development of gynecologic tumors is often correlated with female hormone secretion; however, the development of uterine LMS is not substantially correlated with hormonal conditions, and the risk factors are not yet known. Importantly, a diagnostic-biomarker which distinguishes malignant LMS from benign tumor leiomyoma (LMA) is yet to be established. Accordingly, it is necessary to analyze risk factors associated with uterine LMS, in order to establish a treatment method. LMP2-deficient mice spontaneously develop uterine LMS, with a disease prevalence of ~40% by 14 months of age. We found LMP2 expression to be absent in human LMS, but present in human LMA. Therefore, defective LMP2 expression may be one of the risk factors for LMS. LMP2 is a potential diagnostic-biomarker for uterine LMS, and may be targeted-molecule for a new therapeutic approach.

1. Introduction

The uterus is the female reproductive organ, located at the center of the pelvis between the left and right ovaries. The uterus, the organ in which the embryo grows, is composed of three layers, the uterine endometrium which serves as a bed for the embryo; the myometrium of the wall which protects the embryo; and a serous membrane enveloping the uterus. The myometrium is composed of smooth muscle. In general, the term uterine tumor refers to an epithelial malignant tumor of the uterus, which is roughly classified as a tumor of the uterine cervix or the uterine body. Because of the prevalence of screening, uterine cervix cancer is decreasing in incidence, and usually detected at a very early stage, including stage 0. In contrast, cancer of the uterine body is increasing in incidence, and rarely detected at the initial stages. While most tumors of the uterine body are adenocarcinomas (derived from the subintimal gland), tumors of the uterine cervix are classified into squamous cancer and adenocarcinoma. The myometrium is composed of smooth muscle. Smooth muscle tumors (SMTs) which develop in the myometrium have been traditionally divided into benign LMA and malignant LMS based on cytological atypia, mitotic activity and other criteria. Uterine LMS, one of the most common neoplasms of the female genital tract, is relatively rare, having an estimated annual incidence of

0.64 per 100,000 women [1]. Uterine LMS accounts for 2% to 5% of tumors of the uterine body and develops more often in the muscle layer of the uterine body than in the uterine cervix [2, 3]. Distinguishing uterine LMA from uterine LMS is very difficult, and a diagnosis generally requires surgery and cytoscopy.

The cause of tumors of the uterine cervix has been found to be the human papilloma virus, in combination with other factors. An infection is established by sexual activity. In contrast, a main factor in the development of tumors in the uterine body is the hormonal environment. Patients with uterine body tumors often are unmarried, have never been pregnant, and are taking a hormonal agent. High estrogen levels are considered to significantly influence the development of such tumors. The mechanisms by which uterine LMA and LMS develop are not yet known, though tumor cells that have developed in the myometrium for some reason gradually become larger due to the influence of the female hormone, estrogen, and generate tumors. However, no correlation between the development of uterine LMS and hormonal conditions, and no obvious risk factors have been found. The prognosis of uterine LMS is not good, and the five-year survival rate is approximately 35%, although the five-year survival rate depends on disease stage [2, 3]. It is worth noting that, when adjusted for stage and mitotic count, LMS has a significantly worse prognosis than carcinosarcoma [4]. As uterine LMS is resistant to chemotherapy and radiotherapy, and thus surgical intervention is virtually the only means of treatment [5–7], developing an efficient adjuvant therapy is expected to improve the prognosis of the disease. Although cases accompanied by hypocalcaemia or eosinophilia have been reported, neither clinical abnormality is an initial risk factor for uterine LMS. The identification of a risk factor associated with the development of uterine LMS would significantly contribute to the development of preventive and therapeutic treatments.

2. Biological Roles of the Immunoproteasome

When tissue or an organ is transplanted, the graft is often lost due to an acute rejection caused by the host immune system. This is because the cell surface antigens presented by the major histocompatibility complex (MHC) are intrinsic to an individual and so differ between the donor and recipient. The immunological self markers on cell surfaces are the most important immune system for higher vertebrates such as mammals, protecting the self from invaders. Cytoplasmic proteins are mostly degraded by a protease complex, which has many substrates consisting of twenty-eight 20 to 30-kDa subunits, referred to as the immunoproteasome [8]. The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression, and others. The proteasomal degradation pathway is also essential for the production of peptide antigens which are presented by MHC class I. That is, the immunoproteasome plays a key role in the presentation of immunological self markers on the cell surface by MHC (Figure 1) [8]. Interferon-γ (IFN-)γ) is a critical inducer of

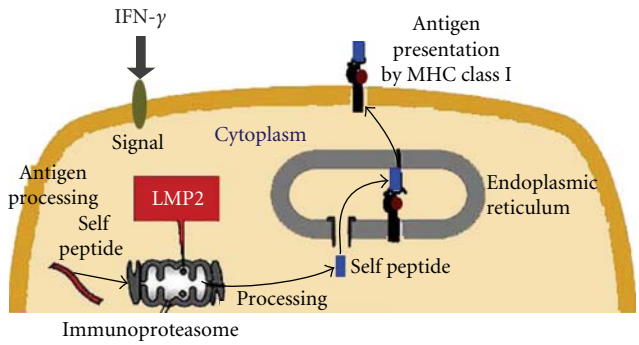

Figure 1: Mediation of the proteasomal degradation pathway to antigen presentation by MHC class I. The immunoproteasomal degradation pathway is essential for antigen presentation by MHC class I. Defecive LMP2 expression results in tissue- and substrate-dependent abnormalities of immunoproteasomal functions. Therefore an impaired proteasome may promote the initial development of disease including tumorigenesis.

the immunoproteasome's expression in immune systems [9]. Recent findings have verified that IFN-γ prevents primary tumor development, thereby showing a tumor suppressor role in the immune response [10, 11]. IFN-γ upregulates the expression of large numbers of responsive genes, also, expression of the immunoproteasome's subunits, that is, low-molecular mass polypeptide (LMP) 2, LMP7, and LMP10, is markedly induced by IFN-γ signaling [9, 12]. The IFN-γ-inducible proteasomal function plays a key role in MHC class I-mediated tumor rejection [11, 13]. Further, a molecular approach to studying the correlation of IFN-γ with tumor cell growth has drawn attention. A deficiency of IFN-γ apparently does not hamper the generation of CTL [10, 11]. Recent reports have demonstrated the multifunctional deficiencies of components of the MHC class I antigen-presentation pathway including LMP2 and TAP-1 in tumor cells [11, 13]. A possible role for the IFN-γ-responsive gene *TAP-1* in tumor recognition was reported [11]. Here we identify LMP2, a single IFN-γ-responsive gene product, as obligatory for tumor surveillance [12] and demonstrate a tissue-specific role for LMP2 in protection from spontaneous neoplasms of the uterus.

3. Development of Malignant Uterine Tumor in LMP2-Deficient Mice

Malignant tumors originate from a single cancerous cell and develop as a result of unlimited cell proliferation. Malignant tumor cells have properties that are biologically different from those of normal cells. Thus, the host immune system should be able to distinguish malignant tumor cells from corresponding normal cells. That is, malignant tumor cells present intrinsic antigens (i.e., tumor-cell-specific antigens that can be the targets of immune responses are referred to as tumor-antigens (TA)) on the cell surface with the aid of MHC. In many cases, however, almost no reaction by the immune system is observed. Also, the incidence of major tumors is not very different between immunodeficient

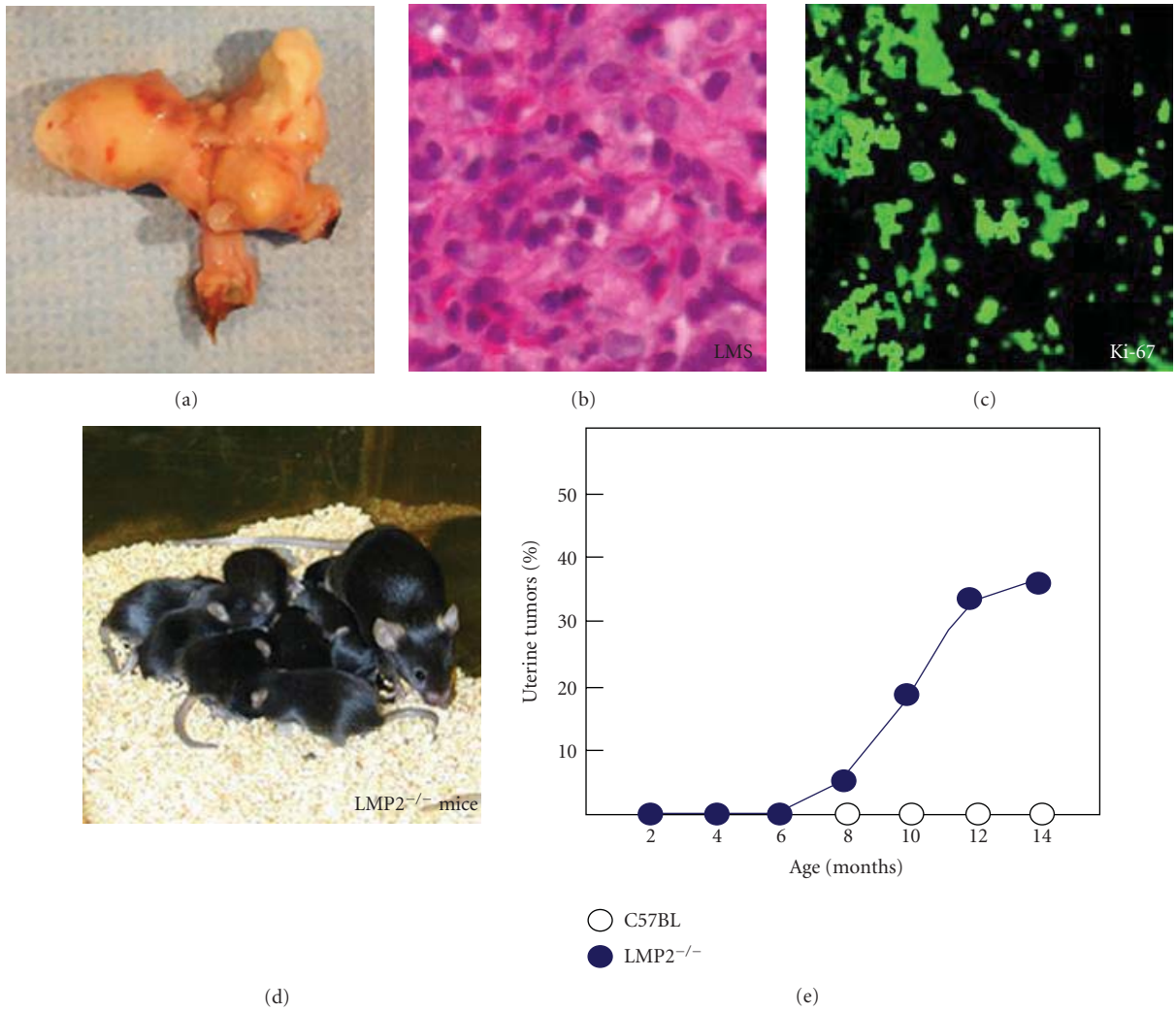

Figure 2: Histological findings of uterine leiomyosarcoma in LMP2-deficient mice. Histological findings of uterine LMS in LMP2-deficient mice ((a) to (c)). Among the histological findings of uterine LMS in LMP2-deficient mice, a cytoskeleton, which is characteristic of uterine LMS, is observed. ((b) and (c) magnification x400) Panel (e), in LMP2-deficient females, uterine LMS is observed at 6 months of age. The incidence at age 14 months is as high as 40% (e). The curve indicating the incidence of mouse uterine LMS is very similar to that indicating the incidence of human uterine LMS, which is observed after menopause. In mice with tumors of the uterus, significant weight loss is observed. Thus, a tumor that develops in the uterus is diagnosed as malignant, that is, uterine LMS.

(i.e., lymphocyte-deficient) mice and control mice having normal immune systems. Specifically, tumor cells can avoid the immune monitoring system via several means [14, 15]. Naturally occurring tumor cells seem to have lost the expression of peptide antigens, TA, or cell adhesion factors intrinsic to tumors. Tumor cells may avoid the host immune reaction due to the absence of MHC expression, although no such mechanism has yet been elucidated. However, it is important to demonstrate how tumor cells evade immune-responses, in order to prevent the development of tumors.

The genes encoding LMP2, LMP7, TAP1, and TAP2, are located in region H-2 which encodes the murine MHC molecule. LMP2-deficient mice show tissue- and substrate-dependent abnormalities in the biological functions of the immunoproteasome, and impaired functioning of the immunoproteasome in the spleen or hepatic cells [16]. Further, LMP2-deficient mice do not show normal immune responses to virus-infected cells, and such immunopathy is known to result from a failure in the presentation of peptide antigens on the cell surface by MHC [16]. We found that uterine LMS occurred in female LMP2-deficient mice at age 6 months or older, and the incidence at 14 months of age was about 40% [17] (Figure 2). The curve indicating the incidence in mice is very similar to that indicating the incidence of human uterine LMS, which occurs after menopause. Histological examinations of LMP2-lacking uterine tumors revealed characteristic abnormalities of LMS [17]. The tumors lacked lymphoid infiltrates, a sign of immune recognition, and consisted of uniform elongated smooth muscle cells arranged into bundles. The nuclei of the tumor cells varied in size and shape; furthermore, mitosis was frequent, in contrast, the uterine smooth muscle cells

of C57BL/6 mice were normal in appearance [17]. Whereas relatively few Ki-67-positive cells, the proliferating cells of solid tumors, were observed in the basal cell layer of the normal uterine smooth muscle, most of the basal cells vividly expressed Ki-67 in LMP2-deficient mice [17]. This immunohistochemical (IHC) staining indicates abnormal proliferation of the LMP2-lacking cells in the basal layer [17] (Figure 2). LMP2-deficient mice that have developed uterine LMS undergo considerable weight loss, and then die by 14 months of age [17]. The LMP2-deficient mice also exhibit skeletal muscle metastasis from uterine LMS. Therefore it is like LMP2-deficient mice with uterine LMS have died of mass effect and metastasis. In general, it is not easy to distinguish uterine LMA from LMS. However, in mice, because of such characteristic pathological findings, significant weight loss, and exhibition of skeletal muscle metastasis, a tumor that develops in the uterus of an LMP2-deficient mouse can be considered malignant, that is, a uterine LMS.

If the *TP53* gene is damaged, tumor suppression is severely reduced. People who inherit only one functional copy of the *TP53* gene will most likely develop tumors in early adulthood, a disease known as Li-Fraumeni syndrome. More than 50 percent of human tumors contain a mutation or deletion of the *TP53* gene [18]. To increase tumor incidence and better assess the role of systemic expression of TP53 in responses to initiation of uterine LMS tumorigenesis, LMP2-deficient mice were bred with TP53-deficient mice to create $Lmp2^{-/-}Tp53^{-/-}$ double knockout mice. Uterine LMS incidence and death rates were similar in $Lmp2^{-/-}Tp53^{-/-}$ mice and closely matched control $Lmp2^{-/-}Tp53^{+/+}$ mice. The correlation of defective TP53 function with uterine LMS tumorigenesis is not clearly understood.

4. Inactivation of the IRF-1 Tumor Suppressor Gene in LMP2-Deficient Mice

Uterine LMS was demonstrated to spontaneously develop in 6-month-old LMP2-deficient mice at high frequency. The expression of LMP2 was significantly induced by IFN-γ as was the expression of other subunits [9, 12]. Accordingly, the expression of cell-cycle regulators that are regulated by the IFN-γ signal cascade or immunoproteasome activity was examined. Signal transducer and activator of transcription (STAT) 1, having been activated by IFN-γ, significantly induced expression of tumor suppressors such as interferon regulatory factor 1 (IRF1) [19, 20]. IRF1 as a transcriptional regulator significantly regulates LMP2 expression [19, 20]. It was examined whether the IFN-γ signal cascade induces the expression of each subunit of the immunoproteasome and IFR1 and IRF2 in LMP2-deficient mice and the parental strain, C57BL/6. No significant difference was observed in the expression of STAT1 and the subunits LMP7, LMP10, CP9, and IRF2. Also, IFN-γ-induced phosphorylation of STAT1 would not be influenced by a lack of LMP2. However, the expression of IRF1 was significantly reduced in splenocytes derived from mice lacking LMP2 in comparison with wild-type mice. IRF1 expression in LMP2-deficient splenocytes was not induced by the IFN-γ signal cascade. In addition, wild type-mouse embryonic fibroblasts (MEFs) that had been treated with the proteasome inhibitor MG-132 exhibited a loss of IFN-γ-inducibility, reproducing a phenotype of the LMP2-deficient mouse. Accordingly, the transcription of *Irf1* mRNA depends on the immunoproteasome's function and is considered to involve the formation of a STAT1 homodimer. Recent reports suggest that proteasomal function contributes to mRNA transcriptional activation [21, 22].

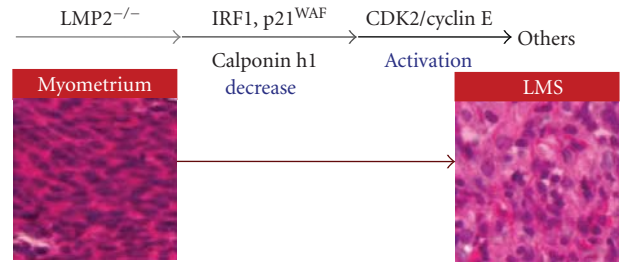

Figure 3: Model of the mechanism for development of uterine leiomyosarcoma. In LMP2-deficient cells, levels of the antioncogenic factor IRF-1, p21^{WAF} are significantly reduced. Reduced expression of the calponin h1 transcript, which contributes to cell proliferation and tumorigenesis in uterine smooth muscle cells, is detected in uterine LMS tissues. Cell cycle regulatory factors, CDK2/Cyclin E, are markedly activated. The inactivation of such antioncogenic factors is considered to transform LMP2-deficient cells into malignant tumor cells.

Primary cultured tumor cells (LMP2-UC) were established from the uterine LMS of LMP2-deficient mice, and then IRF1-overexpressing tumor cells (LMP2-UC-IRF1) were further established by genetic engineering. The LMP2-UC-IRF1 cells were intracutaneously transplanted into immunodeficient mice (BALB/c nu/nu), and significant inhibiting effects of IRF1 on tumor cell proliferation were observed [20, 23]. Thus, a lowered level of IRF1 resulting from a deficiency in LMP2 seemed to be a risk factor for uterine LMS in mice. The effects of IRF1 on tumor cell proliferation are achieved through the expression of p21^{WAF} cell-cycle inhibitors (inhibiting transition from the G1 to S stage) [24]. Whether or not p21^{WAF} expression or activation is affected in LMP2-deficient mice should be examined further. The tumor suppressor, retinoblastoma (Rb) is phosphorylated by a complex of Cyclin E/Cyclin dependent kinase 2 (CDK2) and then inactivated [25]. Also, the activity of CDK2 is negatively regulated via degradation of Cyclin E by the 26S proteasome [26, 27]. A significant level of phosphorylated-Rb is observed in MEFs-lacking LMP2, and the activity of CDK2 for phosphorylation is determined to be stronger than that in normal MEFs. However research overall, including experiments with gene-deficient mouse models and clinical studies, suggests that defective Rb expression does not take part in the onset of uterine LMS [28–30]. In the case of uterine LMS in LMP2-deficient mice, defective IRF1 is considered to be involved in cellular transformation and cell proliferation (Figure 3).

5. Perspectives

Uterine LMS mainly develops in the uterine smooth muscle or endometrial stroma, and menstrual anomalies, such as hypermenorrhea and prolonged menstruation, and symptoms such as abnormal hemorrhage, hypogastric pain, lumbar pain, and abdominal strains, are observed [4]. In the case of gynecological cancers, such as breast cancer, a female hormonal imbalance is often a risk factor for developing tumors. As in the case of uterine LMA, however, a correlation between the development of uterine LMS, the female hormone, and hormone receptors has yet to be elucidated [31, 32]. A recent report showed the expression of *Lmp2* mRNA and protein in luminal and glandular epitheliua, placenta villi, trophoblastic shells, and arterial endothelial cells [33]. These results implicate LMP2 in the invasion of placental villi, degradation of the extracellular matrix, immune tolerance, glandular secretion, and angiogenesis [33]. The present study should help to elucidate the regulatory role of the ubiquitin-proteasome pathway in the implantation of embryos. Unfortunately, it is unclear whether defective LMP2 expression is involved in the onset of uterine LMS. Uterine LMS often seems to develop in individuals exposed to radiation in the pelvis. Risk factors for its development, however, have not been identified because of the absence of a suitable animal model. The LMP2-deficient mouse was the first animal model of spontaneous uterine LMS to be established [17]. Defective LMP2 expression may be one of the causes of uterine LMS [20]. To demonstrate whether LMP2 is a potential biomarker for distinguishing LMS from LMA, we are investigating the reliability and characteristics of LMP2 as a diagnostic indicator with several clinical research facilities. The clinical research is yet to be concluded, and large-scale clinical studies need to be performed. In some cases, uterine LMA may become malignant and develop into uterine LMS. Accordingly, the correlation between the inactivation of LMP2 and the development of uterine LMA needs to be examined. Although LMS usually lacks lymphoid infiltrates recognizable on routine histological staining, further histological examination revealed a few infiltrating $CD56^+$ natural killer cells in human uterine LMS tissues. Definitive histological studies must be performed, including the gene-expression profiling of several known pro-oncogenic factors as well as factors such as brain-specific polypeptide PEP-19 and a transmembrane tyrosine kinase receptor, C-kit [34–36]. The reduced expression of calponin h1 transcripts was reported to be associated with uterine LMS, and calponon h1 might function as a tumor suppressor in uterine LMS [37, 38]. A recent study showed that re-expression of human calponin h1 suppressed cell proliferation and tumorigenesis in uterine LMS cells [38]. Since no spontaneous development of uterine LMS is observed in IRF1-, calponin h1-deficient mice or heterozygous Rb mice, the lack of LMP2 is largely associated with the expression of other known or unknown cell-cycle regulatory factors. Further research is required to demonstrate the correlative functions of LMP2 and other antioncogenic factors with calponin h1 in the tumorigenesis of uterine LMS. Clarification of the correlation between these factors and the development of uterine LMS and the identification of specific risk factors may lead to the development of new treatments for the disease. Uterine LMS is refractory to chemotherapy and has a poor prognosis. The molecular biological and cytological information obtained from LMP2-deficient mice will contribute remarkably to the development of preventive methods, a potential diagnostic-biomarker, and new therapeutic approaches against uterine LMS.

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Review Article

Liposarcoma: Molecular Genetics and Therapeutics

Rachel Conyers, Sophie Young, and David M. Thomas

Sarcoma Genomics & Genetics, Peter MacCallum Cancer Centre, 12 St Andrews Place, East Melbourne, VIC 3002, Australia

Correspondence should be addressed to David M. Thomas, david.thomas@PeterMac.org

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Sarcomas are a group of heterogeneous tumours with varying genetic basis. Cytogenetic abnormalities range from distinct genomic rearrangements such as pathognomonic translocation events and common chromosomal amplification or loss, to more complex rearrangements involving multiple chromosomes. The different subtypes of liposarcoma are spread across this spectrum and constitute an interesting tumour type for molecular review. This paper will outline molecular pathogenesis of the three main subtypes of liposarcoma: well-differentiated/dedifferentiated, myxoid/round cell, and pleomorphic liposarcoma. Both the molecular basis and future avenues for therapeutic intervention will be discussed.

1. Introduction

An estimated 13,000 people were diagnosed with soft tissue and bone sarcoma in 2009 in America, of which liposarcomas constitute 20% [1, 2]. Despite their rarity these tumours have substantial morbidity and mortality, depending on histological subtype, tumour location, and volume with retroperitoneal sarcomas having particularly poor prognosis [3–9]. Liposarcomas may be classified morphologically into 3 main subtypes consisting of: well-differentiated liposarcoma/dedifferentiated liposarcoma (WD/DDLPS), myxoid/round cell liposarcoma (MLPS) and pleomorphic liposarcoma (PLPS) [10]. The morphological diversity of liposarcoma reflects the great variation in biological behaviour ranging from tumours with low metastatic potential, that is, WDLPS, to tumours with high propensity to metastasise, that is, the round cell (RC) variant of MLPS or PLPS [11]. In addition to histological characteristics, anatomical location impacts upon prognosis, given that local control is a prime concern for curative intent.

Treatment is multimodal with surgical removal and radiotherapy used as cornerstones for local control, along with chemotherapy for systemic disease. Few therapeutic options are available for aggressive local or metastatic disease. Chemotherapy sensitivity varies considerably between subtypes with higher response rates in MLPS compared with WD/DDLPS (48% versus 11%) [12]. MLPS tumours are also highly radiosensitive [13, 14]. Given the small subgroup that is chemo-sensitive, and the overriding lack of chemo-curative disease there are avenues and a need for novel molecular therapies.

A recent histological and molecular review of 163 liposarcoma and lipomas at the Netherlands Cancer Institute resulted in 23% of tumours being reclassified based on cytogenetic information. This highlights the importance of molecular classification in these tumours and genetic alterations now considered an integral part of the WHO classification [15]. It is hoped that further insight into the molecular characteristics of liposarcomas will allow for accurate subclassification, whilst providing a platform for molecular therapies to be included in the current treatment approach. This paper will outline the current molecular basis of liposarcoma and potential strategies for therapeutic intervention.

2. Well- and De-differentiated Liposarcoma

WDLPS represents 40%–45% of all diagnosis of liposarcoma [16]. It is classified as a low-grade neoplasm; it is rarely metastatic and has a low recurrence rate (10%) occurring most often in the retroperitoneum and limbs. The World

Health Organization (WHO) classifies WDLPS into three main subtypes: adipocytic, sclerosing, and inflammatory. Adipocytic (lipoma-like) liposarcoma is composed of mature adipocytes, which exhibit variation in cell size and focal nuclear atypia and hyperchromasia [16]. The sclerosing subtype shows scattered distinctive bizarre stromal cells associated with rare multivacuolated lipoblasts set in a fibrillary collagenous background [16]. Finally, the inflammatory subtype shows polyphenotypic lymphoplasmacytic infiltrate, with a B-cell predominance. Less is known about this rare subtype [16–18].

DDLPS represents progression from low grade to high-grade nonlipogenic morphology within a WDLPS. DDLPS is more aggressive and exhibits an increased rapidity of disease in contrast to WDLPS, with a metastatic rate of 10%–20% and overall mortality of 50%–75% [4, 7, 19]. In respect to tumour site, retroperitoneal tumours appear to have a worse prognosis [19]. Histologically, DDLPS consists of a WDPLS with a nonlipogenic component, either high-grade, most often resembling malignant fibrous histiocytoma (MFH), or low-grade resembling fibromatosis or low-grade myxofibrosarcoma. The presence of transition from WDLPS to DDLPS is used to differentiate between DDLPS and these other lesions [4, 7, 11, 19–21].

2.1. Molecular Genetics. A characteristic feature of WD/DDLPS is the presence of supernumerary ring and/or giant rod chromosomes [22]. These chromosomes contain amplified segments from the 12q13–15 region that can be identified with fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) [23]. Intensive research has identified several oncogenes residing in this region including *MDM2, CDK4, HMGA2, TSPAN31, OS1, OS9, CHOP* and *GLI1* [11, 23–25]. The most compelling evidence to date demonstrates an oncogenic role in WD/DDLPS for *MDM2, CDK4, HMGA2* and *TSPAN31*. Additional amplification events may also play a role in liposarcoma genesis, for example, c-Jun in the de-differentiation process [26].

MDM2 amplification is a key feature of WD/DDLPS and is amplified and overexpressed in a number of other cancers, highlighting its importance in tumorigenesis (as reviewed [27]). *MDM2* encodes a negative regulator of the tumour suppressor, p53. MDM2 binds to the transcription activation domain of p53, within an N-terminal hydrophobic pocket [28], blocking p53-dependent transcription [29–33] and recruitment of transcription coactivators [28]. MDM2 also acts as a ubiquitin ligase targeting p53 for proteasomal degradation through both cytoplasmic and nuclear proteasomes [34–36]. MDM2 is involved in its own autodegradation to prevent MDM2 activity inhibiting p53 during times of cellular stress [37]. Thus MDM2 maintains tight control on cellular p53 levels through multiple mechanisms (see Figure 1) [38, 39]. Therapeutically this is important, as MDM2 inhibitors aim to reactivate p53 and thus allow it to actively induce cell death in response to appropriate stressors [40]. In addition to a functional downstream p53 signalling pathway, MDM2 amplification is a predictor of sensitivity to current MDM2 antagonists [40]. Amplification of *MDM2* and mutation of *p53* appear to be mutually exclusive events in WDLPS, but have been reported in DDLPS [41, 42]. *p53* mutations have been associated with the de-differentiation process from WDLPS to DDLPS [41]. Pilotti et al. reported upon a subgroup of WD/DDLPS tumours. Retroperitoneal WD/DDLPS demonstrate mutual exclusivity between *MDM2* amplification and *p53* mutation. In non-retroperitoneal DDLPS, *p53* mutations occur in the absence of *MDM2* amplification suggesting involvement in the de-differentiation process [41].

MDM2 is the most frequent amplification in WD/DDLPS (close to 100%) however *CDK4* is shown to be amplified in over 90% of cases [16, 43, 44]. Given its role in the cell cycle and the frequency of amplification, CDK4 has been well researched in WD/DDLPS. The *CDK4* gene encodes a 33-kD protein that forms complexes with the cyclin D family, to enable G1-S transition [45]. These CDK4/Cyclin D complexes phosphorylate pRb (encoded by *RB1*), with resultant activation of E2F target genes including E-type cyclins (see Figure 2) [46–48]. It has been suggested that CDK4 provides a selection advantage in WD/DDLPS and may contribute to transformation as CDK4 negative WDLPS exhibit more favorable prognostic features [46]. Coamplification of MDM2 and CDK4 is a common feature of WD/DDLPS and may result in proliferation through combined effects upon p53 and the cell cycle [49, 50]. Interestingly, the rearrangements of chromosome 12 on the giant rod chromosome are discontinuous and *MDM2* and *CDK4* may belong to different amplicons [51, 52]. Several studies [43, 53, 54] have suggested that immunohistochemical staining for both CDK4 and MDM2 may provide a useful diagnostic marker, although FISH and quantitative polymerase chain reaction (qPCR) are more effective. Although MDM2 and CDK4 are useful markers to aid in diagnosis, overexpression of these markers is not unique to WD/DDLPS [43, 54]. Further, the amplification and over-expression of *CDK4* and *MDM2* does not distinguish WDLPS from DDLPS [16, 23, 41].

HMGA2 is similarly located on 12q and frequently amplified in WD/DDLPS. This is a member of the high-mobility group of proteins [55, 56]. Previously referred to as *HMGIC*, it encodes an architectural transcription factor capable of remodeling DNA [57–59]. A direct role for HMGA2 in cellular transformation is demonstrated by NIH3T3 neoplastic transformation with the overexpression of HMGA2 [60]. In human sarcomas during chromosomal rearrangement, *HMGA2* is fused to distant sequences, commonly occurring on other chromosomes and loses its 3′ translated end that also contains sites for *Let-7* microRNAS [57]. Further support for HMGA2 involvement in adipogenic neoplasm development includes the xenograft model by Arlotta et al. [55] that showed mice expressing C-terminal truncated HMGA2 developed lipomas. Interestingly HMGA2 is frequently coamplified with MDM2 in human malignant tumours [57, 61], particularly WDLPS and DDLPS [52]. This raises the possibility that HMGA2 and MDM2 have a cooperating role in WD/DDLPS. Also included within the chromosome 12 q13–15 region is the transmembrane

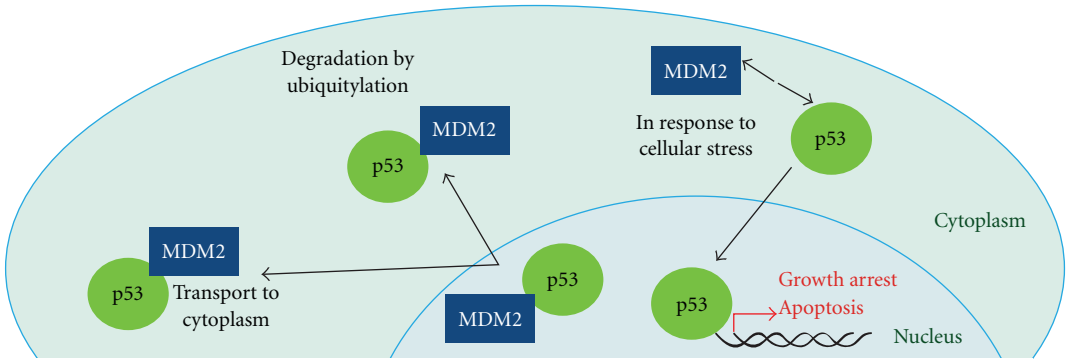

Figure 1: MDM2 binds to the transcriptional activation domain of p53, blocking transcription. MDM2 functions as a ubiquitin ligase, facilitating proteasomal degradation of p53. MDM2 releases p53 in response to cellular stress and p53 translocates to the nucleus where it acts as a transcription factor to enable growth arrest and apoptosis.

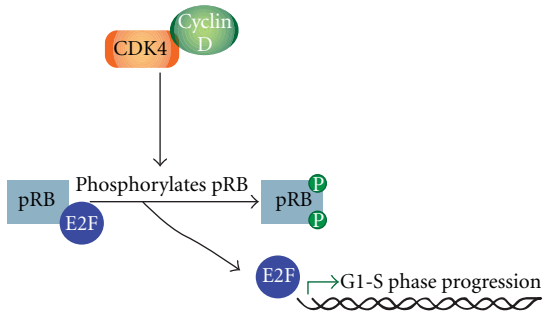

Figure 2: Cyclin dependent kinase CDK4 binds with cyclin D to form active complexes. This results in phosphorylation of Rb and dissociates pRb from the pRb-E2F complex. E2F binds DNA to upregulate transcription of genes required to progress to S phase.

superfamily gene, sarcoma amplified sequence (*SAS* or *TSPAN31*) gene [62, 63]. *TSPAN31* was originally identified and cloned from an amplified sequence in a malignant fibrous histiocytoma [63]. It has been identified in other subtypes of sarcoma, particularly de-differentiated liposarcoma [64, 65], although its precise role in the de-differentiation process is not well delineated. Forus et al. [66] showed *TSPAN31* was as frequently amplified as *MDM2* in 98 sarcomas. Both TSPAN31 and MDM2 were amplified in 8 of 11 liposarcoma samples, with MDM2 amplified alone in one additional tumour. WDLPS and DDLPS have shown co-amplification of 1q21-q22 and/or 12q21-q22 [11, 16, 23], along with amplification of chromosome 1(1q21-q23). Chromosome 1 amplified sequences include *COAS1*, *COAS2* and *COAS3* [67]. Nilsson et al. showed co-amplification of both *COAS* and *MDM2* in 12/18 lipomatous tumours [68]. The biological function of the *COAS* genes remains a subject for study.

Recent studies into the WDLPS de-differentiation process have suggested a role for the c-Jun N-terminal kinase (JNK) pathway. Co-amplification of 1p32 and 6q23, that contain c-Jun, and Apoptosis Signaling Kinase 1 (ASK1), are seen in DDLPS but not WDLPS [69]. The proto-oncogene *c-Jun* encodes part of the activator protein transcription factor (AP-1) complex involved in cell proliferation, transformation and apoptosis [70]. ASK1 activates JNK [71, 72] ultimately leading to c-Jun activation and PPARγ inactivation. PPARγ is involved in the adipocytic differentiation process and its inhibition may result in de-differentiation. A further role for c-Jun in the de-differentiation process is demonstrated by overexpression in a 3T3-L1 adipocytic tumour xenograft model. Transfection of c-Jun into 3T3-L1 cells *in vitro* delays adipocytic differentiation [26].

3. Myxoid Liposarcoma

MLPS is the second most common subtype of liposarcoma and accounts for more than one third of liposarcomas and 10% of all adult soft tissue sarcomas. MLPS is characterized by the presence of spindle or ovoid cells set in a myxoid stroma with signet ring lipoblasts and a distinctive chicken-wire pattern vasculature. The presence of areas with greater cellularity, known as round cell (RC) de-differentiation, is associated with a worse prognosis [73]. Unusual sites of metastasis are common in MLPS with a propensity to metastasize to soft tissue and bone rather than lung [74, 75]. Thirty-one percent of MLPS patients develop metastasis with bone metastases constituting 56% of these [74]. MLPS exhibits inferior survival compared to other low-grade sarcoma subtypes with a 5-year disease survival rate of 85% [76, 77]. MLPS without RC is particularly radiosensitive with good local control rates with patients treated with adjuvant or neoadjuvant radiotherapy approaching 98% 5-year local control [13, 78].

3.1. Molecular Genetics. MLPS is characterised by the recurrent translocation t(12;16)(q13;p11) that results in the *FUS-CHOP* gene fusion that is present in over 95% of cases [79, 80]. In most cases, the amino terminal domain of *FUS* (also known as *TLS*) is fused to C/EBP homologous protein (*CHOP*, also known as *DDIT3* or *GADD153*). In rare cases, an alternative translocation event is found t(12;22)(q13;q12) that results in formation of the novel fusion oncogene where *EWS* takes the place of *FUS* [81, 82]. There is strong evidence for these translocations to be the primary oncogenic event in

MLPS as these tumours have a relatively normal karyotype, the exception being a few recurrent cases of trisomy 8 [83]. In addition, several growth factor pathways have been implicated in MLPS pathogenesis [84–86].

There are currently 11 different *FUS-CHOP* chimeras and 4 different known *EWS-CHOP* fusion genes. In the most common variants, a portion of the amino terminus of *FUS* is fused to the entire coding region of *CHOP*. The *FUS-CHOP* transcript type does not appear to have a significant impact upon clinical outcome, and RC content, necrosis and p53 expression remain stronger predictors of clinical outcome [79, 87]. There is evidence that the fusion transcript type may influence response to therapy although the studies are hindered by sample size [88–90]. Understanding how the FUS-CHOP fusion causes MLPS and uncovering any further molecular abnormalities in the disease will aid in development of novel targeted therapies.

FUS belongs to the FET family of RNA-binding proteins that consists of FUS, EWS, and TAF15 as well as the closely homologous, Drosophila SARFH (Cab) [80, 91, 92]. These structurally and functionally related RNA-binding proteins are composed of an SYGQ-rich amino terminus, an RNA recognition motif, a zinc finger motif, and at least one RGG rich repeat region [93, 94]. FET proteins are expressed in most human tissues and appear to be regulated following differentiation in neuroblastoma cells and spontaneously differentiating human embryonic stem cells [95].

Both FUS and EWS have been shown to localize to the nucleus and the cytoplasm, bind RNA, and are also involved in nucleo-cytoplasmic shuttling [96–98]. The FET family associate with various complexes involved in the induction of transcription, including RNA polymerase II (RNAPII), which regulates transcription and TFIID complexes, that binds DNA as part of the transcriptional machinery [91], implicating both FUS and EWS in transcriptional control. In addition, FUS has recently been shown to repress transcription of RNA polymerase III (RNAPIII), suggesting a broader role in regulation through multiple different mechanisms [99]. Noncoding RNAs are capable of allosterically modifying FUS in response to DNA damage to inhibit the transcription factor CREB-binding protein (CBP) and p300 histone acetyltransferase activity, resulting in transcriptional inhibition at the cyclin D1 promoter in cell lines and shows a further role for FUS in transcriptional control [100]. FUS has also been implicated in the DNA damage response as a downstream target of ATM, which can detect and coordinate DNA repair [101].

CHOP is induced in response to endoplasmic reticular stress and is involved in mediating cell death in response to such stress stimuli [102]. CHOP also plays a role in regulating differentiation in adipocytes by interfering with the process in response to metabolic stress [103]. Adipocytic differentiation is dependent on the coordinated expression of a group of transcription factors, the CCAAT/enhancer-binding protein (C/EBP) family of proteins [104]. The C/EBP family consists of six members from C/EBPα to ζ, and they require dimerisation to bind DNA and can form homodimers or heterodimers. CHOP is capable of binding to the C/EBP family members through their highly conserved leucine zipper domain and inhibiting their function. The leucine zipper dimerization domain and the adjacent basic region in CHOP are required for NIH-3T3 transformation with FUS-CHOP, highlighting the requirement for functional DNA binding and dimerization for FUS-CHOP induced oncogenesis [105].

As C/EBPα and C/EBPβ play an important role in the adipogenic differentiation and are regulated by CHOP, it is possible FUS-CHOP may interfere in cellular differentiation. In support, various studies suggest that FUS-CHOP functions by inhibiting adipogenesis and maintaining immature adipocytes in a continuous cycle of proliferation without differentiation [106–108]. Introduction of FUS-CHOP into mice, where expression of the transgene is driven by the ubiquitously expressed elongation factor 1α (EF1α) promoter, results specifically in liposarcomas with inherent induction of adipocyte specific genes such as PPARγ [109]. Further evidence of adipogenic differentiation block resulting from FUS-CHOP expression was shown *in vitro* where mice expressing FUS-CHOP under the control of the aP2 promoter, which is a downstream target of PPARγ expressed in immature adipocytes, failed to develop liposarcomas, indicating interference between PPARγ and aP2 activation [107].

An emerging clinically relevant targetable pathway in MLPS involves the receptor tyrosine kinases (RTKs) MET, RET, and the PI3K signaling cascade (see Figure 3). RET is overexpressed in MLPS compared to normal fat [84] and high expression has been correlated with poor metastasis free survival in MLPS [108]. RET, IGF1R and IGF2 are highly expressed in MLPS and promote cell survival through both the PI3K/Akt and Ras-Raf-ERK/MAPK pathways [85, 86]. A panel of tyrosine kinases including PDGFRB, EGFR, MET, RET, and VEGFR2 are activated in both treated (with chemotherapy/radiotherapy or Trabectedin) and untreated cases of human MLPS [110]. In addition to activation of MET in clinical MLPS specimens, MET and the ligand HGF are potentially regulated by FUS-CHOP. Both MET and HGF are highly expressed in mesenchymal progenitor cells transfected with FUS-CHOP in a disease mimicking allograft mouse model [111]. In a small clinical cohort, specific Akt phosphorylation was observed in the RC variant and 2 treated cases that harboured *PTEN* mutations, implicating RTK pathways signaling through Akt in MLPS [110]. *FLT1* (that encodes the VEGFR1 protein) is expressed as an indirect downstream effect of FUS-CHOP expression in both FUS-CHOP transfected HT1080 (fibrosarcoma) and MLPS cell lines however, VEGFR tyrosine kinase inhibitors did not have a notable impact on proliferation in MLPS cell lines indicating a separate role in these cells [112, 113].

Akt activation, particularly in the RC variant, suggests a role for phosphoinositide 3-kinases (PI3K) [110]. PI3Ks are activated upon phosphorylation of membrane bound receptor tyrosine kinases. PI3K can activate many proteins including the protein serine-threonine kinase Akt, which when phosphorylated causes downstream activation and ultimately cell growth, cell cycle entry, and subsequently survival. The PI3K holoenzyme complex is composed of both a catalytic and regulatory subunit. The catalytic subunit,

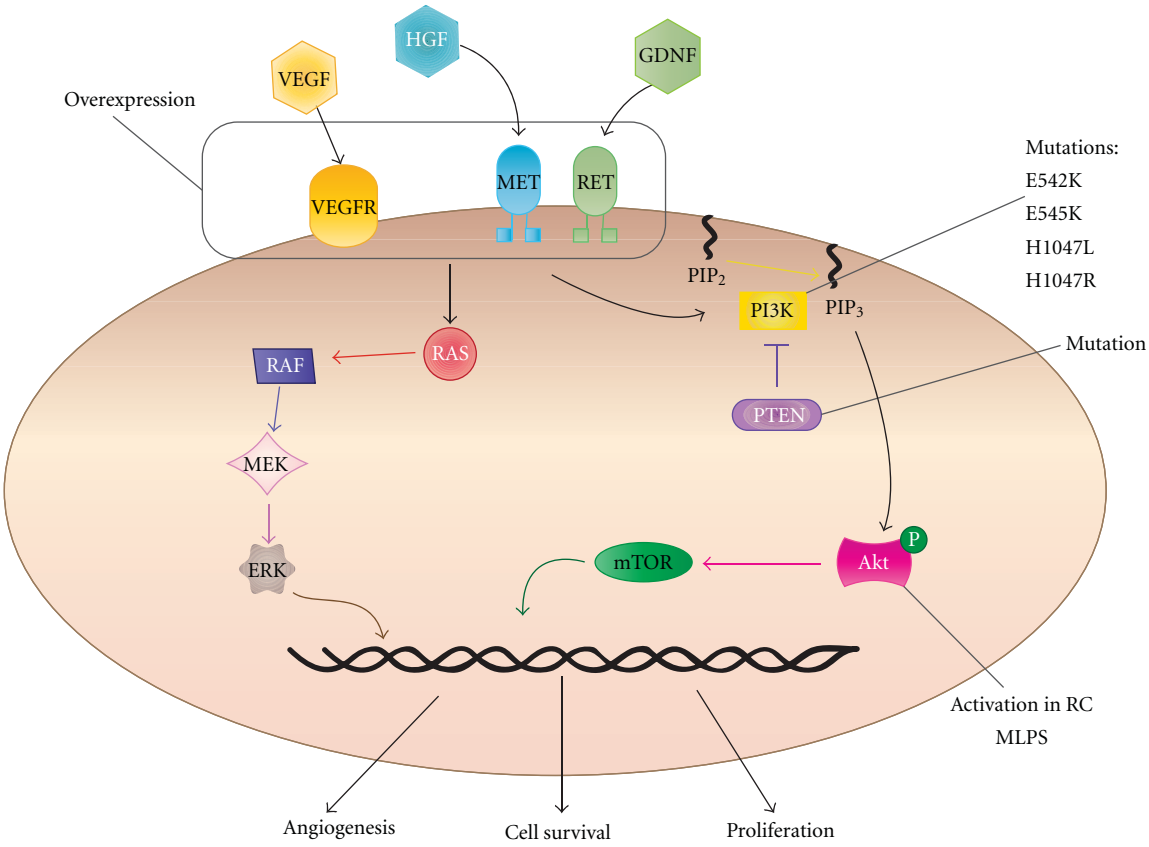

Figure 3: The PI3K pathway is highly active in MLPS, and this is potentiated at least in part by overexpression, and/or activation through RTKs such as MET, RET and VEGFRs. Upon ligand binding, RTKs activate downstream activation of genes involved in multiple cell processes such as cell survival, proliferation, and angiogenesis. These signals are mediated through the PI3K/Akt pathway and also through RAS. PIK3CA and PTEN mutations and Akt activation have also been documented in MLPS.

PIK3CA, encodes the p110α isoform and is commonly mutated in various cancer types including breast, colon, brain and gastric malignancies [114, 115]. A recent study showed 18% of MLPS patients ($n = 71$) had *PIK3CA* mutations in either the helical (E542K and E545K) or kinase (H1047L and H1047R) domain. The presence of a *PIK3CA* mutation was associated with a shortened disease specific survival [116]. Barretina et al. also showed one tumour with a homozygous *PTEN* mutation. PTEN is a tumour suppressor that dephosphorylates phosphoinositide substrates to negatively regulate the Akt signaling pathway [117], demonstrating more mechanisms for perturbation of the pathway.

4. Pleomorphic Liposarcoma

PLPS accounts for only 5% of liposarcomas and occurs mainly within the 55–65 year-old group [8, 118, 119]. PLPS mortality is 40% with no current clinical or pathological predictors of outcome [8, 120]. Histologically PLPS are similar to MFH with the addition of lipoblasts. Histology reveals a disorderly growth pattern, extreme cellularity, and cellular pleomorphism including bizarre giant cells [121]. Lesional cells are polygonal with pale eosinophilic cytoplasm and poorly demarcated boundaries. These lesional cells are interspersed with giant lipoblasts containing enlarged hyperchromatic, angular or globular nuclei [121, 122].

4.1. Molecular Genetics. Molecular studies of PLPS are limited by the scarcity of this disease. Tumours tend to show complex arrangements including gains: 1p, 1q21-q32,2q, 3p, 3q, 5p12-p15, 5q, 6p21, 7p, 7q22 (see reviews) [118, 123, 124]. reported literature shows losses i of 1q, 2q, 3p, 4q, 10q, 11q, 12p13, 13q14, 13q21-qter, 13q23-24, (see reviews) [123–125], Taylor et al. described that 60% of PLPS have a deletion of 13q14.2-q14.3, a region that includes the tumour suppressor *RB1* [123]. Also amplified in PLPS, the mitotic arrest deficient (*MAD2*) may also play a critical role [126, 127]. As reported by Singer et al. [126], MAD2 was found to be over-expressed 13 fold in comparison to normal fat, although small sample size ($n = 6$) must be appreciated. As reported by Taylor et al. [123] additional deletions in PLPS include 17p13 and 17q11.2, where *p53* and the sarcoma associated tumour suppressor gene, neurofibromatosis type 1 (*NF-1*) are located. Consistent with these observations, Barretina et al. [116] showed 16.7% of PLPS cases had mutations identified in *p53*, which are rarely seen in MLPS and WD/DDLPS.

5. Therapeutic Implications in Liposarcoma

The current modalities available (chemotherapy, surgery and radiotherapy) for the treatment of liposarcoma are limited, creating a need to identify novel therapeutics.

5.1. MDM2 Antagonists. Given MDM2 is consistently amplified in WD/DDLPS, and sensitivity to MDM2 antagonists (such as Nutlin-3a) is predicted by MDM2 amplification and an intact wild-type *p53,* it is an appealing therapeutic target [40]. First generation MDM2 inhibitors work via blocking the p53/MDM2 interaction. Nutlin-3a was heralded as one of the most promising MDM2 antagonists when it was shown to activate wild type p53 and induce cell cycle arrest and apoptosis in cancer cell lines [40]. These cell lines included osteosarcoma with amplified MDM2 [40, 128]. Nutlins require wild-type p53 and a functional downstream p53 pathway to be effective [128]. Müller et al. [40] showed downstream p53 dependent transcription and apoptosis in liposarcoma cell lines treated with Nutlin-3a [40].

Translation from *in vitro* to attractive *in vivo* therapeutic intervention requires that drugs pass Phase I requirements. Shangary et al. [129] designed spiro-oxindoles as a new class of inhibitors of the MDM2-p53 complex. Spiro-oxindoles bind to MDM2 with high affinity and activates the p53 pathway, inhibiting the growth of neoplastic cell lines with wild-type p53 [129, 130]. MI-219, the lead compound in this class, demonstrates greater potency along with a superior pharmacokinetic profile than Nutlin-3a [129, 131]. MI-219 has been shown to stimulate rapid p53 activation in tumour xenograft tissues with resultant inhibition of cell proliferation [131]. Studies using both Nutlin-3a and MI-219 show a p53 and p21 dependent cell cycle arrest in normal cells, along with p53 dependent cell death specifically in tumour cells [128, 129, 131, 132]. The ability of Nutlin-3a to induce apoptosis in tumours is variable, and osteosarcoma cell lines lacking MDM2 amplification are resistant to apoptosis [131]. Importantly, Nutlin-3a and MI-219 do not cause visible toxicity to animals, as assessed at necropsy [128, 129, 133].

Two oral MDM2 inhibitors have recently entered the clinical setting [134], JNJ-26854165 (Ortho Biotech; Johnson & Johnson) [135] and R7112 (Hoffmann-La Roche) [136]. Both agents are available in advanced stage or refractory solid tumours Phase I trials [134]. In addition, AT-219 (a derivative of MI-219) is in preclinical studies with phase I trials planned [134]. Of relevant interest, an MDM2 antagonist RO5045337 is about to recruit for a Phase I trial in liposarcoma patients [137].

5.2. CDK4 Antagonists. Targeting CDK4 is an attractive therapeutic strategy given its frequent overexpression in WD/DDLPS [138]. A number of CDK4 inhibitors are in the early pre-clinical development or Phase I and II trials [139]. First generation pan-CDK inhibitors include Flavopiridol and Seleciclib (R-Roscovitine), inhibiting CDK1, CDK2, CDK4, CDK6, CDK7, and CDK1, CDK2, CDK7 and CDK9 respectively [140]. Flavopiridol causes arrest in G1 and G2 phases in a range of solid tumour cell lines [139, 141, 142]. Flavopiridol is more potent if tumour cells are in S phase. Matranga and Shapiro [143] demonstrated recruitment to S phase using hydroxyurea, gemcitabine and cisplatin, followed by flavopiridol resulting in sequence-dependent cytotoxic synergy [143–145]. Flavopiridol and Seliciclib have been investigated in Phase I/II trials for haematological and solid tumours including sarcomas. Trials include Flavopiridol as a single agent and in combination with taxanes where synergism has been noted [141]. Both Flavopiridol and Seleciclib have shown disappointing results relating to clinical outcome and intolerable side effects [146, 147].

Newer generation CDK inhibitors include PD0332991, P27600, ZK 304709, R 547 and P1446A05. All are available in Phase I and II solid tumour trials [146]. PD0332991 is one of two more selective CDK inhibitors specific for CDK4 and CDK6. Preclinical data showed inhibition of cell growth through G1 arrest in pRb-positive tumour cell lines and antitumorigenic effects in xenograft models of colon carcinoma [148]. PD0332991 is available in Phase I and Phase II trials for solid and haematological malignancy. Finally, P1446A05 is the only single CDK4 selective inhibitor available [146]. No pre-clinical data is publicly available for this compound; however, it has been released as a Phase I drug for refractory solid tumour and haematological malignancies [146].

5.3. PPARγ Ligand Agonists. A critical regulator of terminal differentiation for the adipocytic lineage is a nuclear receptor peroxisome proliferator-activated receptor γ [149–151]. PPARγ is an attractive target in undifferentiated lipomatous tumours such as DDLPS and MLPS. PPARγ forms a heterodimeric complex with the retinoid X receptor (RXR). This complex regulates transcription of adipocyte-specific genes by binding sites on DNA. Agonist ligands for the PPARγ receptor have been shown to induce terminal differentiation of normal preadipocytes in human liposarcoma cells *in vitro* [149].

A Dana-Farber Cancer Institute Phase II clinical trial used Troglitazone, a synthetic PPARγ ligand, in patients with high-grade liposarcoma. This trial enrolled three patients. All patients showed histologic and biochemical differentiation *in vivo*, with reduction in immunohistochemical expression of proliferation marker Ki-67 [149]. A more recent study with 12 patients with Rosiglitazone, belonging to the same class of drugs (thiazolidinediones) as Troglitazone, was not as promising, with median progression free survival of 5.5 months. Treatment did not produce any convincing adipocytic differentiation with no correlation between the high expression of differentiation genes that was found in two patients, and clinical response [152].

5.4. Trabectedin (ET-743). Trabectedin (also known as Ecteinascidin or ET-743) is an antitumor drug isolated from the Caribbean marine tunicate, Ecteinascidia turbinata [153]. Trabectedin is an approved second-line agent for advanced soft tissue sarcoma and has been shown to be exquisitely sensitive to Trabectedin in Phase II clinical trials [154, 155].

The drug is a tetrahydroisoquinoline alkaloid whose main mechanism of action is through binding to the DNA minor groove with promoter and sequence specificity; however, it has also been shown to have effects on promoters that are regulated by major groove binding transcription factors [156–158]. Trabectedin does not appear to effect transcription of FUS-CHOP, but has been shown to dissociate the aberrant transcription factor from promoters of its target genes resulting in removal of the differentiation block by activating a differentiation cascade through the C/EBPs [88].

Trabectedin relies on intact nucleotide excision repair (NER) machinery and induces lethal DNA strand breaks in a transcription-couple NER dependant manner [159–161]. It has been suggested that these breaks are repaired by homologous recombination (HR), as HR-deficient cells, such as *BRCA2* mutants, are 100 fold more sensitive to Trabectedin [162]. This effect is specific to HR- mediated double strand break repair as defects in the alternative pathway using nonhomologous end joining do not result in the same degree of Trabectedin sensitivity [161, 162].

FUS-CHOP modulates immune genes by activating NF-κB controlled cytokines IL-6 and IL-8 in a C/EBPβ-dependent manner [163, 164]. Proinflammatory cytokines and growth factors such as CCL2, CXCL8, IL-6, VEGF and PTX3 are highly expressed in both xenograft MLPS models and patient tumours. Trabectedin has been shown to reduce expression and production of these immune modulators, potentially altering the tumour microenvironment in a favorable way [165]. Thus, Trabectedin appears to affect the biological activity of FUS-CHOP and so far shows promise as a therapeutic in MLPS.

5.5. Receptor Tyrosine Kinase Pathway Inhibitors. The high frequency of *PIK3CA* and *PTEN* mutations suggests a role for PI3K inhibitors in MLPS. The nonisoform-specific PI3K inhibitors Wortmannin, and LY294002 have been widely used in biological research but are not particularly suited to clinical work due to their lack of specificity, Wortmannin's instability and LY294002's low potency (as reviewed [166]). GDC-0941 and PX-866 are promising PI3K inhibitors currently in clinical trials that have low nanomolar potency against class I isoforms of PI3K [167–169]. In lung cancer cell lines and xenograft models, *PIK3CA* mutants are more sensitive to GDC-0941 [170]. Similarly, *PIK3CA* mutant and PTEN-null tumours were sensitive to PX-866 in xenograft models, and phase I clinical trials for solid tumours are currently underway [169]. The Rapamycin derivate Everolimus inhibits the mTOR complex-1 (mTORC1), which is a downstream effector of PI3K. Both H1047R and E545K PI3K mutant cells are sensitive to Everolimus [171]. PIK3CA mutated MLPS represents an ideal candidate for PI3K inhibition.

As MET is activated in MLPS and there are many MET pathway inhibitors currently in development and in clinical trials (as reviewed in [172], MLPS may be a good candidate for MET inhibition. For example, the novel and promising inhibitor Foretinib (XL880) inhibits multiple kinases including both MET and VEGFR2 and exhibits extensive biological activity and clinical efficacy in an early Phase I clinical trial in metastatic or unresectable solid tumours [173].

6. Conclusion

Molecular-based therapeutics are not routinely used in liposarcoma, where surgery, radiotherapy, and chemotherapy remain the mainstay of treatment. Translation of targeted molecular therapeutics in sarcoma has been successfully demonstrated with Imatinib mesylate therapy in c-Kit positive gastrointestinal stromal tumour (GIST) [174]. A major challenge with the use of molecularly targeted therapeutics is to translate disease control into disease eradication. One strategy to achieve this goal is to combine two or more independent molecularly targeted agents in a disease where all of the targets are relevant. The dependence of WD/DDLPS on amplification of both *MDM2* and *CDK4* means that this disease represents an important candidate for combination therapy. Recent studies point towards RTK involvement in MLPS oncogenesis, particularly signaling through the PI3K/Akt pathway. This provides an important avenue for new research due to the large number of clinical trials currently underway that target this pathway. Although not considered a molecularly targeted therapeutic, treatment of MLPS with Trabectedin is currently in late stage clinical trials with promising results.

It is hoped that emerging technologies, such as next-generation sequencing, will be fundamental in revealing new molecular targets in liposarcoma. Similarly, advances in drug development should enable improvement of molecular therapies with greater sensitivity, specificity, potency, and limited toxicity. Combining technologies in both areas will allow for efficient clinical translation.

Conflicts of Interest

No potential conflict of interest is disclosed.

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Review Article

The Role of Chemokine Receptor CXCR4 in the Biologic Behavior of Human Soft Tissue Sarcoma

Roger H. Kim, Benjamin D. L. Li, and Quyen D. Chu

Louisiana State University Health Sciences Center - Shreveport and Feist-Weiller Cancer Center, Shreveport, LA 71130-3932, USA

Correspondence should be addressed to Roger H. Kim, rkim@lsuhsc.edu

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The molecular basis of sarcoma remains poorly understood. However, recent studies have begun to uncover some of the molecular pathways involved in sarcomagenesis. The chemokine receptor CXCR4 has been implicated in sarcoma development and has been found to be a prognostic marker for poor clinical outcome. There is growing evidence that overexpression of CXCR4 plays a significant role in development of metastatic disease, especially in directing tumor cells towards the preferential sites of metastases in sarcoma, lung and bone. Although further investigation is necessary to validate these pathways, there is potential for clinical application, particularly in the use of pharmacologic inhibitors of CXCR4 as means of preventing sarcoma metastasis.

1. Introduction

Sarcomas are relatively rare tumors of mesenchymal origin, accounting for less than 1% of malignancies [1, 2]. The American Cancer Society (ACS) estimates that there will be 10,500 new cases of soft tissue sarcoma in 2010 [1]. An estimated 3,920 patients will die in 2010 from sarcoma in the US [1]. In addition to their rarity, sarcomas are a heterogeneous group of malignancies, with over 50 different histologic subtypes with highly variable microscopic appearance and clinical behavior [3]. The combination of rarity and diversity has made scientific investigation into the molecular basis of sarcomas challenging [2]. Indeed, even the cell of origin in sarcomas remains unidentified and a subject of controversy [4]. However, recent studies have started to uncover some of the molecular markers and pathways that contribute to human sarcomagenesis [4]. Among these recent discoveries is the role that the chemokine receptor CXCR4 plays in the pathogenesis of several subtypes of sarcoma. In this paper, we will review the literature on the function of CXCR4 in human sarcomagenesis.

2. Chemokine Receptor 4 (CXCR4)

Chemokines are 8 to 12 kDa peptides that function in cell differentiation, migration, and trafficking by acting as chemoattractant cytokines [5]. There are four groups of chemokine receptors: C, CC, CXC, and CX3C. Chemokine receptor 4 (CXCR4) is a seven-transmembrane G protein-coupled chemokine receptor [6]. CXCR4 is normally expressed on T-lymphocytes, B-lymphocytes, monocytes, macrophages, neutrophils, eosinophils, in addition to being present in brain, lung, colon, heart, kidney, and liver cells [5]. CXCR4 is also expressed on astrocytes, neuronal cells, and smooth muscle progenitors [5]. CXCR4 is also the chemokine receptor most commonly expressed in tumor cells, with increased expression in melanoma, breast, ovarian, gastric, prostate, colorectal, and lung cancer [7–10]. High levels of CXCR4 have been shown to correlate with the presence of metastatic disease in a wide variety of malignancies, including breast, prostate, lung, colorectal cancer, melanoma, and neuroblastoma [8, 10–16]. CXCR4 has also been demonstrated to be involved in cell migration and invasion, as well as angiogenesis.

The activation of CXCR4 by its ligand, CXCL12, initiates multiple intracellular signaling cascades [5]. CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a homeostatic chemokine. CXCL12's major function is in regulating hematopoietic cell trafficking and secondary lymphoid tissue architecture. In malignancy, high expression of CXCL12 has been found in lung and bone, tissues that are preferential sites for certain malignancies, such as breast cancer.

3. Osteosarcoma

Osteosarcoma, also known as osteogenic sarcoma, is the most common primary bone malignancy [4]. CXCR4 is expressed in 67% of osteosarcomas, with high levels of expression correlating with decreased overall survival, event-free survival, and metastasis-free survival [17]. Survival is only 10% in tumor samples that express CXCR4 mRNA, compared to 90% survival in tumor samples that do not express CXCR4 mRNA. CXCR4 expression level also correlates with the presence of metastasis at diagnosis [17]. Human osteosarcoma cell lines also have been found to express high levels of CXCL12 [17].

Osteosarcoma preferentially metastasizes to lung and bone, tissues with high levels of CXCL12 [10]. Osteosarcoma cells expressing CXCR4 migrate towards a CXCL12 gradient [18]. Adhesion of osteosarcoma to endothelial and bone marrow stromal cells is also promoted by CXCL12. In addition, there is a significant correlation in osteosarcoma between CXCR4 and expression of vascular endothelial growth factor (VEGF), a critical mediator of angiogenesis and tumor proliferation [19].

The role of CXCR4 in osteosarcoma metastasis has been further validated in animal models. The T134 peptide, a CXCR4 inhibitor, was found to prevent the development of lung metastasis after the injection of osteosarcoma cells in a mouse model [18]. In another study, administration of CTCE-9908, also a CXCR4 inhibitor, resulted in a 50% decrease in the number of metastases in mice injected with osteosarcoma cells [20].

4. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue malignancy in children [21]. CXCR4 is highly expressed on the surface of RMS cells, with higher expression in the more clinically aggressive alveolar subtype of RMS compared to the embryonal subtype [21, 22]. High CXCR4 expression also correlates with unfavorable primary sites, advanced stage, marrow involvement, decreased overall survival, and event-free survival in RMS [23].

CXCL12 has no effect on the proliferation or survival of RMS cells, but does stimulate processes related to cell invasion and metastasis [22]. CXCL12 increases adhesion of RMS cells to endothelium. RMS cells also follow a directional chemotaxis towards bone marrow stroma, a CXCL12-rich environment, which may indicate a role of CXCR4 in tendency of RMS to preferentially metastasize to bone marrow [21, 22]. However, CXCL12 did not increase the survival of RMS cells exposed to radiation or chemotherapy, indicating that CXCR4 may not play a role in the development of treatment resistance [24].

5. Chondrosarcoma

Chondrosarcoma is the second most common primary bone malignancy, after osteosarcoma [25]. CXCR4 and CXCL12 expressions have been found to be increased in both chondrosarcoma tissue and cell lines [26], with the expression of CXCR4 correlating with tumor grade. CXCR4 signaling regulates the expression of matrix metalloproteinase 1 (MMP1), a marker of chondrosarcoma tissue invasion, metastasis, and poor prognosis [25]. In addition, CXCR4 signaling appears to partially mediate hypoxia-induced increases in MMP1 expression [25].

6. Ewing's Sarcoma

Ewing's sarcomas are poorly differentiated tumors and have high metastatic potential [4, 27]. A subset of Ewing's sarcoma tumors and cell lines predominately express CXCR4. High expression of CXCR4 correlates with metastatic Ewing's sarcoma and with poor patient survival [27]. Also, CXCL12 has been demonstrated to be a potent stimulator of invasion by Ewing's sarcoma cells [28].

7. Malignant Fibrous Histiocytoma

Malignant fibrous histiocytoma (MFH), also termed as high-grade undifferentiated pleomorphic sarcoma, is one of the highest-grade soft tissue sarcomas [4]. CXCR4 expression has been shown to be upregulated in MFH tumor cell lines [29].

8. Other Soft Tissue Sarcomas

In a heterogeneous group of malignant nonround cell tumors, which included synovial sarcoma, malignant peripheral nerve sheath tumor, leiomyosarcoma, MFH, liposarcoma, fibrosarcoma, angiosarcoma, clear cell sarcoma, epithelioid sarcoma, osteosarcoma, and chondrosarcoma, high CXCR4 mRNA expression was an independent predictor of poor prognosis by univariate and Cox multivariate analysis [30]. There was also a significant correlation between CXCR4 and expression of VEGF in this group of soft tissue sarcomas [30].

9. CXCR4 Inhibition

Because of the wealth of evidence implicating CXCR4's role in metastatic disease for a variety of malignancies, CXCR4 inhibition has been investigated for its potential for clinical application in cancer therapy. Plerixafor (AMD3100) was initially discovered as an anti-HIV agent and later found to be a potent selective inhibitor of CXCR4 [31]. Recently, it has been utilized in multiple myeloma and non-Hodgkin's lymphoma as a hematopoietic stem cell mobilizer [32].

The use of plerixafor as a chemotherapeutic agent has been suggested for nonhematologic malignancies, including glioblastoma, various gastrointestinal cancers, melanoma, and lung cancer [14, 20, 33, 34].

In regards to CXCR4 inhibition for sarcoma, as mentioned previously, CXCR4 inhibitors have been validated in two animal models of osteosarcoma metastasis. The CXCR4 inhibitors T134 peptide and CTCE-9908 have both been shown to decrease or prevent the development the osteosarcoma metastases in mice [18, 20]. Also, inhibition of CXCR4 by plerixafor resulted in decreased directional cell migration of a rhabdomyosarcoma cell line [21].

10. Summary

CXCR4 appears to be a useful prognostic marker for multiple histologic subtypes of soft tissue sarcoma. High expression levels of CXCR4 are correlated with poor outcomes and also predict metastatic disease. There is evidence that CXCR4 and its ligand, CXCL12, play a critical role in the preferential targeting of sarcoma metastases towards lung and bone. Finally, in vivo data indicate the potential of CXCR4 as a target for chemotherapy agents and the possible use of CXCR4 inhibitors in preventing the development of metastasis from sarcomas. Further studies will be necessary to translate this potential into application in the form of clinical trials.

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