Universidade de Aveiro Secção Autónoma das Ciências da Saúde
2015

JOANA DE MATOS RODRIGUES

FROM GENES TO RADIORESISTANCE IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

DOS GENES À RADIORRESISTÊNCIA NO CANCRO DA CABEÇA E PESCOÇO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Isabel Marques Carreira, Professora Associada com Agregação da Faculdade de Medicina da Universidade de Coimbra e co-orientação da Professora Doutora Ana Gabriela Henriques, Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro.

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."

William Bragg

O júri

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Palavras-chave

Cancro da Cabeça e Pescoço; Radioterapia; Radioresistência; Variação do Número de Cópias; Metilação do DNA; MS-MLPA; aCGH; Citogenética convencional

Resumo

Cancro da Cabeça e Pescoço refere-se a um grupo de tumores que aparecem no trato aerodigestivo superior, sendo que o carcinoma das células escamosas da cabeça e pescoço (CCECP) corresponde a mais de 90% de todos os casos de cancro nesta região. Foi considerado o sexto tumor mais maligno em todo o mundo e, apesar de todos os avanços tecnológicos e clínicos, a taxa de sobrevivência a cinco anos não melhorou significativamente nas últimas décadas. Atualmente sabe-se que o CCECP é uma doença bastante heterogénea que se desenvolve devido à acumulação de alterações genéticas e epigenéticas. Alguns dos grandes problemas associados a este tipo de cancro são o diagnóstico em fase tardia da doença e os poucos resultados terapêuticos. Uma das escolhas terapêuticas para o CCECP é a radioterapia, no entanto, esta tem diversos inconvenientes, como a radioresistência adquirida por algumas células tumorais, que se associam a piores prognósticos. Um aumento do conhecimento na área da biologia da radiação é necessário para melhorar esta opção terapêutica, evitando futuros efeitos tóxicos e fornecendo uma melhor qualidade de vida nos anos subsequentes ao tratamento. Desta forma, a identificação de marcadores moleculares associados quer a uma resposta à radioterapia, quer a possíveis alterações celulares após tratamento com radiação, é essencial para melhorar o diagnóstico, taxa de sobrevivência e qualidade de vida destes doentes. Adicionalmente, existe uma grande falha no conhecimento em relação aos efeitos da radiação nas células, como tal, o principal objetivo deste estudo foi o de contribuir para um conhecimento mais alargado do efeito da radiação em doentes com CCECP. Para isso foram utilizadas duas linhas comerciais celulares, HSC-3 (derivada de um tumor metastático da língua) e BICR-10 (derivada de um tumor da mucosa bucal), que foram caracterizadas com recurso a aCGH, MS-MLPA e citogenética convencional. Estas linhas foram submetidas a diferentes doses de radiação e as alterações genéticas e de metilação pós tratamento foram determinadas. Estes resultados demonstraram uma grande variação de resposta à radiação para estas duas linhas celulares, permitindo a conclusão que a linha HSC-3 é mais radiossensível que a linha BICR-10. Tendo isto em mente, procedeu-se a análise da morte celular, ciclo celular e danos no DNA de forma a tentar compreender esta diferença.

A caracterização genética de ambas as linhas celulares permitiu corroborar que a linha HSC-3 era derivada de um tumor metastático e sugeriu que a linha celular BICR-10 estaria associada a um estado de displasia. Para além disto, foi possível analisar alterações genéticas e epigenéticas ocorridas após irradiação e associar determinados perfis genéticos a uma melhor ou pior resposta à radiação.

Em suma, os nossos resultados contribuiram para um conhecimento mais aprofundado dos efeitos da radiação no CCECP possibilitando, no futuro, melhores opções de tratamento e uma melhor qualidade de vida para estes doentes.

Keywords

Head and Neck Cancer; Radiotherapy; Radioresistance; Copy Number Variations; DNA Methylation; MS-MLPA; aCGH; Karyotyping

Abstract

Head and Neck Cancers (HNC) are a group of tumours located in the upper aero-digestive tract. Head and Neck Squamous Cell Carcinoma (HNSCC) represent about 90% of all HNC cases. It has been considered the sixth most malignant tumour worldwide and, despite clinical and technological advances, the five-year survival rate has not improved much in the last years. Nowadays, HNSCC is well established as a heterogeneous disease and that its development is due to accumulation of genetic events.

Apart from the majority of the patients being diagnosed in an advanced stage, HNSCC is also a disease with poor therapeutic outcome. One of the therapeutic approaches is radiotherapy. However, this approach has different drawbacks like the radioresistance acquired by some tumour cells, leading to a worse prognosis. A major knowledge in radiation biology is imperative to improve this type of treatment and avoid late toxicities, maintaining patient quality of life in the subsequent years after treatment. Then, identification of genetic markers associated to radiotherapy response in patients and possible alterations in cells after radiotherapy are essential steps towards an improved diagnosis, higher survival rate and a better life quality. Not much is known about the radiation effects on cells, so, the principal aim of this study was to contribute to a more extensive knowledge about radiation treatment in HNSCC. For this, two commercial cell lines, HSC-3 and BICR-10, were used and characterized resorting to karyotyping, aCGH and MS-MLPA. These cell lines were submitted to different doses of irradiation and the resulting genetic and methylation alterations were evaluated. Our results showed a great difference in radiation response between the two cell lines, allowing the conclusion that HSC-3 was much more radiosensitive than BICR-10. Bearing this in mind, analysis of cell death, cell cycle and DNA damages was performed to try to elucidate the motifs behind this difference.

The characterization of both cell lines allowed the confirmation that HSC-3 was derived from a metastatic tumour and the hypothesis that BICR-10 was derived from a dysplasia. Furthermore, this pilot study enabled the suggestion of some genetic and epigenetic alterations that cells suffer after radiation treatment. Additionally, it also allowed the association of some genetic characteristics that could be related to the differences in radiation response observable in this two cell lines.

Taken together all of our results contribute to a better understanding of radiation effects on HNSCC allowing one further step towards the prediction of patients' outcome, better choice of treatment approaches and ultimately a better quality of life.

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ABBREVIATIONS

aCGH	Array Comparative Genomic Hybridization
AKT	Protein kinase B
APBA1	Amyloid beta precursor protein-binding
APC	Adenomatous polyposis coli
ATM	ATM serine/threonine kinase
ATR	ATR serine/threonine kinase
AV	Annexin-V
B4GALT5	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5
BER	Excision repair
BLC6	B-cell Lymphoma 6
bp	base pair
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BRCA3	Breast cancer 3
BRIP1	BRCA1 Interacting Protein C-terminal Helicase 1
C	centromere
CADM1	Cell Adhesion Molecule 1
cAMP	Cyclic adenosine monophosphate
CASP1	Caspase 1
CASP8	Caspase 8
CASR	Calcium-sensing receptor
CCNA1	Cyclin A1
CCND1	Cyclin D1
CD44	CD44 molecule (Indian blood group)
CDH1	E-cadherin
CDH13	Cadherin 13
CDK	Cyclin-dependent kinase
CDK6	Cyclin-dependent kinase 6
CDKN1B1	Cyclin-dependent kinase inhibitor 1B
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2A^{ARF}	Alternative open reading frame (ARF) of INK4a locus
CDKN2B	Cyclin-dependent kinase inhibitor 2B

CFTR	Chloride channels, ATP-gated CFTR
CHEK1	Checkpoint kinase 1
CHFR	Checkpoint with forkhead and ring finger domains
CIN	Chromosomal instability
CMA	Chromosomal microarray analysis
COL1A1	Collagen, Type I, Alpha 1
COL1A2	Collagen, Type I, Alpha 2
CREM	cAMP responsive element modulator
CRK	V-crk avian sarcoma virus CT10 oncogene homolog
CSMD1	CUB and Sushi multiple domains 1
Cy3	Cyanine 3
Cy5	Cyanine 5
DAPK1	Death-associated protein kinase 1
DCC	DCC netrin 1 receptor
DDR	DNA Damage Response
DDX27	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27
DLC1	DLC1 Rho GTPase activating protein
DLEC1	Deleted in lung and esophageal cancer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
DSB	Double-strand DNA break
EDNRB	Endothelin receptor type B
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth factor receptor
ESR1	Estrogen receptor 1
FANCD2	Fanconi anemia, complementation group D2
FISH	Fluorescence *in situ* Hybridization
FBLIM1	Filamin Binding LIM Protein 1
FBS	Fetal Bovine Serum
FC	Flow Cytometry
FHIT	Fragile histidine triad
FRA11F	Fragile site, aphidicolin type, common, fra (11)(q14.2)
G	Giemsa

GATA4	GATA binding protein 4
GATA5	GATA binding protein 5
GPR39	G Protein-coupled Receptor 39
GSTP1	Glutathione S-transferase pi 1
Gy	Gray
H2AFX	H2A histone family member X
HER	Human epidermal growth factor receptor
HIC1	Hypermethylated in cancer 1
HIF-1	Hypoxia-inducible transcription factor 1
HIP1R	Huntingtin interacting protein 1 related
HNC	Head and neck cancer
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human papillomaviruses
HRE	Hypoxia-response elements
HS3ST3A1	Heparan Sulfate (glucosamine) 3-O-Sulfotransferase 3A1
hsr	Homogeneously staining regions
IFN	Interferon
IL12A	Interleukin 12A
IL2	Interleukin 2
ING1	Inhibitor of growth family member 1
INTS2	Integrator Complex Subunit 2
IRF5	Interferon Regulatory Factor 5
kDA	Kilo Dalton
KIF1A	Kinesin family member 1A
KIF26B	Kinesin Family Member 26B
KLK3	Kallikrein-related peptidase 3
KLK10	Kallikrein-related peptidase 10
KLLN	Killin, p53-regulated DNA replication inhibitor
LD50	Median lethal dose
LET	Linear energy transfer
LOH	Loss of heterozygosity
LQ	Linear Quadratic
MAPK	Ras-mitogen-activated protein kinase
MBD	MethylCpG binding protein

MDM2	MDM2 proto-oncogene
MED13	Mediator Complex Subunit 13
MGMT	O(6)-methylguanine-DNA methyltransferase
MIR183	microRNA 183
MLH1	mutL homolog 1
MLPA	Multiplex Ligation-dependent Probe Amplification
MME	Membrane metallo-endopeptidase
MRE11A	MRE11 meiotic recombination 11 homolog A
MSH2	mutS homolog 2
MSH6	mutS homolog 6
Ms-MLPA	Methylation-Specific Multiplex Ligation-dependent Probe Amplification
MSX1	Msh Homeobox 1
mTOR	Mechanistic target of rapamycin (serine/threonine kinase)
mTORC1	Mammalian Target of Rapamycin complex 1
MTUS1	Microtubule associated tumour suppressor 1
MU	Monitor Units
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MYF6	myogenic factor 6
NAALADL2	N-acetylated Alpha-linked Acidic Dipeptidase-like 2
NACA2	Nascent Polypeptide-associated Complex Alpha Subunit 2
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NFkB	Nuclear factor kappa B
NSCLC	Non-small-cell lung carcinoma
nt	Nucleotides
OSCC	Oral squamous cell carcinoma
p	Short arm of chromosome
$p14^{ARF}$	Tumour suppressor ARF
$p16^{INKa}$	Cyclin-dependent kinase inhibitor 2A
p53	Cellular tumor antigen p53
PAH	Phenylalanine Hydroxylase
PAX5	Paired box 5
PAX6	Paired box 6

PCR	Polymerase Chain Reaction
PE	Plate Efficiency
PFDN4	Prefoldin subunit 4
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3KCA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PMP22	Peripheral Myelin Protein 22
PTCH1	Patched 1
PTEN	Phosphatase and tensin homolog
PTK2	Protein kinase 2
PYCARD	PYD and CARD domain containing
q	Long arm of chromosome
Q	Quinacrine
R	Reverse
RAD51	Rec A recombinase
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1
RARβ	Retinoic acid receptor beta
RASSF1	Ras association (RalGDS/AF-6) domain family member 1A
RASSF2	Ras association (RalGDS/AF-6) domain family member 2
Rb	Retinoblastoma-associated protein
RB1	Retinoblastoma
RERE	Arginine-glutamic Acid Dipeptide (RE) Repeats
RIGI	Radiation-induced genomic instability
RNF114	Ring finger protein 114
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
SCGB3A1	Secretoglobin, family 3A, member 1
SD	Standard Deviation
SERPINB13	Serpin peptidase inhibitor, clade B (ovalbumin) member 13
SF	Surviving Factor
SFRP1	Secreted frizzled-related protein 1
SKY	Spectral Karyotyping
SLC22A18	Solute Carrier Family 22, Member 18
SSB	Single-strand DNA break

STAT	Signal transducer and activator of transcription
STK11	Serine/threonine kinase 11
TAF4B	TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor, 105kDa
TBX4	T-box 4
TGFRB2	Transforming Growth Factor-β receptor
THBS1	Thrombospondin 1
TIMP3	Tissue inhibitor of metalloproteinases 3
TNM	Tumour-node-metastasis
TP53	Tumour protein p53
TP63	Tumour protein 63
TP73	Tumour protein p73
TSC2	Tuberous Sclerosis 2
TSG	Tumour suppressor gene
UCHL1	Ubiquitin carboxylterminal esterase L1 (ubiquitin thiolesterase)
VHL	von Hippel-Lindau tumour suppressor, E3 ubiquitin protein ligase
WIF1	Wnt inhibitory factor 1
WISP1	WNT1 inducible signaling pathway protein 1
WNT1	Wingless-type MMTV integration site family, member 1
WRAP73	WD Repeat Containing, Antisense to TP73
WT1	Wilms tumour 1
WHO	World Health Organization
ZFP64	ZFP64 zinc finger protein
ZMYND10	MYND-type containing 10

1 INTRODUCTION

1.1 CANCER

Willis, in 1960, defined neoplasm as "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after the cessation of the stimuli which evoked the change" (Willis, 1960). Considering this, it can be said that neoplastic cells are able to replicate indefinitely and are almost autonomous. The term that usually substitutes neoplasm is tumour, which can be benign or malign according to its potential clinical behaviour. A tumour is considered benign when is tough that it will remain localized, not spreading to other locals in the body and that it is susceptible to be local surgical removed. Malignant tumours are usually called cancers, that are able to invade and destroy other structures, metastasize and even lead to death (Kumar *et al.*, 2007).

Cancer is an enormous global health challenge, as it is one of the leading causes of death worldwide. According to GLOBOCAN, in 2012, 32.6 million people were living with cancer and 8.2 million died due to this disease. It is estimated that, in 2035, almost 15 million people for year will die from cancer (GLOBOCAN, Accessed: 15-12-2014; WHO, Accessed: 15-12-2014).

1.1.1 Hallmarks of Cancer

In 2000, Hanahan and Weinberg introduced the term "Hallmarks of Cancer". These hallmarks referred to processes that must be deregulated for the development of carcinogenesis and metastasis. There were six hallmarks: (i) Sustaining proliferative signalling; (ii) Evading growth suppressors; (iii) Activating invasion and metastasis; (iv) Enabling replicative immortality; (v) Inducing angiogenesis; (iv) Resisting cell death. Meanwhile, in the last years, two more hallmarks have been added: reprogramming of energy metabolism and evading immune destructions. Underlying these hallmarks are two important characteristics of cancer cells: genome instability, which is responsible for the genetic diversity that leads to the development of the hallmarks, and inflammation, which promotes many of the hallmarks functions (Figure 1) (Hanahan and Weinberg, 2011).

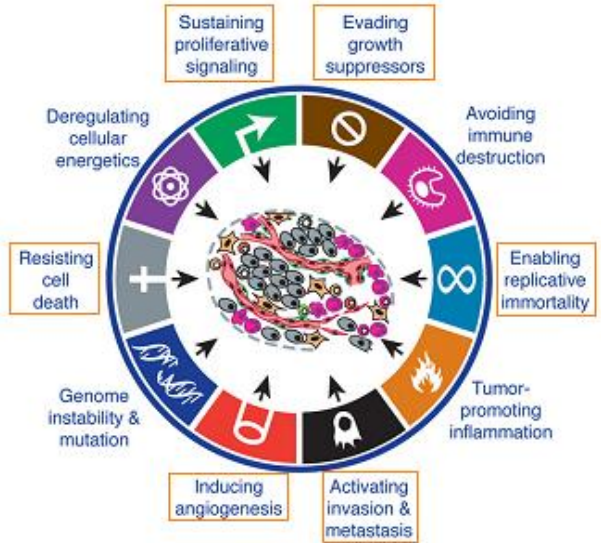

Figure 1 – The Six Hallmarks of cancer (Highlighted) alongside with the two new emerging hallmarks and the enabling characteristics. Adapted from: Hanahan and Weinberg et al. (2011)

1.2 HEAD AND NECK CANCER

Head and neck cancer (HNC) refers to the malignant tumours that arise from different anatomic sub sites of the head and neck region: Nasal Cavity/Paranasal, Oral Cavity, Salivary Gland, Trachea, Thyroid, Nasopharynx, Oropharynx, Hypopharynx, and Larynx (Figure 2). Squamous cell carcinoma represent more than 90% of all HNC and arise in the oral cavity, oropharynx, larynx or hypopharynx. This way, the term "Head and Neck Squamous Cell Carcinoma" (HNSCC) is frequently used to refer to these anatomical sites (Stadler *et al.*, 2008).

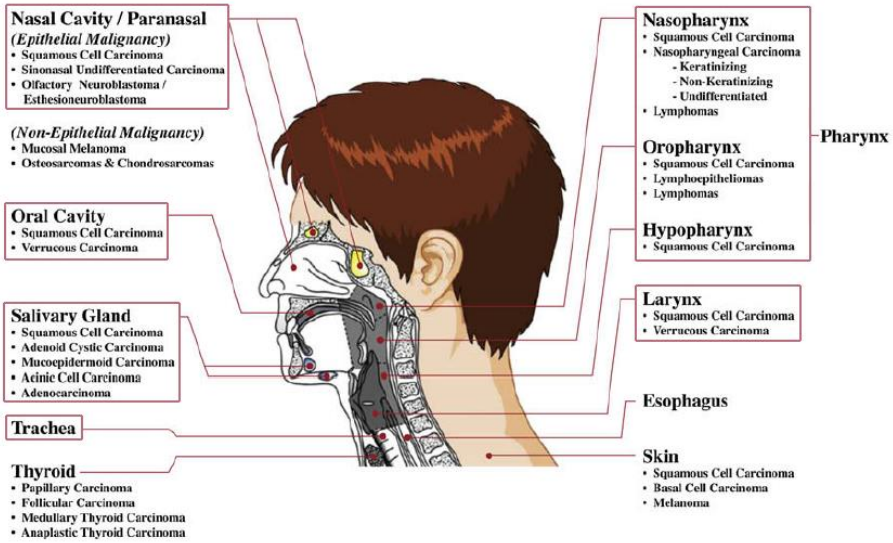

Figure 2 - Diversity of head and neck cancer and histopathologic diagnosis that present at the various subsites in the head and neck. HNSCC regions are marked. Adapted from: Stadler et al. (2008)

Squamous cell carcinoma, which arises from normal squamous epithelium through a stepwise process called dysplasia, is a neoplasm that penetrate through the basement membrane and extend to the connective tissue underline it (Radosevich, 2013). HNSCC results from the accumulation of

many genetic and epigenetic alterations in various signalling pathways, which can lead to tumour development and invasive carcinoma (Tan *et al.*, 2013).

The fact that more than 90% of all types of HNC are squamous cell carcinomas could lead to a wrong conclusion: HNSCC is a homogenous disease. However, scientists have proven this theory wrong. HNSCC is a very heterogeneous disease, which makes the treatment planning and the identification of the etiologic genes much harder (Leemans *et al.*, 2011).

Despite technological and clinical advances, the five-year survival rate of these cancer patients does not have improved much in the last years, since this disease is still diagnosed at an advanced stage (Llewellyn *et al.*, 2001; Smeets *et al.*, 2006).

1.2.1 HNSCC Histology

All parts of the oral cavity are lined by a protective mucous membrane, called oral mucosa. The oral cavity is lined with stratified squamous epithelium, which, according to the region, can be keratinized or not. The nonkeratinezed epithelium appears in the soft palate, lips, cheeks and the floor of the mouth, whereas the keratinezed one is present on the gingiva and hard palate. The basement membrane, which regulates the differentiation and migration of the epithelial cells and function as a barrier to stromal invasion, is the interface between the keratinocytes and the lamina propria. The latter is responsible for the support of the oral epithelium (Pai and Westra, 2009; Young *et al.*, 2013).

World Health Organization (WHO) defined squamous cell carcinoma of the oral cavity as "an invasive epithelial neoplasm with varying degrees of squamous differentiation and a propensity to early and extensive lymph node metastases" (Barnes *et al.*, 2005). The first steps for the development of oral malignancies are cellular disorganization of the epithelium, enhancement of mitotic activity, nuclear enlargement and pleomorphism. In posterior phases, the basement membrane is disrupted and the carcinoma can invade into the connective tissue. At last, it can reach skeletal muscle, craniofacial bones and facial skin, possible through perineural invasion and the lymphatic spaces (Barnes *et al.*, 2005; Pai and Westra, 2009).

1.2.2 Epidemiology and Risk Factors

In 2012, HNSCC was considered the sixth most malignant tumour, with an estimative of 600 000 new cases every year. Particularly, in Western European countries the incidence rate is estimated to be 15/100 000 (Wittekindt *et al.*, 2012; Tornesello *et al.*, 2014).

Accordingly to the GLOBOCAN 2012, Portugal was in the highest group of incidence for lip and oral cavity cancer, alongside with Australia, India and other Asian countries (Figure 3) (GLOBOCAN, Accessed: 22-07-2014). Incidence rates of oral cavity and laryngeal cancers have

been decreasing worldwide over the past years, however, oropharyngeal cancer rates have been increasing about 2.9% *per* year since 2001, most likely due to changes in people's lifestyle (Michaud *et al.*, 2014).

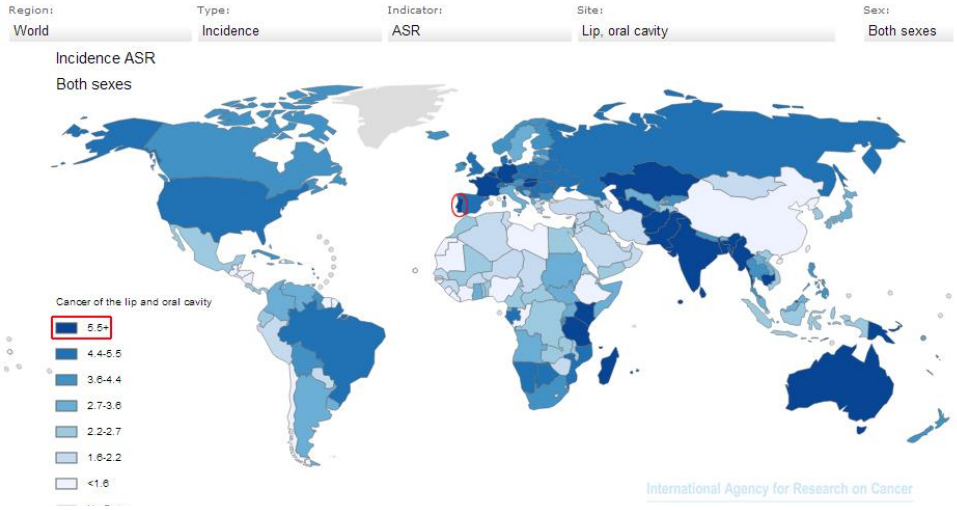

Figure 3 – Schematic representation of the incidence of lip and oral cavity worldwide. Portugal isin the higher incidence group. Adapted from: GLOBOCAN 2012

It was in 1957 that Wynder and Bross identified tobacco and alcohol as etiologic factors in oral and oropharyngeal cancer. Later, the use of tobacco and alcohol consumption were confirmed to be causes for the development of HNSCC and, nowadays, it is known their implication in 75% of all HNSCC cases (Argiris *et al.*, 2008). Moreover, some investigators concluded that they have a synergistic effect in causing this type of tumours (Ragin *et al.*, 2007; Argiris *et al.*, 2008) .

Tobacco smoke induces carcinogenesis manly due to its carcinogens, like nitrosamine and polycyclic hydrocarbons, which have genotoxic effects. Those agents can induce structural changes in DNA, being the majority associated to oxidative damage. Although those changes can be repaired by the cellular mechanisms of base excision repair (BER) or nucleotide excision repair (NER), many studies have proved that changes in these pathways are common and they may have a part in the higher risks observed for tobacco smoking-related cancers (Stadler *et al.*, 2008). Alcohol consumption has been considered a risk, especially for the hypopharynx cancer, and its carcinogenic effect is due to its ability to act as a solvent for the components of tobacco smoke. Besides, genetic polymorphisms in enzymes that metabolize tobacco and alcohol have also been linked to their elevated risk in inducing carcinogenesis (Argiris *et al.*, 2008; Wittekindt *et al.*, 2012).

Despite the decrease of the tobacco smoking-related cancers, the number of HNSCC has not followed this decline. This phenomenon is attributed to an increase of oropharyngeal squamous cell carcinoma (OPSCC) in younger individuals due to infections with human papillomavirus (HPV) (Marur *et al.*, 2010). However, HPV is not the only virus associated to HNSCC. The presence of Epstein-Barr virus (EBV) in nasopharyngeal carcinoma (NPC) have also been identified (Yoshizaki *et al.*, 2007).

Furthermore, there are some inherited disorders that predispose to HNSCC. One of the most documented is Fanconi Anemia, an autosomal recessive disorder. The cancer susceptibility aspect of this disease is associated with an incapacity to maintain the integrity of the genome, leading to an elevated stage of chromosomal instability (CIN). Hereditary non-polyposis colorectal cancer, Li-Fraumeni syndrome and ataxia telangiectasia were also associated with a higher risk of developing HNSCC (Butturini *et al.*, 1994; Argiris *et al.*, 2008; Polanska *et al.*, 2014).

Other risk factors for this type of cancer include poor oral hygiene, environmental contaminants (gasoline and paint fumes, for example), diet and marijuana consumption. These factors do not have such huge impact as the previously ones described (Mao *et al.*, 2004).

1.2.3 Therapy for HNSCC

Nowadays, HNSCCs are classified according to the T (size of tumour) N (involvement of loco-regional lymph nodes) M (evidence of distant metastasis) system, which is based on morphology and anatomic distribution of the tumour. However, it is obvious that this classification lacks important markers, such as the biological and molecular ones, leading to the same treatment approach for different tumours (Prince *et al.*, 2010). It is important to mention that patients that suffer from HNSCC are at risk of developing subsequent primary tumours. Those with recurrent or metastatic disease have a worse prognosis, with a median survival of six to eight months (Goerner *et al.*, 2010).

Treatment for HNSCC requires a delicate balance between complete eradication of cancer and the preservation of the anatomical form and function of the organs of this sensible region. Organ preservation and quality of life are critical factors in the treatment planning, since the organs affected by this type of cancer are extremely important to the patient life (Ko and Citrin, 2009; Prince *et al.*, 2010).

At the time of diagnosis, only 15-30% of the patients are in an early-stage disease (stages I/II), which is usually curable with surgery or radiotherapy. However, the majority of the HNSCC patients is diagnosed with locally advanced disease (stages III/IV). For these patients is required a multidisciplinary approach that incorporates chemotherapy, radiotherapy and surgery. This approach brings significant toxicity rates, however most of the patients dies from loco-regional progression (Langer, 2008; Goerner *et al.*, 2010). Response to treatment therapy is independent of the TNM stage and has been proposed that patients with weak responses to primary therapy (radiotherapy or chemoradiotherapy) were more likely to present distant metastasis and, consequently, a lower survival (Akervall *et al.*, 2014).

Reirradiation, with or without chemotherapy for recurrent and second primary head and neck cancers can bring local control, but usually also leads to dead related to the treatment. Moreover,

many patients often develop resistance to the treatments, chemotherapy or radiotherapy, and there is some variation in the response to the treatments, probably due to biological factors of each case in particular. Following this line of reasoning, one great aim nowadays is to try to predict beforehand which patients will benefit from certain types of therapy, in order to minimize the toxicity rates (Begg, 2012).

The identification of specific molecules or pathways directly associated to tumourigenesis can provide opportunities to develop new strategies to treat and cure HNSCC with less toxic effects to the patient. Various techniques have been developed and studied for targeting cancer cells (Goerner *et al.*, 2010; Prince *et al.*, 2010). These new targets could improve the treatment outcome and patient's quality of life (Skvortsov *et al.*, 2014).

1.2.4 Carcinogenesis

Nowadays, it is known that HNSCC is a heterogeneous disease, subdivide in, at least, two different groups: tumours caused by HPV infection and tumours caused by other mechanisms (Tornesello *et al.*, 2014).

1.2.4.1 HPV Carcinogenesis

HPV-induced carcinogenesis is thought to be present in about 20-25% of HNSCC cases and the patients tend to be younger, with no historical of tobacco or alcohol consumption. The majority of the cases occurs in the economically developed countries and are associated with a better prognosis (Polanska *et al.*, 2014; Tornesello *et al.*, 2014).

Human Papillomaviruses belong to the family Papillomaviridae, 8 kb circular double-stranded DNA viruses that infects basal cells of the epithelial mucosa. About 90% of HPV-related head and neck cancers harbour HPV 16 (Dufour *et al.*, 2012).

The HPV life cycle is related to the stage of differentiation of the epithelial cells that are infected. The infection starts in undifferentiated proliferating cells of the basal epithelial layer, leading to the expression of the early viral genes, particularly the E6 and E7 oncogenes. These target cellular tumour antigen p53 (p53) and Retinoblastoma protein (Rb) pathways, respectively. The E6 protein binds to p53 and targets the protein to degradation, which leads to the inhibition of p53-mediated apoptosis. The E7 protein binds to Rb and inactivates it. This action originates an enhancement of cyclin-dependent kinase inhibitor 2A (CDKN2A) and, consequently, abnormal cell proliferation (Figure 4). Furthermore, the E7 protein induces defective centrosome duplication, originating multipolar and abnormal mitoses, aneuploidy and genomic instability (Leemans *et al.*, 2011).

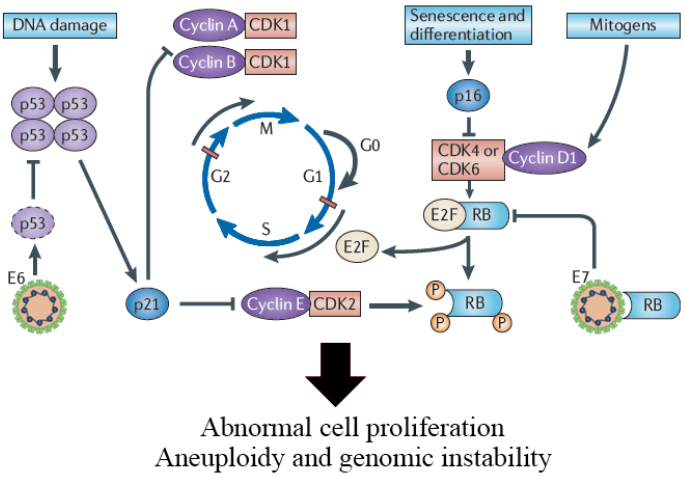

Figure 4 – HPV genome contains two viral oncoproteins: E6 and E7. Whereas E6 binds to p53, leading to the elemination of this protein, E7 is able to bind and inactivate Rb protein. These alterations lead to inhibition of apoptosis and entry in cell cycle, allowing the vírus to replicate. The replication of the vírus will eventually lead to abnormal cell proliferation, aneuploidy and genomic instability. Adapted from: Leemans et al. (2011) and ViralZone

1.2.4.2 Non-HPV Carcinogenesis

The majority of the knowledge about the carcinogenesis of HNSCC is due to oral cancers, mostly because precursor's lesions are in the top of the most frequently diagnosed, being the commonest oral leucoplakia, a white lesion in the oral mucosa (Leemans *et al.*, 2011).

As in all cancers, there is a variety of mutations in the basis of the development of HNSCC. Those mutations can be inactivation of tumour suppressor genes (TSGs), activation of proto-oncogenes, or both. However, tumour progression in HNSCC is not only a consequence of an accumulation of inactivating/activating mutations. Nowadays, it is known that many of these alterations crosstalk and interact with each other, integrating one major circuit in order to maintain the malignant phenotype of cancer. It is suggested that HNSCC results from a multistep development with characteristic alterations of cellular and molecular pathways occurring in the stratified squamous epithelium. With the help of molecular techniques, was proposed a progression model for the HNSCC carcinogenesis (Figure 5) (Argiris *et al.*, 2008; Haddad and Shin, 2008).

A few studies determined some cytogenetic alterations that may lead to dysplasia, carcinoma *in situ* and invasive tumours (Haddad and Shin, 2008; Pai and Westra, 2009; Rothenberg and Ellisen, 2012). Chromosomal alterations occur mostly at critical components of a few genetic pathways that regulate important cell functions, as cell growth, motility, squamous epithelial differentiation and stromal interactions (Rothenberg and Ellisen, 2012). Carcinomas that develop in large pre-neoplastic fields usually extend into the surgical margins of tumour's excision, leading to recurrences and second primary tumours. Besides local invasions, HNSCC is also characterized for cervical nodes dissemination, leading to the conclusion that it is a disease associated with metastatic and invasion progression (Koontongkaew, 2013).

The metastatic and invasion progression of tumour cells appears to be closely related to the interactions between the cells and the microenvironment that surrounds them, which include cell adhesion, cytoskeletal rearrangements, cell migration and degradation of the basement membrane, intravasion, survival in the blood vessel, extravasion, growth of the tumour cells at the distant site and promotion of angiogenesis (Koontongkaew, 2013).

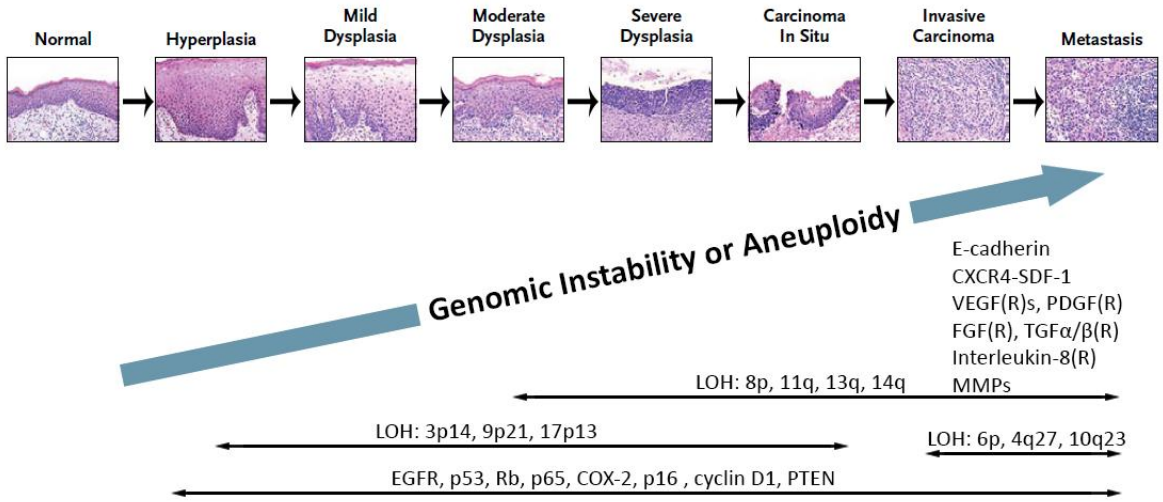

Figure 5 - Models of Genetic Instability and Progression in Head and Neck Cancer. The histologic evolution of HNSCC starts with normal characteristics and goes through hyperplasia, dysplasia, carcinoma in situ, invasive carcinoma and, finally, metastasis. According to this epithelium dysregulation, there is a progressive enhancement of genomic instability or aneuploidy and some molecular alterations are associated to different stages of the multistep progress. LOH: Loss of Heterozygosity. Adapted from: Haddad & Shin (2008)

1.2.5 Changes in Signalling Pathways

Rather than point mutations, genetic alterations in HNSCC are frequently associated to different pathways involved in key regulatory events such as: proliferation, squamous epithelial differentiation, cell survival, invasion and metastasis (Rothenberg and Ellisen, 2012).

1.2.5.1 Cellular proliferation - p53 and Rb pathways

There are several proteins implicated in cellular proliferation. One of the most studied is p53, which is a short-lived protein, activated through different stress pathways. At low levels of stress, p53 can lead to a blockage of the cellular proliferation, whereas at enhanced levels of stress, p53 induces apoptosis. Tumour protein *p53* (*TP53)*, a TSG, is frequently mutated in HNSCC patients and it is associated to an early event in carcinogenesis for this type of cancer. The inactivation of *p53*, not only stimulates cellular proliferation, but also gives rise to abnormal responses to DNA damage. Independent of the mechanisms, data studies appoint to the downregulation of the p53 pathway in more than 80% of all HNSCC cases (Partridge *et al.*, 2007; Rothenberg and Ellisen, 2012).

The Rb protein is involved in the initiation of cellular proliferation and is one of the most important proteins of the G1 checkpoint. The important role of Rb pathway is also evidenced through

the axis *CCND1-CDK4/CDK6-RB*, where oncogene *CCND1* is amplified in more than 80% of HNSCC. This protein promotes cell cycle progression through activation of G1 phase cyclin-dependent kinases (CDKs) 4 and 6. Together with the alterations in the p53, these changes lead to cellular immortalization (Rothenberg and Ellisen, 2012).

1.2.5.2 Terminal differentiation – NOTCH pathway

The NOTCH pathway has been associated to several cellular functions, such as regulation of self-renewal capacity, tumour angiogenesis, cell cycle exit and cell survival. Furthermore, NOTCH pathway is involved in squamous epithelium terminal differentiation promotion that can occur either through direct effects, such as the activation of suprabasal keratins, or through indirect effects, which involves the Wnt, Interferon and Hedgehog pathways. The *NOTCH* gene is inhibited in the basal epithelial cells by the transcription factor p63 and activation of p63 expression was observed in the dysplasic stage of HNSCC. Additionally, alterations in the Tumour protein 63 (*TP63*) that induced activation of p63, were observed in almost all of HNSCCs invasive cases (Rothenberg and Ellisen, 2012; Sun *et al.*, 2014).

1.2.5.3 Cell survival – PI3KCA and EGFR pathways

Several studies demonstrated that PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) pathway is commonly activated in HNSCC patients. PI3Ks are a family of enzymes, which function has been linked to the regulation of numerous cellular processes, like apoptosis, proliferation, cell cycle progression, cytoskeletal stability, motility, and energy metabolism. Many of these functions are related with the ability of PI3Ks to activate protein kinase B (AKT). Alterations in two genes that code for regulators of this pathway, phosphatase and tensin homolog (*PTEN*) and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PI3KCA*), are common. In the first one, loss of heterozygosity (LOH) is described in about 40% of all HNSCC cases. In the case of *PI3KCA,* gene mutations are activating and are thought to occur in 6-11% of the cases (Bussink *et al.*, 2008; Stadler *et al.*, 2008; Rothenberg and Ellisen, 2012).

Epidermal Growth factor receptor (EGFR) is a member of the ErbB family, which is described as overexpressed in more than 90% of HNSCC cases. When the ligand binds to EGFR, it produces a conformation change that allows a homodimerization or a heterodimezition with another EGFR or another Human epidermal growth factor receptor (HER) family member, respectively. This action induces auto-activation. After phosphorylation, these molecules are able to bind to numerous molecules associated with many different signal transduction pathways. The EGFR overexpression is considered to be an early event in HNSCC progression and it is associated to a worse prognosis (Stadler *et al.*, 2008; Leemans *et al.*, 2011; Rothenberg and Ellisen, 2012).

The alterations mentioned are all associated with activities that prevents cell death indirectly. However, there are also alterations that are able to prevent cell death directly, like mutations in components of the apoptotic cascade. An example is mutations in caspase 8 (*CASP8*), which codes for the enzyme that is responsible for the initiation of the cascade (Rothenberg and Ellisen, 2012).

1.2.5.4 Adhesion and Invasion – TGF-β/SMAD pathway

Changes that lead to the inactivation of Transforming growth factor (TGF)-β pathway are common in all types of cancer, including HNSCC. This inactivation is mostly common through the loss of TGF-β receptor (*TGFRB2*) and the *SMAD* genes. These genes are mapped at chromosome 18q, which is a region that is frequently lost in HNSCC patients. The inactivation of this pathway components via mutations is associated with tumour initiation and the activation of the pathway with metastasis promotion (Leemans *et al.*, 2011; Rothenberg and Ellisen, 2012). Cohen *et al.* (2009) demonstrated that the loss of the TGF-β pathway was associated with the activation of nuclear factor kappa B (NFkB), a transcription factor that is important to the survival of the cells.

1.2.6 Cytogenetic Alterations and Genes Common Altered in HNSCC

Cytogenetic alterations have been considered useful for diagnosis and prognosis, not only for cancers, both also for other several malignancies. Additionally, they can point to specific genes that may be involved in the disease's pathogenesis, helping in the understanding of the disease. However, classical cytogenetic analysis can be difficult because of many factors, including low mitotic index or small specimen size. In the specific case of the HNSCC, there is a small percentage (30%) of tumours that grow in culture and yield metaphases that are able to be analysed (Gollin, 2001).

Cytogenetic analysis of HNSCC samples have revealed consistent chromosomal breakpoints, structural chromosome rearrangements and homogeneously staining regions (hsr), which involve the centromeric region or juxtacentromeric bands and may result in whole-arm and Robertsonian translocations, and formation of isochromosomes. Isochromosomes have been identified for almost every chromosome. However, analysing karyotypes of HNSCC samples has proven to be a very difficult situation, not only by the points mentioned above, but also because they frequently range between diploid to pentaploid, with a large number of numerical and structural chromosome abnormalities (Gollin, 2001; Bockmuhl and Petersen, 2002; Martin *et al.*, 2008).

Recurring to different cytogenetic techniques it was possible to associate specific alterations to a particular stage in HNSCC development (Figure 5) (Haddad and Shin, 2008).

1.2.6.1 Region 3q

The presence of an isochromosome 3q is common in HNSCC and gain of the distal part, seems to be one of the most frequent genetic gains. A few studies also considered it one of the most frequent alterations in HNSCC patients (Gollin, 2014; Ribeiro *et al.*, 2014b). Gains in this region may suggest the presence of a cancer-related gene, such as *ATR* (ATR serine/threonine kinase), encoded at bands 3q22-q24. *ATR* is responsible for the checkpoint activation after stresses that damage the DNA. Previous studies showed that the overexpression of this gene could lead to tumour initiation and/or progression by promoting CIN. Other genes, like membrane metallo-endopeptidase (*MME*) (3q25.2), B-cell Lymphoma 6 (*BLC6*) (3q27), *Hs.570518* (3q28), interleukin 12A (*IL12A*) (3q25.33) and *TP63* (3q27-q29), were noticed to have extra copy numbers and are thought to be related to oral carcinogenesis (Gollin, 2001; Martin *et al.*, 2008; Gollin, 2014; Ribeiro *et al.*, 2014b). Another important proto-oncogene mapped in this chromosome arm is the *PI3KCA* (3q26.3). As described above, this pathway is commonly altered and it is part of the carcinogenesis process in HNSCC. In a study develop by Kozaki *et al.* (2006), mutations in the *PI3KCA* were associated to an advanced stage in HNSCC pathogenesis.

1.2.6.2 Region 3p

3p losses have been observed in dysplastic oral lesions, suggesting that they are associated to an early event in HNSCC carcinogenesis. One gene candidate in this region is the fragile histidine triad (*FHIT*) gene, which is a TSG that it is frequently lost in samples of HNSCC. It is mapped to 3p14.2 and it is suggested that its loss of function plays a key role in the development and progression of this type of cancer. Loss of FHIT protein causes DNA damage and genetic instability. Other gene mapped in this region is the retinoic acid receptor beta (*RARβ*), that could help distinguish normal tissue from potential malignant lesions, since previous studies showed that 60% of the latter did not express this gene (Lotan *et al.*, 1995; Gollin, 2001; Ribeiro *et al.*, 2014b).

1.2.6.3 Region 7p

One of the major genes mapped in this region is the *EGFR* gene, which maps to band 7p12.3-p12.1. The EGFR pathway is highly involved in HNSCC carcinogenesis, so it was foreseeable that the region where it is mapped showed alterations. There are several possible mechanisms that could explain *EGFR* overexpression, such as increased gene copy number, increased mRNA synthesis, decreased downregulation and expression of EGFRvIII. Studies have demonstrated that the increased expression of its protein and its ligand, TGF-α, could be a statistically significant predictor for disease recurrence and decreased overall survive (Gollin, 2001; Martin *et al.*, 2008; Gollin, 2014).

1.2.6.4 Region 8q

Like for other chromosomes, there are also reports of isochromosome formation for this region in HNSCC samples (Jin *et al.*, 2001). However, there are also gains and losses on chromosome 8 reported. Bands 8q24.12-q24.13 are very important in these tumours, since they encode the v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) gene, an alteration observed in 70% of all cases in Ribeiro *et al.* study. Its amplification and overexpression has been observed in 10-40% of human oral tumours. *MYC* gene is also associated with poor survival rates. Other overexpressed genes encoded in the 8q chromosomal region are *PTK2* (protein kinase 2) and *WNT1* inducible signalling pathway protein 1 (*WISP1*). *PTK2* is mapped in 8q24-qter, plays an important part in adhesion and growth-regulatory signal transduction and its overexpression has been associated with invasiveness. Gains of *MYC* and *WISP1* genes are thought to be associated with tumours localized in the floor of the mouth (Gollin, 2014; Ribeiro *et al.*, 2014a; Ribeiro *et al.*, 2014b). When Bhattacharya *et al*. (2009) were studying the amplification of *MYC* gene in HNSCC samples, they proposed that *MYC* could be a predictor of the progression of dysplasia to hyperplasia in HNSCC Furthermore, gains involving bands 8q21.1-8q24.22, are thought to be an early event in oral dysplasia (Gollin, 2014).

1.2.6.5 Region 8p

The short arm of chromosome 8 is frequently lost in many tumours, including HNSCC. Losses are most frequent in three regions: 8p23.3, 8p22-p23 and 8p21. The genes that are usually deleted are CUB and Sushi multiple domains 1 (*CSMD1*), GATA binding protein 4 (*GATA4*) and microtubule associated tumour suppressor 1 (*MTUS1*). *CSMD1*, mapped at 8p23, is present in other epithelial cancers and it is associated with a poor prognosis, with reduced overall survival and a short interval without disease. Although *GATA4* has been thought to be a tumour suppressor gene (TSG), its function in human cancer is not yet fully understood. *MTUS1* gene has a potential role as tumour suppressor in HNSCC carcinogenesis and reduction of its expression could be associated with an advance stage in oral tongue squamous cell carcinoma (Gollin, 2001; Ribeiro *et al.*, 2014a; Ribeiro *et al.*, 2014b).

1.2.6.6 Region 9p

Associated with an early event in HNSCC development, loss of chromosome 9p is very common, in particularly breakpoints at 9p21, 9p13 and 9p11. Formation of isochromosomes 9q are also frequent (Gollin, 2014). LOH at 9p21-p22, is a common event, with a frequency of 72%. Band 9p21 contains several TSGs, including *CDKN2B*, *CDKN2A*, *CDKN2C* and *CDKN2D*. All these genes encode for CDK inhibitors, which block cell cycle progression after the checkpoint of G1

phase. Inactivation of these genes may lead to loss of cell cycle checkpoints, which result in deregulation of cellular proliferation (Martin *et al.*, 2008; Ribeiro *et al.*, 2014b).

1.2.6.7 11q13 Band

This amplification has been very studied not only because it harbours a few oncogenes, but also because this amplification is very heterogeneous (Szyfter *et al.*, 2014). 11q13 amplification usually occurs as a result of a breakage-fusion-bridges cycles initiated by a break at one chromosomal fragile site, *FRA11F*. This chromosomal change has been seen in about 50% of all HNSCC tumours. The 11q13 band contains 13-14 genes, most of all are overexpressed in HNSCC. This band contains the *CCND1,* considered one of the most important target genes in 11q13, which is thought to play a direct role in oral cancer. Overexpression of this protein can make the passage through the G1-S phase faster, leading to accumulation of genetic alterations, therefore, may leading to cancer. In HNSCC, CCND1 protein expression is correlated with less recurrence time, higher stage disease, lymph node involvement and reduced overall survival (Gollin, 2001; Martin *et al.*, 2008; Gollin, 2014). So, the overexpression of this gene has been associated with an aggressive tumour behaviour (Argiris *et al.*, 2008). This amplification is considered as an early change in HNSCC and it is involved in the transition from moderate to severe dysplasia (Gollin, 2014).

1.2.6.8 Region 11q

Beside amplification of 11q13 band, there is also a common loss on chromosome 11. This loss is distal to the amplified 11q13 band. This deletion is thought to occur as a first step for the characteristic amplification, between the dysplasia stage and the carcinoma *in situ* stage. Together, amplification and loss, happens in about 50% of the cases of HNSCC. In this region are mapped several DNA damage responsive (DDR) genes, including caspase 1 (*CASP1*) (11q23), MRE11 meiotic recombination 11 homolog A (*MRE11A*) (11q21), ATM serine/threonine kinase (*ATM*) (11q23.3), H2A histone family member X (*H2AFX*) (11q23.3) and checkpoint kinase 1 *(CHEK1*) (11q24.2) (Gollin, 2001; 2014; Ribeiro *et al.*, 2014b).

Moreover, amplification of 11q13 and loss of distal 11q have been associated with tumours of the tongue, retromolar trigone and buccal mucosa and provide a decreased survival. It has been pointed out that these combination could be used as biomarker for prognosis (Gollin, 2014).

1.2.6.9 Region 13q

Chromosomal deletion on region 13q is also common in HNSCC patients, especially at two bands: 13q12.11 (44%) and 13q14.2 (41%). One important gene mapped at 13q is the retinoblastoma gene, *RB1* (13q14.2). Rb protein function was described above. Other gene that has also been considered is the inhibitor of growth family member 1 (*ING1*) gene mapped at 13q34. This is also a

TSG as it is proposed that the mutations occurred would lead to the suppression of its normal function (cooperates with p53, preventing cell growth) (Gollin, 2014; Szyfter *et al.*, 2014).

1.2.6.10 Region 17p

Mapped at 17p13.1 is *TP53* gene. Disruption of the p53 pathway is one of the most frequent alterations in HNSCC, which happens through mutations, LOH or interaction with viral proteins. Mutations in this gene are often present in 50-80% of HNSCC tumours and are associated to an early stage of carcinogenesis. They can even be detected in premalignant dysplasic lesions and in the surgical margins considered to be histopathological negative for tumours. *TP53*, a TSG, is often called "the guardian of the genome" due to its involvement in cell cycle arrest, DNA repair and apoptosis. Furthermore, alterations of p53 seem to be particular relevant for HNSCC development since two major risk factors, tobacco carcinogens and HPV, target this protein (Blons and Laurent-Puig, 2003; Loyo *et al.*, 2013).

There is some controversial about the prognostic significance of *TP53* mutations among the science community, however, *TP53* mutations are frequently associated with reduced survival, at least after surgical treatment (Argiris *et al.*, 2008). However, mutations in the gene are not the only way that p53 expression could be altered. It can also be a consequence of the tumour development process, radiation, among others (Scully *et al.*, 2000).

Other genes have also been found to be lost in HNSCC samples that are mapped at chromosome 17, like breast cancer 1 (*BRCA1*) (17q21) and v-crk avian sarcoma virus CT10 oncogene homolog (*CRK*) (17p13.3) (Ribeiro *et al.*, 2014b).

1.2.6.11 Region 18q

Loss of the q-arm of chromosome 18 has been well documented as a cytogenetic alteration common in HNSCC, however, the relevant TSG of this region has not yet been determined. Nevertheless, it is known to be associated with an advanced stage of the tumour development and poor prognosis (Scully *et al.*, 2000; Gollin, 2014).

DCC netrin 1 receptor (*DCC*) and serpin peptidase inhibitor, clade B (ovalbumin) member 13 (*SERPINB13*) are examples of genes in this location that are frequently deleted. LOH of *DCC* gene has been observed and associated to a decreased survival, however, it is only altered in 12-24% of the cases. *SERPINB13* is normally expressed in normal oral mucosa, oral keratinocytes and skin but it is poorly expressed in HNSCC, which suggest that its reduced expression may be involved in HNSCC development (Gollin, 2001; Martin *et al.*, 2008; Ribeiro *et al.*, 2014b).

1.2.7 Epigenetic Alterations

Epigenetic changes, which occur more frequently than gene mutations, are modifications in gene function that are mitotically and/or meiotically heritable without alterations of the DNA sequence. They can persist for the entire cell life or even for multiple generations.

Several differentiation processes are regulated through epigenetic mechanisms. Then, disruption of epigenetic mechanisms can lead to gene expression alterations, which can induce carcinogenesis and other diseases (Table 1). Since they can be reversible, epigenetic changes are more attractive therapy targets than genetic alterations (Mascolo *et al.*, 2012; Arantes *et al.*, 2014).

Table 1 - The most frequent epigenetic alterations. Adapted from: Mascolo et al. (2012)

Epigenetic Change	Putative Mechanism	Biological Consequence
DNA hypomethylation	Activation of cellular oncogenes Activation of transposable elements	Increased proliferation, growth advantage Genomic instability, transcriptional noise
DNA hypermethylation	*De novo* hypermethylation of CpG islands within gene promoters leading to silencing of TSG and cancer-associated genes	Genomic and chromosomal instability, increased proliferation, growth advantage
Loss of imprinting	Reactivation of silent alleles, biallelic expression of imprinted genes	Expansion of precursor cell population
Relaxation of X-chromosome inactivation	Mechanisms is unknown but it appears to be age-related	Altered gene dosage, growth advantage
Histone acetylation	Gain-of-function Loss-of-function	Activation of tumour promoting genes Defects in DNA repair and checkpoints
Histone deacetylation	Silencing of TSG	Genomic instability, increased proliferation
Histone methylation	Loss of heritable patterns of gene expression (“cellular memory”)	Genomic instability, growth advantage
MicroRNAs (miRNAs) amplification in cancer	Function as oncogenes	Neoplastic transformation
miRNAs deletion in cancer	Function as tumour suppressors	Neoplastic transformation

1.2.7.1 DNA Methylation

DNA methylation, the most common and studied epigenetic modification, is a known mechanism for silencing gene expression and maintenance of genomic stability. The DNA methylation machinery in mammals is composed of DNA methyltransferases (DNMTs) and methylCpG binding proteins (MBDs). The function of the first ones is to establish and maintain DNA methylation patterns, where the second ones are capable of detecting the methylations marks. This epigenetic alteration occurs more frequently at repetitive genomic regions. DNA methylation is the

covalent addition of a methyl group to the 5-carbon position of cytosine to bases that are located 5' to a guanosine base in a CpG dinucleotide. CpG dinucleotides are often found in clusters, were they are called CpG islands (Robertson, 2005; Mascolo *et al.*, 2012).

Methylation can affect gene expression by two principal mechanisms: (1) it can interfere directly with the ligation of factors that are sensible to methylated CpG islands; (2) cytosines methylated can be recognised by the methyl binding domain (MBD) protein family which is responsible for the recruitment of enzymes that remodel the chromatin, like histone deacetylases. The result will be condensation of chromatin and, consequently, the silencing of the neighbouring gene (Delpu *et al.*, 2013).

Several studies through the years showed that DNA methylation has a crucial role in the development of the cancer genome (Figure 6), since this particular epigenetic mutation is considered an early event in carcinogenesis. There are, at least, three different ways by which CpG methylation could lead to cancer: hypomethylation of the cancer genome, hypermethylation of the promotors of TSG and direct mutagenesis. CpG islands are usually localized at the promoter of TSG and this event can contribute to carcinogenesis, since CpG islands are often hypermethylated in tumours (Hatziapostolou and Iliopoulos, 2011).

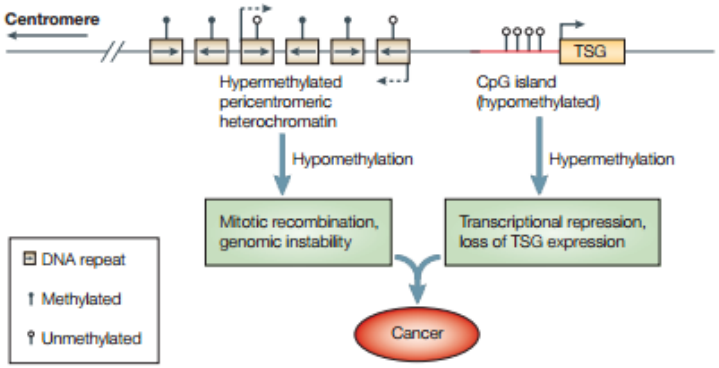

Figure 6 - DNA methylation and cancer. The diagram shows a representative region of genomic DNA in a normal cell. The region shown contains hypermethylated pericentromeric heterochromatin and an actively transcribed tumour suppressor gene associated with a hypomethylated CpG island (indicated in red). Adapted from: Robertson (2005)

Hypermethylation results in alterations in chromatin structure, which leads to the silencing of the TSG transcription, since it blocks the accessibility of the transcription factor to the promoter. The genes most frequently hypermethylated reside in chromosome regions that usually shows signs of LOH (Mascolo *et al.*, 2012).

Besides hypermethylation, hypomethylation can also occur in tumour cells, mainly due to the loss of methylation from repetitive regions of the genome, which could lead to the genomic instability often seen in cancer. Furthermore, hypomethylation of the genome can also induce re-expression of silenced genes. It is important to refer that hypomethylation was the first epigenetic

alteration identified in tumours (Robertson, 2005; Hatziapostolou and Iliopoulos, 2011; Delpu *et al.*, 2013).

In HNC, methylation is the epigenetic change most studied and promoter methylation has been found in a large number of genes. One of the most important risk factors for HNSCC, tobacco, has been linked to non-specific hypomethylation. Contrarily, alcohol consumption has been associated to an enhancement of hypermethylation at CpG islands. Methylation has been well studied in oral squamous cell carcinoma (OSCC) and genes that are methylated in OSCC are involved in various cellular processes, including cell cycle control, apoptosis, DNA repair and cell to cell adhesion (Viswanathan *et al.*, 2003; Gasche and Goel, 2012; Mascolo *et al.*, 2012) .

A lot of studies have, nowadays, focused in analyse the promoter methylation of TSGs panels that play a role in vital cellular mechanisms. As a result, there is knowledge of numerous genes that are methylated in head and neck cancer (Table 2) (Demokan and Dalay, 2011).

Recently, Worsham *et al.* (2014) have tried to delineate an epigenetic progression model for HNSCC based on different reports of genes hypermetylated (Figure 7). Hypermethylation of *CDKN2B*, *CDKN2A*, *APC* and *BRCA2* were proposed to occur as epigenetic events leading to the progression to a stage of carcinoma.

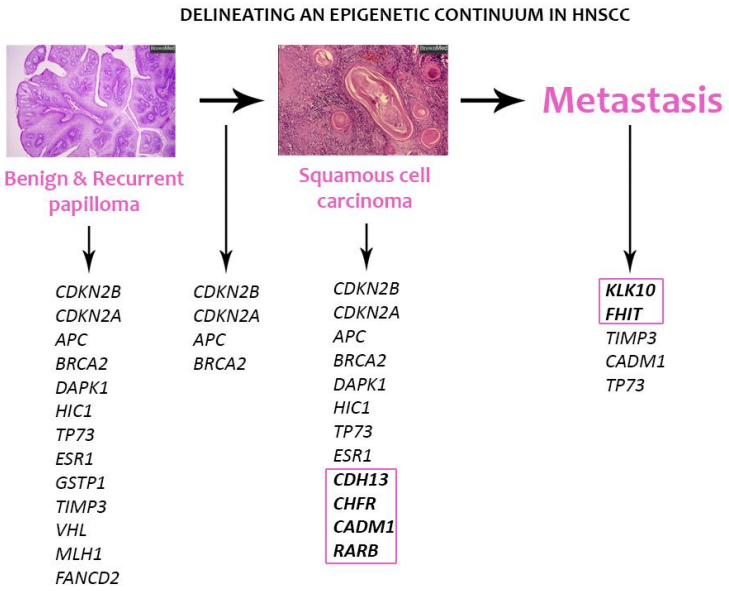

Figure 7 – Possible epigenetic model of HNSCC regarding DNA hypermethylation. They suggested that DNA hypermethylation of CDKN2B, CDKN2A, APC, BRCA2, DAPK1, HIC1, TP73, and ESR1 were early events in tumorigenesis, whereas CDH13, CHFR, CADM1 and RARβ were primary tumour-specific events. Finally, KLK10 and FHIT were associated to metastasis devolpment. Adapted from: Worsham et al. (2014). Histological images were taken from Digital Pathology – Brown Medical School

Table 2 – Some candidate genes frequently methylated in HNC. Adapted from: Demokan and Dalay (2011)

Gene	Gene name	Gene function	Methylation range (%)
APC	Adenomatous polyposis coli	Tumour suppression	21-34
ZMYND10	MYND-type containing 10	Tumour suppression	34-74
CCNA1	Cyclin A1	Cell cycle	45-53
CDH1	E-cadherin	Cell adhesion	23-85
CHFR	Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase	Early G2/M checkpoint	19-61
DAPK1	Death-associated protein kinase 1	Apoptosis	7-77
DCC	DCC netrin 1 receptor	Tumour suppression	50-75
DLC1	DLC1 Rho GTPase activating protein	Tumour suppression	43-89
DLEC1	Deleted in lung and esophageal cancer	Tumour suppression	60-86
EDNRB	Endothelin receptor type B	Signalling mechanism	60-97
SCGB3A1	Secretoglobin, family 3A, member 1	Tumour suppression	7.3-77%
MLH1	mutL homolog 1	DNA repair	32.5-50
MSH2	mutS homolog 2	DNA repair	30-50
KIF1A	Kinesin family member 1A	Axonal transport of synaptic vesicles and cell division	35-98
MGMT	O(6)-methylguanine-DNA methyltransferase	DNA repair	6-56
APBA1	Amyloid beta precursor protein-binding	Synaptic vesicle exocytosis	23-48
CDKN2A^{ARF}	Alternative open reading frame (ARF) of INK4a locus	Tumour suppression	14-34
CDKN2B	Cyclin-dependent kinase inhibitor 2B	Tumour suppression	22-65
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Tumour suppression	5-68
UCHL1	Ubiquitin carboxylterminal esterase L1 (ubiquitin thiolesterase)	Processing of ubiquitin precursors and of ubiquitinated proteins	60-82
RARβ	Retinoic acid receptor beta	Tumour suppression	15-80
RASSF1	Ras association (RalGDS/AF-6) domain family member 1A	Tumour suppression	2.4-84
RASSF2	Ras association (RalGDS/AF-6) domain family member 2	Tumour suppression	22-51
SFRP1	Secreted frizzled-related protein 1	Inhibition of Wnt signalling	24-58.6
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	Response to retinoic acid receptor	51-91
TIMP3	Tissue inhibitor of metalloproteinases 3	Inhibition of angiogenesis and tumour growth	40.5-71.8
WIF1	Wnt inhibitory factor 1	Inhibition of Wnt signalling	85-89.7

1.3 RADIOTHERAPY IN CANCER

In 1961, Munro and Gilbert published a paper in which they mentioned the goal of using radiotherapy to treat cancer: "the object of treating a tumour by radiotherapy is to damage every single potentially malignant cell to such an extent that it cannot continue to proliferate" (Munro and Gilbert, 1961).

Although surgery is the primary form of treatment for the majority of the tumours, it is estimated that half of all cancer patients are submitted to radiotherapy at some point during their treatment. Surgery gives good treatment results in tumours early detected and non-metastatic, however radiotherapy is a good alternative for the long-term control of several tumours, such as head and neck cancers (Joiner and van der Kogel, 2009).

The principle of radiation treatment of cancer is that cells with high proliferative rates are more sensitive to radiation, meaning that cancer cells are more sensitive to radiation than normal cells (Kelsey *et al.*, 2013). Radiation therapy uses low and high linear energy transfer (LET – number of ionizations which radiation causes per unit of distance as it trespasses the living cells or tissue) radiations in order to kill the most possible malignant cells, while minimizing the effects on normal tissues and trying to prevent toxicity on them. Over the years, improvements have been made in radiotherapy treatment, increasing the number of cancer survivors. However, tissue toxicities due to late effects induced by radiation in normal cells are a major problem, especially in children (Figure 8) (Baskar *et al.*, 2014; Barker *et al.*, 2015).

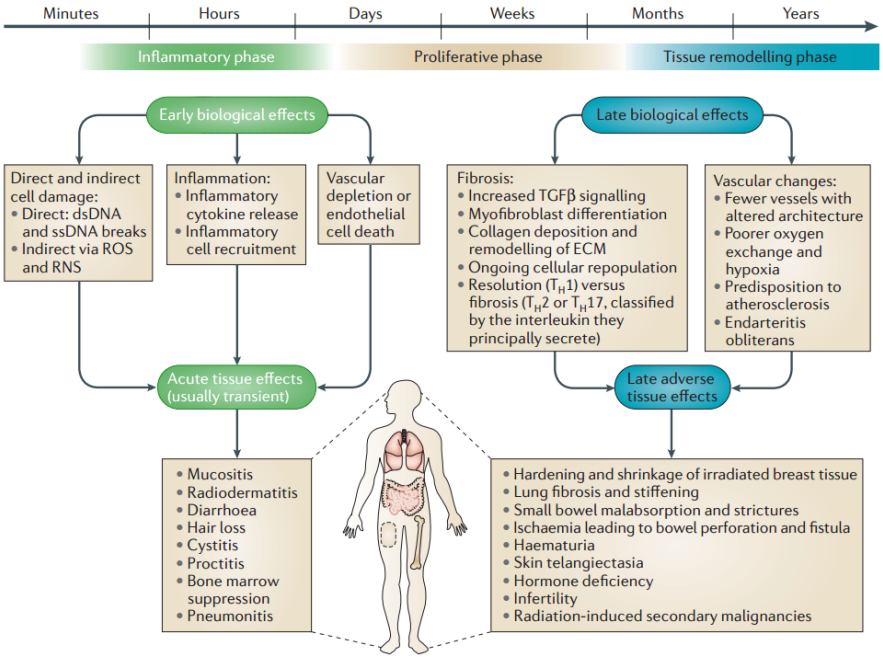

Figure 8 - Biological effects and normal tissue toxicity after radiotherapy. The early biological effects of irradiation lead to acute tissue effects that usually are transient. However, these can also lead to late biological effects than can be notice in tissues or by the appearance of secondary malignancies. Moreover, both early and late effects affect therapeutic efficacy and patients' quality of life. Adapted from: Barker et al. (2015)

1.3.1 Effects of Radiation on Tissue

There are four effects of radiation on tissues: direct effects, indirect effects, bystander effects and adaptive response.

The major effect of ionizing radiation is the direct cell killing, which occurs mostly by producing DNA damage. When the interaction of the energetic charged particles occurs directly with the target in cells, the process is referred as direct action, producing direct effects. When the interaction occurs with water molecules in the cell, the radiation produces free radicals. Free radicals interact strongly with biomolecules, producing damaging chemical reactions, which leads to damage in cells. This process produces indirect effects, which correspond to 60% of the damage cause by irradiation (Kelsey *et al.*, 2013; Baskar *et al.*, 2014).

Recently, radiobiologists found that radiation not only affect adjacent cells of the tumour but it is also able to affect cells far away, due to the transmission by gap-junctions, cytokine-mediated cellular toxicity or by the involvement of cellular and microenvironmental signalling cascades. This phenomenon has been called bystander effect. The radiation-induced bystander effect refers to the ability of radiation to induce alterations, commonly reported in directly irradiated cells, in non-irradiated cells, like induction of cell death, sister chromatid exchanges, formation of micronuclei, mutations and delay in cell cycle. Chromosomal aberrations can arise *de novo* in cell progeny several generations after irradiation (Baskar *et al.*, 2014).

Finally, ionizing radiation also triggers cancer cells adaptive responses. In response to irradiation, several resistant signal transduction pathways become activated. This resistance can be an intrinsic characteristic of the cell or can be acquired due to fractionated radiation treatment. This will give those cells a selective advantage, since they end up with a higher proliferative ratio or with capability to evade cell death. These adaptive responses are major clinical problems nowadays and there are several molecular events that allow tumours to become radioresistant, such as ligand-independent activation of signal transduction pathways. The greater example are those regulated by membrane-bound receptor tyrosine kinases (RTK), such EGFR (Baskar *et al.*, 2014).

1.3.2 Irradiation-induced damage

Ionized molecules affect the cells randomly, causing damage to the molecules. However, there are several copies from the most of the molecules, and the majority undergo a continuous and rapid turnover. So, the damage to these molecules turns up to be very small. Contrary, the DNA only has two copies, turning it to the most affected molecule on a cell by irradiation. 1 Gy (Gray) of irradiation can cause, in each cell, approximately 10^5 ionizations, more than 1000 damages to DNA bases, about 1000 single-strand DNA breaks (SSBs) and 20-40 double-strand DNA breaks (DSBs).

However, due to efficient DNA repair, 1 Gy will only kill about 30% of cells (Joiner and van der Kogel, 2009).

Regarding SSBs, they are easy to repair, since the intact strand serves as template. DSBs are more difficult and more likely to result in deficient repair, once the molecule may come apart. Most DSBs are lethal to the cells because they will trigger DNA checkpoints in cell cycle. Taking this into consideration, DSBs are the greater source of cell killing due to radiation (Kelsey *et al.*, 2013).

Moreover, radiation can originate chromosomal aberrations, of which there are two types: Chromosome aberrations and chromatid aberrations. The first ones happen early in interphase, before DNA duplication and the last ones happen later in interphase, after S phase of cell cycle. Many different types of aberrations can occur and, generally, three of them are considered to be lethal for the cell, two of which are chromosome aberrations and one is a chromatid aberration. Dicentrics, a type of chromosome aberration, happens when two different chromosomes suffer breaks and re-join, being subsequently replicated during S phase. The dicentric chromosome will have two centromeres, causing problems during anaphase. The other type of lethal chromosome aberration is the ring formation, that happens when one chromosome has breaks in both arms and is able to reconnect itself. It is replicated, however the problems happen during mitotic spindle in anaphase. The anaphase bridge is the lethal chromatid aberration and it happens after the damage sister chromatids fail to separate properly, going to one of the poles of the cell. Regarding non-lethal aberrations, examples are translocations and small interstitial deletions (Kelsey *et al.*, 2013).

1.3.3 Irradiation-induced cell death

Cells respond to irradiation by the activation of the DNA damage response and cell cycle arrest, leading to senescence or apoptosis of the cells irradiated. Nowadays, it is still not know precisely which pathways are responsible for these mechanisms (Partridge *et al.*, 2007; Eriksson and Stigbrand, 2010). It is, however, important to highlight that, in the context of radiobiology, cell death is referred to any process that results in loss of clonogenic capacity (Joiner and van der Kogel, 2009).

The effects of radiation on cells depends on the LET, total dose, fractionation rate and radioresistance/radiosensitivity of the target cells (Eriksson and Stigbrand, 2010). Cell death to irradiation is due to autophagy, senescence, apoptosis, necrosis and mitotic catastrophe (Joiner and van der Kogel, 2009).

Radiation-induced apoptosis was considered one of the main cell death mechanism after radiation therapy and the central characteristics of this controlled mechanism of cell death are often seen: pyknosis, cell shrinkage and internucleosomal breakage of chromatin. As described extensively, apoptosis is due to the activation of caspases, a family of proteases. These proteins are activated when the cell receive external and internal stimuli's, one example is radiation. Radiation

can induce apoptosis either via the extrinsic and intrinsic pathways. The endpoint of both these pathways is the activation of the effectors caspase-3, caspase-6 and caspase-7, which play important roles in apoptosis. Apoptosis is the major cell death mechanism, however, the majority of cancer cells lose their pro-apoptotic mechanisms during tumorigenesis. So, there have to be other types of cell death mechanisms involved in the death of cancer cells after radiation (Partridge *et al.*, 2007; Eriksson and Stigbrand, 2010).

Nowadays, mitotic catastrophe is considered to be the major radiation-induced cell death mechanism for solid tumours. Mitotic catastrophe is used to refer to a cell death that occurs as a result of an aberrant mitosis and can be induced by several agents that are able to damage the DNA, radiation included. Two important mechanisms have been proposed for the initiation of this type of cell death. The first one is related with problems in the cell cycle checkpoints and DNA damage. Since the majority of cancers have alterations in the cell cycle checkpoints, the mitotic catastrophe is the cancer cells response to DNA damage. Secondly, it is proposed that hyperamplification of centrosomes could lead to mitotic catastrophe. Centrosomes are important during mitosis in order to organize the microtubule centres and in the formation of bipolar mitotic spindles. The hyperamplification of centrosomes could result in abnormal chromosome segregation and originate cells with nuclei alterations. This will lead to mitotic catastrophe (Eriksson and Stigbrand, 2010).

Other type of cell death induced by radiation is senescence, referred as a condition of permanent cell cycle arrest, and can occur after extensive cellular stress induced by a number of DNA damaging agents. DDR are induced after radiation, they sense the damages of the DNA and transmit information in order to activate a transient cell cycle arrest wherefore the DNA will be repaired. DNA damage not repaired will induce cell death, by apoptosis or mitotic catastrophe, or activate a persistent and chronic DDR signalling, leading to senescence. This will prevent DNA damage to be passed for the next cell generations (Eriksson and Stigbrand, 2010).

Autophagy describes a process in which cells are able to digest parts of their own to generate small macromolecules and energy. This process has been observed after treatment, however it is currently unknown if it represents an attempt by the cell for survival or if it is an induced type of cell death. As for necrosis, it is generally thought to be an uncontrollable type of cell death, however proves have appear that this could also be regulated by the cell. Necrosis is also commonly observed in tumour after treatment with radiation (Joiner and van der Kogel, 2009).

1.3.4 Radiotherapy in HNSCC

Radiotherapy is one option for HNSCC's treatment. Radiotherapy alone results in high tumour control and cure rates for early stage glottis, base of tongue, and tonsillar cancer (Argiris *et al.*, 2008).

The amount of radiation given in the treatment of HNSCC usually is 2 Gy in daily fractions, 5 days a week, reaching up to total of 70 Gy through 7 weeks. When radiotherapy is interrupted for a long time it can have a harmful effect because of repopulation of cancer cells (Argiris *et al.*, 2008).

Two major fractionation variants have been tested to make possible to delivery multiples fractions *per* day: hiperfractionation and accelerated fractionation. The first one was designed to improve effectiveness by delivering two or three fractions every day, but with reduce dose *per* fraction. The second one was designed to increase radiation dose intensity in a short period of time. Phase III trials showed that these approaches improve loco-regional control with enhance infield toxic effects, but with greater effects on survival, when compared with conventional radiotherapy (Argiris *et al.*, 2008). The toxicities originated from radiotherapy include mucositis, xerostomia, dermatitis and dysphagia, which leads to reduced quality of life and morbidity (Bhide and Nutting, 2010).

1.3.5 Radioresistance in HNSCC

The terms "radiosensitive" and "radioresistant" are common to describe tumours that regress rapidly or slowly, respectively, after radiation treatment. Resistance to radiotherapy is very common, and it is related with a worse prognosis. Radioresistance can have three different results: (1) no treatment response at all, (2) only a partial response to treatment or (3) it can cause that recurrences appear too early. Radioresistance is a major clinical problem, since affects the probabilities of tumour control and the patient quality of life (IAEA, 2010; Perri *et al.*, 2014). The response rate to radiotherapy from a tumour is highly dependent on the proliferative capability of cells, because the majority die due to cell cycle checkpoints activation, leading to promotion of cell death. So, a tumour that has a high proliferative ratio will regress quickly, because its cell will die faster (IAEA, 2010).

There are a few mechanisms proposed as potential causes for radioresistance, however, the three major biological factors associated with this adverse effect are (1) intrinsic radiosensitivity, (2) hypoxia and (3) capacity of cancer cells to repopulate after the treatment (Baskar *et al.*, 2014).

Furthermore, it is important to highlight that the infection of HPV can influence tumour behaviour, such as the tumour vascularity. Since these patients respond better to the treatment than HPV-negative patients, it is hypothesized that the virus can modify one of the three factors highly related to radioresistance (Begg, 2012).

1.3.5.1 Intrinsic Radiosensitivity/Radioresistance

Several scientists worldwide have been hypothesized about a lot of genes and chromosomal regions that could be involved in intrinsic radiosensitivity/radioresistance to radiotherapy. However, as many predictor studies, these results lack independently validation and possible mechanisms that explain how they are involved (Begg, 2012).

One of the examples of how molecular alterations can be involved in radiation response is the ligand-independent activation of signal transduction pathways, as RTKs, such as EGFR, which plays an important role in the regulation of several downstream signalling pathways. Examples are the PI3K and its downstream effectors AKT and mTOR, signal transducer and activator of transcription (STAT) pathway and Ras-mitogen-activated protein kinase (MAPK) pathway. These pathways are involved in almost all hallmarks of cancer: cell cycle and cell survival, invasion, metabolism, angiogenesis and genomic instability. The PI3K-AKT-mTOR signalling pathway has been studied as one of the most important pathways involved in the resistance to cancer treatment (Bussink *et al.*, 2008; Baskar *et al.*, 2014).

Regarding chromosomal regions, it has been previous described an association between distal 11q loss and radioresistance (Parikh *et al.*, 2007). This loss is in the neighbouring of *ATM* gene, and the protein ATM is essential for survival of cells after irradiation. It was proposed that a chromatin protein, H2AX, plays a role in the induction and repair of DSBs. This protein, H2AX is phosphorylated, minutes after irradiation-induced DSB, by ATM. However distal loss of 11q is associated with decreased expression of *ATM* and *H2AXF*. The proteins ATM and H2AX are important members of ATM-CHECK2 DNA damage repair pathway and, since their levels are reduced, the cell upregulate ATR-CHECK1 pathway, resulting in a prolonged S and G2/M checkpoint arrest. The cells with this loss seem to "repair" the damage DNA in this prolonged arrest, enabling them to progress through the cell cycle without suffer mitotic catastrophe. This enables survival and result in decreased sensitivity to irradiation (Begg, 2012; Sankunny *et al.*, 2014).

Moreover, a lot of studies have been developed in order to discovery a panel of biomarkers responsible for the radioresistance seen in HNSCC tumours (Higo *et al.*, 2005; Toulany *et al.*, 2005; Ishigami *et al.*, 2007; Eckers *et al.*, 2014; Skvortsov *et al.*, 2014; Van Limbergen *et al.*, 2014).

1.3.5.2 Repopulation of cancer cells

The first evidence of repopulation of cancer cells came from a study that showed that local tumour control was decreasing when the overall duration of radiotherapy was increasing. The main reason behind this event was the enhancement of tumour cell proliferation due to the loss of cells during treatment (Bussink *et al.*, 2008).

HNSCC cells have a high capacity of rapid repopulation after irradiation treatment and that is one of the reasons that leaded to the study of other type of therapies, like accelerated fractionation. Cell proliferation could be affected by many factors such as differentiation stage, cell-cycle regulation and even microenvironmental factors, like hypoxia (Bussink *et al.*, 2008; Begg, 2012).

In the majority of cancers, high proliferation rate has been considered a bad prognostic factor, however, in HNSCC patients, the proliferation signatures were not predictive. It was proposed that the response capacity to HNSCC therapy could be a more important factor to predict the tumour

population rate than proliferation. This response capacity is hypothesized to correlate with the differentiation stage (Begg, 2012).

1.3.5.3 Hypoxia

The term hypoxia refers to a reduction in the normal level of oxygen tensions, being hypoxia a condition where tissues are not sufficient oxygenated, usually due to a deficient oxygen concentration in the blood. It is considered to be an important factor that leads to the metabolism reprogramming seen in cancer cells. When severe or protracted, it could result in cell death. Tumours become hypoxic because they develop new blood vessels that are aberrant and have a decreased blood flow. Hypoxic is toxic to both normal and cancer cells, since it is able to alter the DNA replication and genomic instability; however, the last ones undergo a series of genetic alterations that enable them to proliferate and survive in these extreme conditions. This effect is associated with an aggressive tumour behaviour and, thus, to a more malignant phenotype (Weinberg, 2013).

Solid tumours which undergoes rapid growth, exceed vascularization and the result is a lack of nutrients and oxygen supply. Within these conditions, the hypoxia-inducible transcription factor 1 (HIF-1) pathway becomes activated. In the lack of oxygen, HIF-1 binds to hypoxia-response elements (HREs). This ligation induces the expression of a series of target genes, involved in angiogenesis, glycolysis, growth-factor signalling, immortalization, genetic instability, tissue invasion and metastasis, apoptosis and pH regulation (Harris, 2002; Pereira *et al.*, 2013; Weinberg, 2013; Suh *et al.*, 2014).

Radiation creates DSBs in the DNA and these breaks can trigger cell cycle arrest and cell death. Oxygen can potentiate the production of reactive oxygen species (ROS)/free radicals, which are essential for the occurrence of the DSBs. With the growth of the tumours, the microenvironment lacks blood supply, leading to hypoxic areas. Since this hypoxic areas lack oxygen, the breaks in the dsDNA will not occur so easily and the desired effects of radiation would not happen (Hsu *et al.*, 2014). Furthermore, it is been reported that the DNA damage produced by free radicals can be repaired in hypoxic environments and, in opposite, at normoxic conditions, the oxygen is able to react with the DNA radicals and produce stable organic peroxides. Also, hypoxia is able to alter the expression and function of proteins that are involved in the DSB repair, such as the number of phosphorylated histone H2AX foci and it is also able to decreases the levels of the Rec A recombinase (RAD51) protein. By decreasing the levels of this protein, hypoxia inhibit homologous recombination DNA repair (Peitzsch *et al.*, 2014).

Hypoxia is frequent in HNSCC. It is important to highlight that hypoxia has been considered a negative prognosis factor for radiotherapy, chemotherapy and also surgery. It is well established that solid tumours may contain oxygen-deficient hypoxic areas and that cells in those areas can make tumours radioresistant. (Bussink *et al.*, 2008; Begg, 2012). In HNSCC, hypoxic areas are often

present and it is thought that they could be associated with the resistance to ionizing radiation that is often seen in patients (Koontongkaew, 2013; Perri *et al.*, 2014).

1.4 LAB TECHNIQUES

1.4.1 Cell Culture and Karyotyping

Primary cultures is the term used to refer to cultures that were prepared directly from a tissue or an organ. They can be subcultured for a certain amount of time, being called secondary cultures. The use of trypsin only become common in the 50s after Dulbecco described its utilization in monolayer cultures for viral plaque assays (Freshney, 2005; Alberts *et al.*, 2007). Grey, in 1952, was the responsible for a huge breakthrough in the field: he was able to establish the first continuous human cell line, the known HeLa cells (Freshney, 2005).

Cell culture have developed much and this is due especially to two branches of medical research: the demand for knowledge of neoplasia and the production of antiviral vaccines. Nowadays, tissue culture has a spectrum of applications (Freshney, 2005).

Commercial cell lines are an attractive option to be used as models for cancer research, since they are promptly and economically available. Furthermore, several of them are well described in literature regarding biochemical, pharmacological and genetic characteristics. However, one of the most important reasons is the fact that various cancer cell lines are well suited to allow the characterization of the pathophysiological properties of individual tumours. For example, the detailed study of cancer cell lines allows the forethought of several biomarkers and responses to target inhibiton (Dudley *et al.*, 2011).

It was in the XIX century that the chromosomes were, for the first time, identified. However, the right chromosome complement was only established in 1956. After this, the association between Down's syndrome and the presence of an extra chromosome was a big step for the utilization of karyotyping in research. In 1969, the development of chromosome banding allowed the detection of more subtle structural chromosomal abnormalities. Furthermore, the enhancement of the knowledge regard to malignant diseases and the development of the cell culture methods led to the first discovery of chromosomal abnormalities in malignant tumours. This discovery was a specific characteristic of chronic myelogenous leukemia, the Ph1 chromosome (Faas *et al.*, 2011).

Almost all of the chromosome banding methods are made by staining of the chromosomes in mitosis. This is achieved by treating cells with tubulin inhibitors, such as colchicine or colcemid. These tubulin inhibitors, inhibit microtubule polymerization by binding to the tubulin, arresting the cells in metaphase. After, the use of dyes, like Giemsa, allows distinction of AT-rich chromosomal regions from GC-rich chromosomal regions in a unique banding pattern for each chromosome. The utilization of dyes makes possible the association between chromosome abnormalities and diseases.

The most common chromosome banding methods are the G (Giemsa) banding, the R (Reverse) banding, the C (centromere) banding and the Q (Quinacrine) banding (Bickmore, 2001).

Conventional karyotyping is a highly reliable method to diagnose any aneuploidy and genomic structural rearrangement with about 5-10 million base pairs (bp) of length. Also, it allows visualization of all chromosomes. However, it also have some limitations associated. It is time consuming, since cell culture is an obligatory step. To the current days, its resolution is quite limited, which can lead to interpretation difficulties. It is necessary to have personnel with specific formation in order to analyse correctly the karyotype, leading to an increase in the costs. Finally, it also is hardworking and expensive (Boormans *et al.*, 2008; Faas *et al.*, 2011).

1.4.2 Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA)

The Multiplex Ligation-dependent Probe Amplification (MLPA) was developed in 2002 and is an extension of the Polymerase Chain Reaction (PCR). In opposite of the common PCR, MLPA is a technique were the DNA is not the one that is amplified, but instead the probes are. They are amplified using only one PCR prime pair, which allows a simultaneous and semiquantitative amplification of up to 50 MLPA probes in just one reaction (Schouten *et al.*, 2002).

MLPA comprises four steps: Denaturation and hybridization; Ligation; PCR amplification; Capillary electrophoresis and fragment analysis. Each MLPA probe is constituted with two oligonucleotides, one synthetic and other derived from the single chain bacteriophage M13. These oligonucleotides hybridize to adjacent locations from the target sequence. After successful hybridization to their target sequences, the ligase is added. At this point, the probe can be amplified by PCR. Every probe has identical final sequences, which allows the amplification by a universal primer pair labelled with a fluorophore. Each probe is able to produce an amplification product of unique length that will ranges from 130-480 bp. This is possible because one of the oligonucleotides has a stuffer sequence of unique length for each probe, allowing fragment separation and quantification accordingly to their length and fluorescence intensity by capillary electrophoresis (Figure 9) (Schouten *et al.*, 2002; MRC-Holland, Accessed: 22-05-2014).

MLPA is a method capable of detect gene copy number status, DNA methylation and point mutations simultaneously. In order to detect promotor hypermethylation, the variant of MLPA, MS-MLPA is used. In this variant, the methylation-sensitive restriction enzyme HhaI is the crucial component. However, there is an obvious limitation, this detection only happen in hot spots of methylation that contains the recognition site (GCGC) for HhaI. MS-MLPA is quite similar to MLPA, however, the samples are divided in two after the ligation step. One half is submitted to the endonuclease HhaI and the other remains undigested. If the sample is not methylated in the

recognition sequence of HhaI, it will cut the probe-sample DNA hybrid and the fragment would not be amplified. If the DNA is methylated, the enzyme would not be able to cut and the fragment will be amplified. In the analysis step, the uncut and the cut samples will be compared, allowing the determination of status' methylation (Homig-Holzel and Savola, 2012; MRC-Holland, Accessed: 02-01-2015).

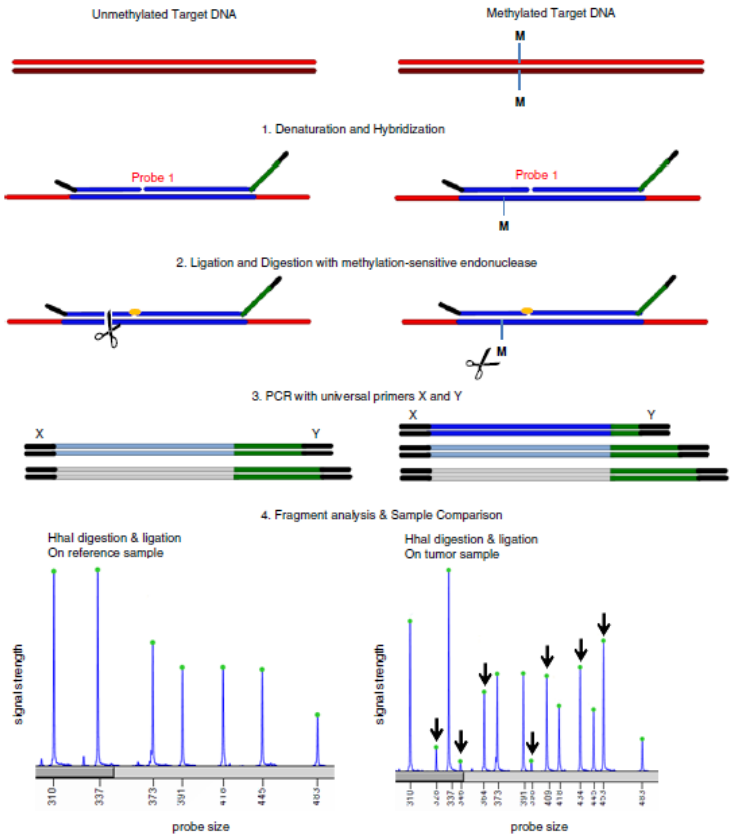

Figure 9 – The four steps of MS-MLPA reaction. Since the reaction is divided in two, two electropherograms are the final result, being the comparison result with sample references. At left we have a control sample, where the reference probes were amplified and the target probes are not (there is no signal), since the DNA is normally unmethylated. At right we have a tumour sample: the arrows are pointing for the probes that were not digested, since target was methylated. Adapted from: Hömig-Hölzel and Savola (2012)

1.4.3 Array Comparative Genomic Hybridization (aCGH)

In the last years, chromosomal microarray analysis (CMA), like comparative genomic hybridization, aCGH and single nucleotide polymorphism array, have become widely used in both research and diagnostic. CMA combines a high resolution analysis of variations in the copy number of all the genome with a short time of procedure. Also, it has a high income and allow the observation of alterations with 1kb of resolution (Armengol *et al.*, 2012; Evangelidou *et al.*, 2013).

The principle associated with aCGH is based in the detection of chromosomal deletions and duplications, by comparison of equal genomic DNA quantities between a patient and a healthy control. In aCGH, equal genomic DNA quantities from a control and a sample are labeled. One of them is labeled with Cyanine 3 (Cy3) whereas the other is labeled with Cyanine 5 (Cy5). They are

co-hybridized in an array with the DNA fragments immobilized. The analytic principle involve competition between de DNA fragments differently labelled. The slides are, then, transformed in images by a microarray scanner. The intensity of the spots is measured and the images are quantified by a software. The resulting ratio of fluorescence intensity is proportional to the ratio of the copy number of DNA sequences in the sample and in the controls. If the intensity of both fluorescence dies is equal for a probe, the corresponding region of the genome is considered to have the same amount of DNA between the sample and the control. If an alteration in the ratio Cy3:Cy5 is detected, the interpretation is that the sample as a gain or a loss of DNA in that region, when compared to the control (Figure 10) (Shinawi and Cheung, 2008).

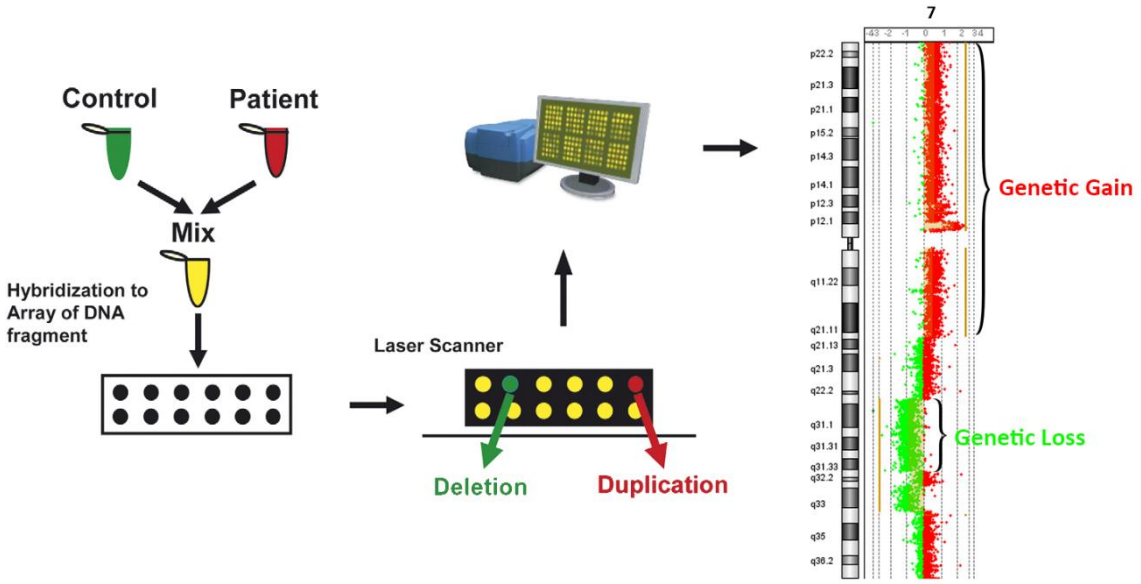

Figure 10 - Principles of the aCGH technology. Sample of DNA is labeled with the red dye (Cy5) and the control DNA with the green one (Cy3). Both samples are mixed and co-hybridize in an array with the genomic DNA targets. The ratio of intensity is analyzed. A Scanner of samples is responsible for the transformation of the results into images. At left we have an Array profile. Adapted from: Shinawi & Cheung (2008)

1.4.4 Flow cytometry

Flow cytometry (FC) is a multiparametric technique that allows detailed analysis of single cells in a fluid suspension regarding their intrinsic light-scattering properties, being evaluated for one or several extrinsic properties with resource of fluorescent probes. The fluorescent probe may bind directly to the target or a fluorescent dye may be coupled with an antibody probe, in order to detect some specific protein. After the labelling of cells, they are funnelled while passing through lasers of specific wavelengths. The flow rate of cells in the flow cytometer is rapid, allowing the analysis of thousands of cells in seconds. As the cells are travelling and passing through the different lasers, the fluorescent probes are excited, producing a specific light that passes through several filters and mirror sets, being finally collected and amplified by photomultiplier tubes. The last step in the conversion

of this light into digital signals that are visualized on a computer screen (Coleman and Tsongalis, 2006; Barnard, 2012).

One of the cellular events that is possible do analyse with FC is cell cycle. For this, propidium iodide (PI), is the most commonly used dye, that emits red fluorescence when excited with blue light (488 nm). PI is capable to bind to DNA by intercalating in the double strand. Furthermore, it also binds to RNA, being necessary to remove the RNA with a nuclease treatment (RNase) in order to achieve optimal DNA resolution. Since there is different amounts of DNA in the different cell cycle phases, it is possible to assess the amount of cells in each phase, once the quantity of fluorescence is proportional to the quantity of DNA. It is possible to categorize the cell in four different phases: the G0/G1 phase, the S phase, the G2/M phases (that have double DNA content) and the pre-G0 phase. This latter phase is associated to the fractional DNA content of apoptotic cells (Darzynkiewicz *et al.*, 2001).

Other cellular event that is commonly analysed by FC is cell death. This is possible due to the existence of characteristic markers for apoptosis and necrosis, as for annexin-V (AV) and IP. This allows the distinction between live and death cells and is even possible to distinguish apoptosis from necrosis (Darzynkiewicz *et al.*, 2001).

There is an alteration on the distribution of the membrane phospholipids during apoptosis. In the first steps of apoptosis, phosphatidylserine (a negative charged phospholipid) is translocated from the inner surface to the outside surface. AV is an anticoagulant protein that binds phosphatidylserine with high affinity in the presence of calcium. Binding of AV to a fluorophore allows the determination of the localization of phosphatidylserine on cellular membrane, leading to the identification of cells in early apoptosis. During necrosis lysis occurs after cellular swelling, causing the cellular components to be exposed to the intercellular space, allowing IP to intercalate with DNA (Darzynkiewicz *et al.*, 2001).

This combination allows the identification of four categories in cell death: live cells (AV and PI negative); early apoptotic cells (AV positive and PI negative) and late apoptotic as well as necrotic cells (PI positive) (Darzynkiewicz *et al.*, 2001).

1.4.5 Comet Assay

The comet assay is a simple method that allows the measure of DNA strand breaks in eukaryotic cells (Collins, 2004). The basic principle associated to this mini-gel electrophoresis technique is the DNA migration in agarose in electrophoretic conditions (Speit and Rothfuss, 2012). The cells are embedded in a tiny agarose layer on a slide, are lysed with detergent plus a high salt concentration in order to form nucleoids with supercoiled DNA loops attached to the nuclear matrix. After they are subjected to electrophoresis, which can be performed in neutral, alkaline or super-

alkaline conditions. The electrophoresis is responsible for the DNA migration of the loops which contain DNA breaks, leading to the formation of a comet that is observable with fluorescence microscopy (Collins, 2004).

2 AIMS

In spite of major advances in technological and molecular fields in regard of head and neck cancer study, the 5-year survival rate for these patients remains very low. One of the biggest problems is the late diagnosis of the disease, wherein 70-85% of all HNSCC patients are presented with a late stage (stages III/IV) at diagnostic's time (Langer, 2008). These patients are normally treated with a complex therapeutic approach that includes chemotherapy, surgery and radiotherapy. However, this approach has a high toxicity rate and several patients develop resistance to therapy.

Radiotherapy is widely applied for cancer treatment, however, late tissues toxicity are a major problem in normal cells after this approach. Furthermore, resistance to radiotherapy is very common and it is related with a worse prognosis. All sum up, the use of radiotherapy, in spite its major benefits in treating cancer, is also associated to several problems, some of them appearing many years after the treatment. With this in mind, the need to find biomarkers associated to radiotherapy response in patients it is an essential step towards an increased 5-year survival rate for HNSCC patients, minimizing the damages that patients often suffer.

Thus, it is essential to understand the effects of radiation, particularly the differences between radiation responses, to try to prevent the major toxicity problems in patients that are more sensitive to radiation. Then, identify some genetic alterations that could help to distinguish radiosensitive from radioresistant tumours is of utmost importance to access this major problem. Furthermore, little is known about the alterations that happen in cells after they were submitted to different doses of radiation. For example, adaptive response to low doses are described to be other of the radiation problems, but the alterations behind this response are unknown.

The goal of this study is to unveil some genetic and epigenetic alterations that could be responsible for different responses of the tumour to different radiation doses, in order to improve the knowledge of this clinical problem in radiobiology. Furthermore, it is our aim to contribute to the understanding of radioresistance in HNSCC, accessing some alterations that could be associated to better or to worse responses to this kind of treatment. For this, two commercial cell lines were used: HSC-3 and BICR-10 cell lines.

Due to possible differences induced by hypoxia in response to radiation treatment, once the lack of oxygen is considered to be a relevant cause of radioresistance in tumours, we also studied these cell lines after they were submitted to hypoxic conditions for a further acknowledge of the different responses that irradiation can have on hypoxic cells, frequently observed in solid tumours.

In order to achieve this goals, the epigenetic and genetic characterization of the two HNSCC cell lines was performed using MS-MLPA, aCGH and karyotyping techniques. This analysis was

conducted before and after the two cell lines were treated with the same type of radiation usually applied to HNSCC patients. We intend, this way, to analyse the differences in the copy number and methylation patterns induced by radiation to the cells.

At the same time, since commercial cell lines are one of the most used models in research and clinical investigation, we will contribute to a better genetic and epigenetic characterization of both cell lines, in order to understand and study the characteristics of cancer cells and their development. A better characterization of commercial cell lines improve the translation of the results obtained from experiences to what happens with the patient's cells, leading to a better understanding of the disease, better therapeutic approaches and better survival rates.

3 MATERIAL AND METHODS

3.1 CELL LINES AND CULTURE CONDITIONS

The cell lines used in this study are the commercial cell lines BICR-10 and HSC-3.

BICR-10 is an adherent keratinocyte cell line derived from a recurrent squamous cell carcinoma of the buccal mucosa of a caucasian female, with markers for keratin and involucrin. Known mutations of this line are in *TP53*, *CDKN2A* and *CDKN2A*^{ARF} (Sigma, Accessed: 02-02-2015). This cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco®, Life Technologies, California, USA) containing 10% Fetal Bovine Serum (FBS) (Gibco®, Life Technologies, California, USA), 1% of hydrocortisone (Gibco®, Life Technologies, California, USA) and 1% of penicillin and streptomycin (Gibco®, Life Technologies, California, USA).

HSC-3 is a human oral squamous cell carcinoma cell line and it was derived from a Japanese male with 64 years old. It was from a primary tumour from the tongue with lymph node metastasis and was moderately differentiated (JCRB, Accessed: 02-02-2015). This cell line was cultured in DMEM medium containing 10% of FBS and 1% of penicillin and streptomycin.

Controls were obtained from healthy patients undergoing surgical removal of wisdom teeth. They were cultured in DMEM medium containing 1% of FBS, 1% of hydrocortisone and 1% of penicillin and streptomycin.

Both cultures and controls were maintained in an incubator at 37ºC in a 5% CO_2 atmosphere with 65% humidity. After reaching confluence, the samples were subcultured by incubation with a solution of trypsin (Gibco®, Life Technologies, California, USA) and seeded into fresh tissue culture flasks.

In order to further analyse the effects of irradiation on hypoxic cells, both cell lines were incubated for 48 hours in a controlled environment chamber (Plas-Labs, Lansing, MI) at 37ºC in a 93% N_2, 2% O_2, and 5% CO_2 atmosphere.

3.2 MORPHOLOGY CHARACTERIZATION

The photographs of the cells were accessed with the help of an inverted microscope (Motic AE31, Ted Pella, California) and the camera Axio Cam ERc55 (Zeiss, Germany). The software utilized to process the photographs was ZEN 2011 (Zeiss, Germany).

3.3 RADIATION TREATMENT

The irradiation was performed at Serviço de Radioterapia do Centro Hospitalar e Universitário de Coimbra by experts on Medical Physics, on a Varian Clinac 600C linear accelerator (Varian Medical Systems) with a photon beam 4 MV used in clinical practice for treatment. The cells were irradiated and packaged in a irradiation box specially designed for this purpose in the Departamento de Física da Faculdade de Ciências e Tecnologia da Universidade de Coimbra. The box was constructed of acrylic material with walls with 1 cm of thickness. The dimensions of the box and the positioning references that the box has guarantee positioning conditions and reproducible packaging as well as ensure uniformity of radiation dose. Initially it was conducted a dosimetric study led by computed tomography, thereby generating a three-dimensional planning with the planning system Eclipse™ (Varian Medical Systems), in order to guarantee homogeneity in the distribution of radiation dose. The dimensions of the irradiation box and of the irradiation field (40 cm x 40 cm), as well as the distance from the beam source to the box center allowed the calculation of the irradiation time required to administer the required dose, which is measured in Monitor Units (MU).

To carry out the cells irradiation, the irradiation box was placed on the treatment table containing the vials, and the remaining empty space was filled with distilled water at a temperature of 37ºC. The positioning of the box was driven through a reference system of orthogonal lasers. The dosing was carried out using two side fields, by rotation of the gantry at 90º and 270º, to ensure a homogeneous dose distribution in accordance with planning. For all doses the collimator and the table were placed at 0º, with all multileaf collimator blades collected.

In HSC-3 cell line case, the cells were exposed to 0,5 Gy, 1 Gy, 2 Gy, 3 Gy, 5 Gy and 10 Gy. In BICR-10 cell line case, the cells were exposed to 0,5 Gy, 2 Gy, 3 Gy, 5 Gy, 10 Gy and 15 Gy. In Table 3, there is an example of the MU required for two of the irradiation doses applied.

Table 3 - Two of the doses applied to the samplesin the irradiation box.

Dose	**1 Gy**	**3 Gy**
***Gantry* at 90º**	46 MU	139 MU
***Gantry* at 180º**	47 MU	140 MU

3.4 CLONOGENIC ASSAY

Clonogenic assay is an *in vitro* study used to determine the cellular survival of a population. It is based on the capacity of a single cell to grow and divide in order to originate colonies after the population has been submitted to some treatment/effect.

After irradiation, the cells, BICR-10 and HSC-3, were seeded into six-well plate and cultured for approximately 14 and 21 days, respectively, in order to allow colonies formation. The base protocol was the one published by Franken *et al.* (2006). After colonies formation, they were stained with a solution of crystal violet (Sigma M2128, USA; 0.5% diluted in methanol) and counted (Figure 11).

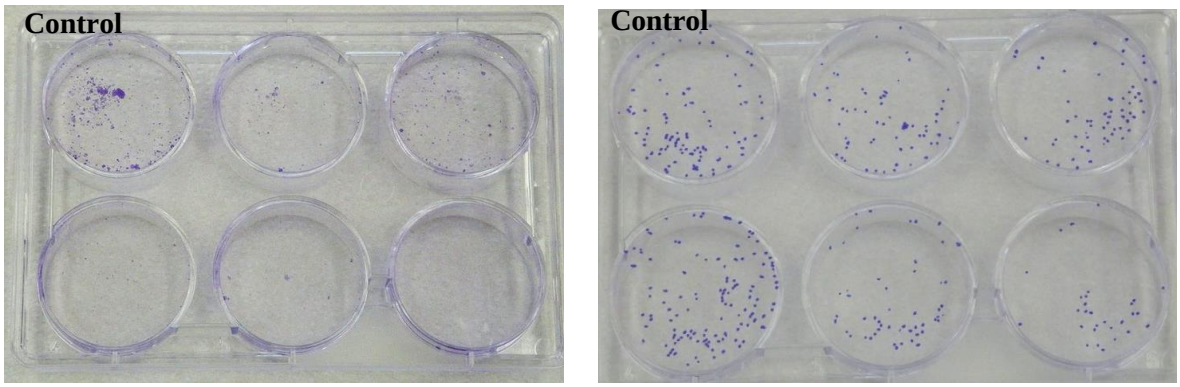

Figure 11 – Aspect of the six wells after the performing of Clonogenic Assay. At left is a representation of HSC-3 results and at right is a representation of BICR-10 results. The first well represents, in both cases, the control cells.

The plate efficiency (PE) was determined as the ratio between the number of colonies and the number of cells seeded and the survival factor (SF) as the ratio of plating efficiencies for irradiated and nonirradiated cells.

$$PE = \frac{Number\ of\ Colonies\ Formed}{Number\ of\ cells\ seeded} \times 100$$

$$SF = \frac{Number\ of\ Colonies\ Formed\ after\ treatment}{Number\ of\ cells\ seeded \times PE} \times 100$$

These experiments were performed three to four times for each condition and a survival curve for each cell line was delineated. Furthermore, the median lethal dose (LD50) of each cell line was calculated. The colonogenic assay was performed to both cell lines in both, normoxic and hypoxic conditions. However, the survival curve and LD50 was only calculated for normoxic conditions (Figure 22).

3.5 COMET ASSAY

To access the damage in cell DNA, an alkaline single-cell gel electrophoresis was performed. The procedure was applied to both cell lines before and after radiation treatment and in normoxic and hypoxic conditions. The procedure was carried out accordingly to Olive and Banath (2006) . Briefly, a cellular suspension of 5×10^4 cells was prepared in PBS for each condition. The positive control was prepared from the untreated cells, by the administration of 20 ηM of hydroxide peroxide (Sigma Aldrich, USA) for 15 minutes.

Cellular suspensions were diluted 1:1 in 1% low melting point agarose and applied in Starfrost slides. These slides were previously precoating with 1% normal melting point agarose. After application of the cellular suspension on the slides, they were maintain at -4ºC for 30-45 minutes in order to allow agarose to gel. The slides were then submerged in alkaline lysis solution (2,5M NaCl, 100mM EDTA, 10mM Tris, 10% DMSO and 1% Triton x-100) (Sigma Aldrich, USA) for about 20 hours at 4ºC. Afterwards, slides were equilibrated in alkaline electrophoresis buffer (300mM NaOH and 1mM EDTA, pH>13) (Sigma Aldrich, USA) and submitted to a potential difference of 25V and current of 1A for 15 minutes. After this slides were incubated with neutralizing buffer (0,4M Tris, pH 7,4) and stained with 25ηg/mL of ethidium bromide for 20 minutes. The slides were then washed with destilated water and visualized in an epifluorescent microscope (Eclipse-400, Nikon) with excitation at 546nm with 100W mercury lamp with emission at 580/10. Image acquisition was performed with a Texas Red filter (G-2A, EX510-560 - Nikon) using Cytovision software (Applied Imaging System).The comet images obtained were then analysed using the software CometScoreTM v1.5, in order to calculate tail moment.

These experiments were realized three times in an independent way, 38-39 hours after irradiation. The comet assay was applied to several conditions: the untreated cells, cells submitted to LD50 dose of radiation, cells in hypoxic conditions and cells in hypoxic conditions submitted to LD50 dose of radiation.

3.6 FLOW CYTOMETRY

Flow cytometry (FC) was used to evaluate the effects of X-radiation in cell viability, types of induced cell death and changes in cell cycle on both cell lines in normoxic and hypoxic conditions. The conditions to which it was apllied were to the untreated cells, cells submitted to LD50 dose of radiation, cells in hypoxic conditions and cells in hypoxic conditions submitted to LD50 dose of radiation.

The analysis was performed using a six parameter, four-color FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA). For each assay, ten thousand events were collected using CellQuest software (Becton Dickinson, San Jose, CA) and analysed through Paint-a-Gate software (Becton Dickinson, San Jose, CA) and Modfit™ software (Verity Software House).

These experiments were realized two times in an independent way, 37h30'-38h after irradiation.

3.6.1 Cell death evaluation

By FC is possible to analyse cell death due to the use of two different markers, annexin-V (AV) and Propidium Iodide (PI). These two markers allowed the distinction between live and death

cells. Furthermore, they make possible to distinguish which cells are suffering apoptosis and/or necrosis. So, both cell lines in all the conditions described earlier were stained simultaneously with Annexin-V – Allophycocyanin (BD Biosciences) and with PI (BioLegends). This assay discriminates amongst intact cells (AV-/PI-), early apoptotic cells (AV+/PI-) and late apoptotic or necrotic cells (AV+/PI+).

To analyse the influence of X-radiation on cell viability and on the types of induced cell death, 5×10^5 cell per probe were used. The cellular suspension was washed in PBS by centrifugation (1000xg for 5 minutes) and then the pellet was resuspended in 100µl of binding buffer. Cells were incubated during 15 minutes with 1 µL of AV and 5µL of PI (Kit Immunotech, Marseille, France). Then 300µL of binding buffer was added and finally the cells were analyzed on the cytometer. The emission wavelength was 650nm and the excitation wavelength was 660nm. The results are presented as percentage ± standard deviation (SD) according to the double staining results.

3.6.2 Cell cycle analysis

For the cell cycle analysis, the PI/RNase detection probe panel (Immunostep, Salamanca, Spain) was applied. 1×10^6 cells were used to study the influence of irradiation on cell cycle on both cell lines, in normoxic and hypoxic conditions.

The cells were fixed with 200µL ice-cold ethanol (70%) while vortexing. They were then, incubated on ice during 30 minutes for fixation purposes. Afterwards they were washed with PBS and incubated for 15 minutes with 300µL of PI/RNase solution, at room temperature. Finally, cells were excited at a wavelength of 488nm, with an emission filter of 617nm. The data was analysed by the Modfit™ software. The results were presented as a percentage mean ± SD of cells in each cell cycle's phase.

3.7 KARYOTYPING

Semi confluent cell cultures were incubated for 1 hour with colcemid (Gibco®, Life Technologies, California, USA). After incubation, the cells were harvested and the slides were banded according to routine protocols used in the Cytogenetic and Genomic Laboratory. Chromosomes of 10 metaphases were counted and analysed. Metaphases were digitally imaged and karyotyped resorting to a microscope (Eclipse-400, Nikon) and Cytovision software (Applied Imaging System). Karyotype description followed the International System for Human Cytogenetic Nomenclature 2013 recommendations. The analysis was performed on control cell lines, before irradiation, and cell lines submitted to irradiation.

3.8 DNA EXTRACTION, QUANTIFICATION AND ASSESSMENT OF PURITY

DNA was extracted from the culture cell lines using High Pure PCR Template Preparation Kit (Roche GmbH, Manmheim, Germany), according to the manufacture's recommendations (Roche, Accessed: 02-02-2015).

The next step was DNA quantification (ng/µL). This was performed with the use of the spectrophotometer NanoDrop-1000 (Thermo Scientific, Wilmington, USA). This method is also able to determine the DNA purity, which was evaluated using the ratio between absorbance at 260 nm and 280nm (A260/A280) and between absorbance at 260nm and 230nm (A260/A230). The DNA is considered to be pure when value of the ratio A260/A280 is approximately 1,8 and the value of the ratio A260/A230 is between 1,8 and 2,2 (Thermo, Accessed: 02-02-2015).

3.9 ARRAY-CGH

aCGH was the technique used for Copy Number Variations (CNV) analysis, through Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (Agilent Technologies, Santa Clara, California, USA), according to the manufacture's recommendations (Agilent, Accessed: 30-04-2015). aCGH was carried out using an Agilent SurePrint G3 Human Genome Microarray 18K (Agilent Technologies, Santa Clara, California, USA), an oligonucleotide microarray containing approximately 180 000 probes scattered throughout the genome.

Briefly, DNA from each sample was labelled with Cy5 by random primer labelling. The control used was labelled with Cy3. After the labelling, DNA was purified and the degree of labelling was obtain resorting to NanoDrop-1000 (Thermo Scientific, Wilmington, USA). The expected specific activity of Cy3 or Cy5 labelled samples with a 1µg input of gDNA is 25-40pmol/µg and 20-35pmol/µg, respectively. Excess of primers and nucleotides were removed using Amicon 30-kDA individual filters (Millipore, Billerica, MA, USA). Afterwards, both DNAs (sample and control) were combined with Human Cot-1 DNA (Kreatech Diagnostics, Amsterdam, Netherlands), treated with Agilent blocking agent and 2x Hi-RPM buffer and hybridized in a 4x180K oligonucleotide slide, at 65°C for 24h in a hybridization oven (Agilent Technologies, Santa Clara, California, USA) at a constant rotation of 20rpm. The slides were then scanned with Scanner C (Agilent Technologies, Santa Clara, California, USA) and the data was processed with the Feature Extraction Software v10.7. These data were analysed resorting to an aberration calling algorithm, ADM-2, and a threshold filter that requires at least three contiguous probes. The last step was the analysis of the results with Agilent Genomic Workbench v6.5.

aCGH was applied to both cell lines untreated and to both cell lines submitted to 0,5 Gy and 10 Gy of irradiation.

3.10 MS-MLPA

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), a modification of the conventional MLPA assay, allows for the simultaneous detection of changes in methylation status as well as in copy number variations. The MS-MLPA technique was performed as previously described by Nygren *et al*. (2005). The probe panel used was the MRC-Holland SALSA MS-MLPA probemix ME002-C1 Tumour Suppressor-2 (MRC-Holland, Accessed: 05-03-2015). It was applied to the samples previous and after the radiation treatment, in both normoxic and hypoxic conditions. All the reagents utilized were acquired by MRC-Holland (Amsterdam, The Netherlands), except for the restriction enzyme *HhaI* (Promega, Madison, USA).

This probe panel contains 41 MS-MLPA probes, 27 of those recognize different *HhaI* restriction sites in their regions, and it allows the analysis of the methylation status of the promoter region of 25 different tumour suppressor genes. Such genes are frequently silenced by methylation in tumour cells. Besides, it includes 14 methylation reference probes that are not affected by the restriction enzyme *HhaI*. Furthermore, all 41 probes are able to determined copy number variations of the samples (Figure 12).

Briefly, the DNA (100ηg) was denatured for 10 minutes at 98ºC and subsequently cooled to 25ºC. After the addition of the probe mix, the samples were heated to 98ºC for 1 minute and maintained at 60ºC during 15-16 hours, allowing hybridization. The thermocycler used was the ABI 2720 (Applied Biosystems, Foster City, USA).

After hybridization, the samples were divided into two: half of the sample was only incubated with the ligase enzyme, whereas the other half was combined with the restriction enzyme while ligation occurred. This latter digestion resulted in ligation of only the methylated sequences. This reaction was performed at 54ºC during 15 minutes. After inactivation of ligase enzyme, the multiplex PCR reaction (35 cycles of 30 seconds at 95ºC, 30 seconds at 60ºC and 1 minute at 72ºC) was carried on. After the PCR reaction, 0,70μL of the sample were combined with 9,4μL of a solution with *Rox™* (Applied Biosystems, Foster City, USA) and highly deionized formamide (Applied Biosystems, Foster City, USA) to allow fragment separation. The capillary electrophoresis was performed using the capillary sequencer ABI PRISM™ 3130 (Applied Biosystems, Foster City, USA). Three reference controls and one negative control were used for each experiment. The results were analysed with the help of the software GeneMapper v4.1 (Applied Biosystems, Foster City, USA), which allows the analysis of the electropherograms. Automated fragment and data analysis was performed with the program Coffalyser.Net (MRC-Holland, Amsterdam, The Netherlands). This program determined the ratio between tumour samples and controls. The interpretation of the copy number status is described in Table 4. According to literature and lab experience, aberrant methylation was considered when the calculated methylation ratio was 0,20 or greater.

Table 4- Interpretation of Copy Number Status obtained from Coffalyser.Net.

Ratio	Copy number status
≥ 1,2	Numerical Gain/ Amplification
0,8 - 1,2	Normal
< 0,8	Numerical Loss

MS-MLPA was applied to both cell lines in normoxic and hypoxic conditions before and after irradiations, accordingly to Table 5.

Table 5 – Conditions to which the MS-MLPA was applied.

	Untreated	0,5 Gy	1 Gy	2 Gy	3 Gy	5 Gy	10 Gy	Hypoxia	Hypoxia + 1 Gy	Hypoxia + 3 Gy
HSC-3	✓	✓	✓	✓	-	✓	✓	✓	✓	-
BICR-10	✓	✓	-	✓	✓	✓	✓	✓	-	✓

3.11 STATISTICAL ANALYSIS

The graphics for clonogenic assay and LD50 calculation were obtain with resource to the software GraphPad Prism 5.

For analysis of the results obtained with comet assay we used the software SPSS Statistics for Mac v21.0. The non-parametric Kruskal-Wallis test was apllied for comparison of quantitative variables in more than two goups and the Bonferroni correction was applied. It was considered a significance level of 5%.

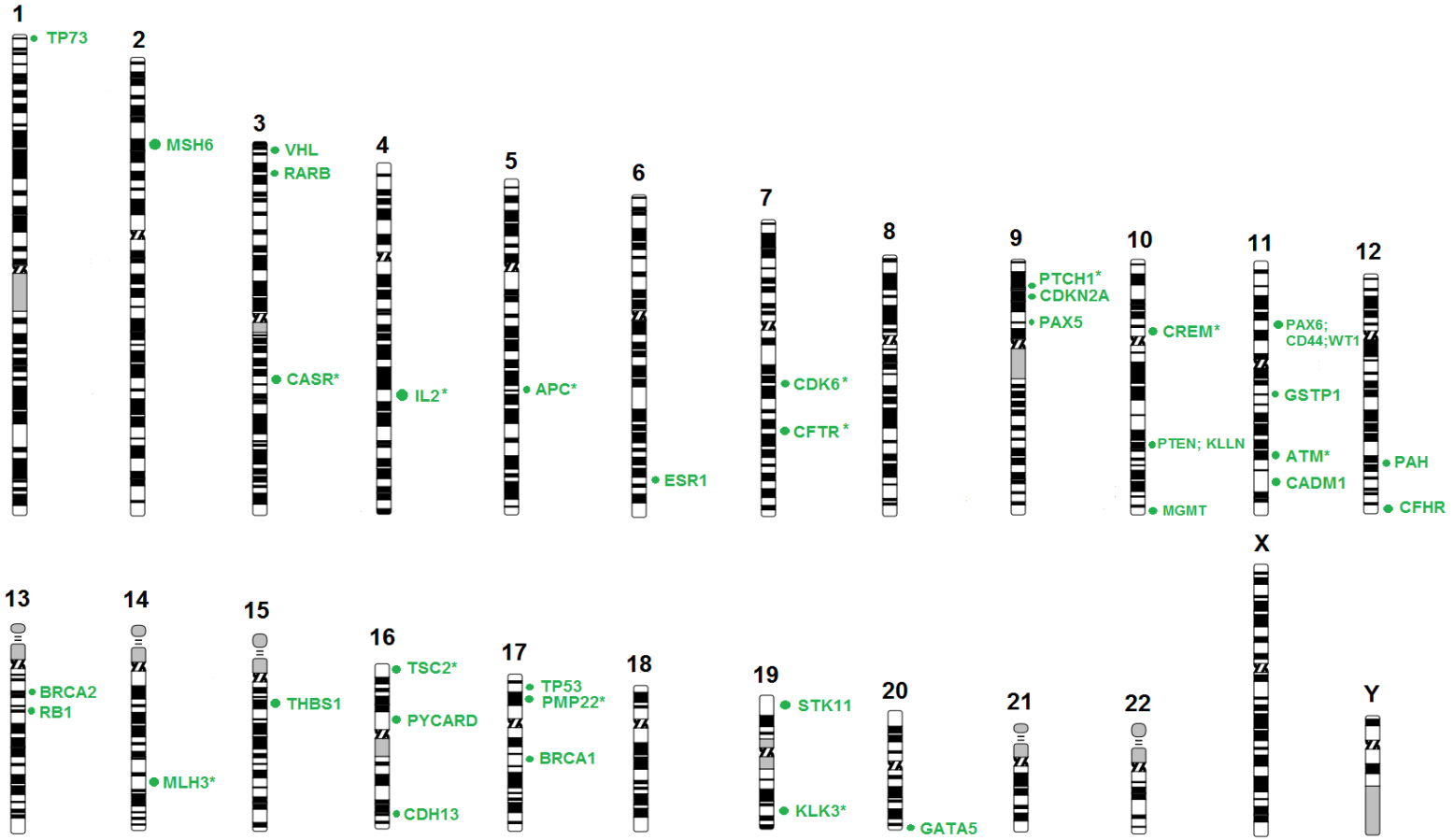

Figure 12 - ***Chromosomal distribution of the genes studied using SALSA MS-MLPA ME002-C1 tumour suppressor-2 probemix****. Reference probes are represented by *. APC - Adenomatous polyposis coli; ATM - ATM serine/threonine kinase; BRCA1 - Breast cancer 1; BRCA2 - Breast cancer 2; CADM1 - Cell Adhesion Molecule 1; CASR - Calcium-sensing receptor; CD44 - CD44 molecule; CDH13 - Cadherin 13; CDK6 - Cyclin-dependent kinase 6; CDKN2A - Cyclin-dependent kinase inhibitor 2A; CFTR - Chloride channels, ATP-gated CFTR; CHFR - Checkpoint with forkhead and ring finger domains; CREM - cAMP responsive element modulator; ESR1 - Estrogen receptor 1; GATA5 - GATA binding protein 5; GSTP1 - Glutathione S-transferase pi 1; IL2 - Interleukin 2; KLK3 - Kallikrein-related peptidase 3; KLLN - Killin, p53-regulated DNA replication inhibitor; MGMT - O(6)-methylguanine-DNA methyltransferase; MLH3 – mutL homolog 3; MSH6 - mutS homolog 6; PAH - Phenylalanine Hydroxylase; PAX5 - Paired box 5; PAX6 - Paired box 6; PMP22 - Peripheral Myelin Protein 22; PTCH1 - Patched 1; PTEN - Phosphatase and tensin homolog; PYCARD - PYD and CARD domain containing; RARβ - Retinoic acid receptor beta; RB1 – Retinoblastoma; STK11 - Serine/threonine kinase 11; THBS1 - Thrombospondin 1; TP53 – Tumour protein 53; TP73 - Tumour protein p73; TSC2 - Tuberous Sclerosis 2; VHL - von Hippel-Lindau tumour suppressor; WT1 - Wilms tumour 1.*

4 RESULTS

4.1 CHARACTERIZATION OF HSC-3 AND BICR-10 CELL LINES

4.1.1 Cell Line's Morphology

In spite of the fact that both cell lines are from the oral cavity, they are derived from different anatomical sites. BICR-10 is derived from buccal mucosa, whereas HSC-3 cell line is derived from the tongue. Since they are both squamous cell carcinomas, from different histological places, it is normal that they differ in their histological appearance (Figure 13). While BICR-10 cell line appears more elongated, HSC-3 cell line has a more rounded appearance. It is noteworthy that, even on the cellular size, they are both very dissimilar. The BICR-10 cell line has bigger cells than the HSC-3 cell line.

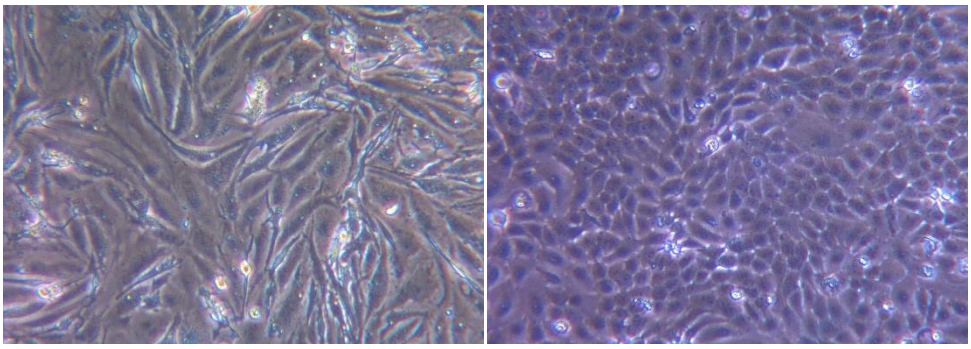

Figure 13 - At left it is represented an image of BICR-10 cell line and at right an image of HSC-3 cell line. Photographes were taken by an Axio Cam ERc55 Camera (Zeiss, Germany).

4.1.2 Karyotyping

Karyotyping analysis of BICR-10 and HSC-3 cell lines showed a big difference between the number of chromosomes in the different cells analysed: BICR-10 cell line presented on average 41 chromosomes whereas HSC-3 cell line showed 57 chromosomes, on average.

The most common alterations observable in BICR-10 cell line were structural alterations. It was possible to notice der(1), der(2)t(1;2), t(3;5)(p10;q10), i(5)(p10) i(7)(p10), der(7)t(7;11), der(8;8), der(8)t(5;8), del(9)(pter), der(15)t(X;15)(q10;q10) and der(17)t(7;17) (Figure 14).

In HSC-3 cell line, it was possible to observe not only structural abnormalities, but also numerical ones. Regarding the latter, in addition to the monosomy of 10 and 18 that appeared frequently, we also found the trisomy of 9 and 20 to be common events. The structural abnormalities appeared in a higher number and the most common were der(X)t(X;8)(q10;q10), i(1)(q10), del(1)(p10), t(2;15)(q10;q10), del(2)(q10), del(5)(q?), del(7)(q?), t(11;13), dup(11q), der(13;13)(q10;q10) and der(14)t(14;17)(q10;q10). The loss of chromosome Y was also very

frequent in this cell line (Figure 15 and Figure 23). The aberration on chromosome 4, 4q$^-$, was also identified, although it only appear in 30% of the metaphases analysed.

Besides the differences between the number of chromosomes, it is possible to observe that HSC-3 has a greater number of abnormalities in comparison to BICR-10 cell line.

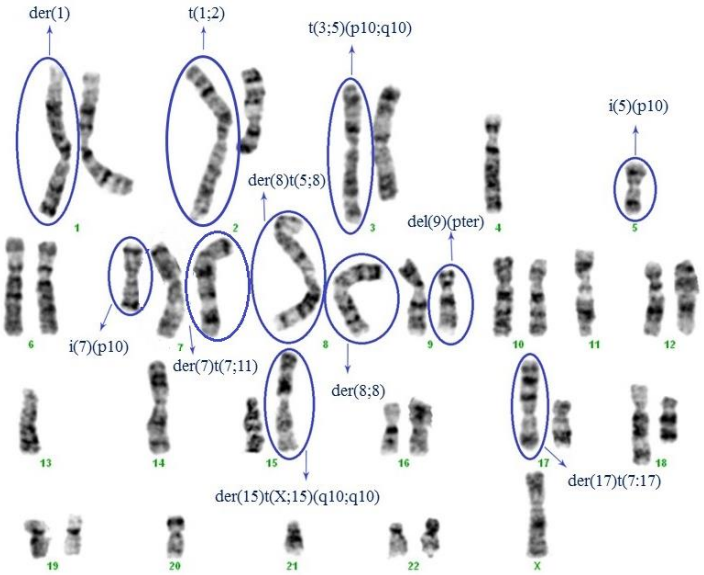

Figure 14 - Karyogram from BICR-10 cell line with the most common aberrations identified.

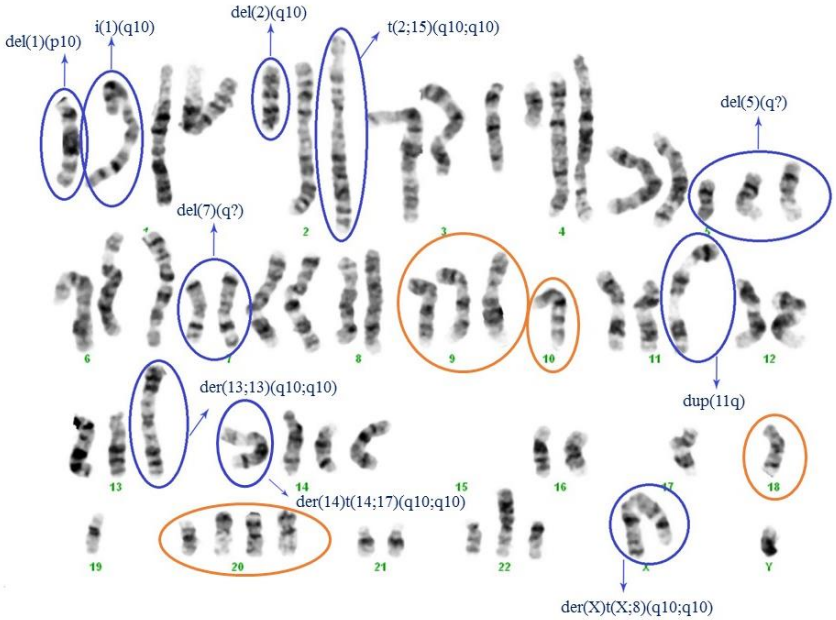

Figure 15 – Karyogram from HSC-3 cell line with the most common aberrations identified. Blue: Structural Aberrations. Orange: Numeric Aberrations.

4.1.3 MS-MLPA

Both cell lines were genetically and epigenetically characterized by MS-MLPA using *SALSA MLPA probemix ME002-C1 Tumour Suppressor-2*. The software GeneMapper v4.1 allowed to obtain electropherograms with a peak pattern for each sample.

MS-MLPA probe sets contain quality control fragments that are able to signalize problems that may alter the results obtained (Table 6).

Table 6- Quality control fragments of MS-MLPA.(MRC-Holland, Accessed: 08-07-2015)

Name	Length (nt)	Interpretation
92 nt benchmark probe	92	Normal probe, which forms a benchmark that allows the comparison for other quality control fragments.
Q-fragments	64, 70, 76, 82	These fragments are high when the amount of DNA is too low or the ligation step has failed. When all Q-fragments have signals ≥ 33% of the 92 nt control fragment, the DNA quantity in the sample is insufficient.
D-fragments	88, 96	These fragments are low when a poor sample denaturation occurred. When the signal is ≤ 40% of the 92 nt control fragments, the denaturation of DNA had problems.
X & y fragments	100, 105	These two fragments are the control for sample swapping.

In each reaction, three control samples and one negative control were used. The negative control used was water and, since there is no DNA present in water, the Q-fragments signals were greater than 33%, in relation to the 92 nucleotide (nt) control fragment.

Regarding the control samples, they presented an electrophoresis peak pattern without any genetic or epigenetic abnormalities (Figura16a and Figure16c). This is also evidenced by the ratio charts that have the analysed data arranged by genomic location, since all the probes are localized between the limit lines. Above the blue line represent gain of genetic material and under the red line means loss of genetic material (Figura16b). Regarding the methylation pattern, when the probes are localized above the blue line, the gene is methylated (Figura16d).

On the other hand, the electropherograms and ratio charts for the samples analysed had alterations. Using BICR-10 cell line as an example (Figure 17) we have a few probes outside the limit lines and we have increased and decreased peaks on the electropherogram. Regarding the CNV, for example, we have gain of genetic material of the *VHL1* (3p25.3), *RARβ* (3p24.2) and *APC* (5q22.2) genes, since the probes for this genes have higher peaks on the electropherogram and are above the blue line on the ratio chart. BICR-10 shows loss of *CFTR* (7q31.2), *CDKN2A* (9p21.3), *BRCA2* (13q13.1), *RB1* (13q14.2) and *TP53* (17p12) genes, since the probes have decreased peaks

on the electropherogram and are underneath the red line on the ratio chart (Figure 17a and Figure 17b).

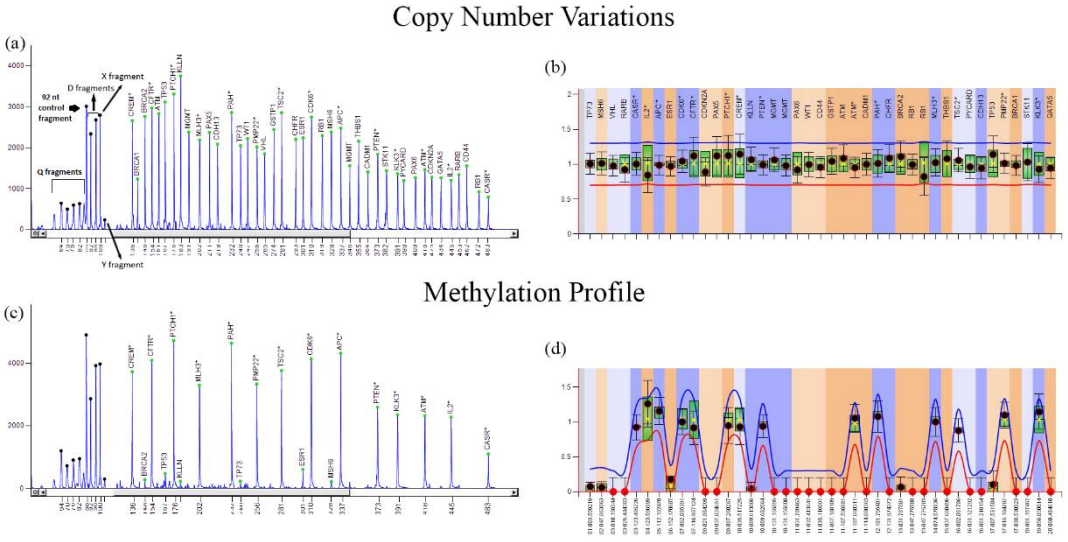

Figure 16 - Electropherograms and ratio chart with the data from copy number variations(a), (b) and methylation profile (c), (d) of a control sample. Obtained with the software GeneMapper v4

It is important to highlight that concerning the methylation analysis, only the gene probes which do not have restriction site for HhaI enzyme, or the ones that are methylated, can be amplified in the PCR reaction. This will lead to less peaks and probes on the electropherogram in comparison with the data from CNV. In both ratio chart and electropherogram of BICR-10 cell line is possible to distinguish which genes were methylated in the sample seeing as they are the ones with higher peaks or the ones above the blue line, as, for example, *RARβ* (3p24.2) and *WT1* (11p13) (Figure 17c and Figure 17d).

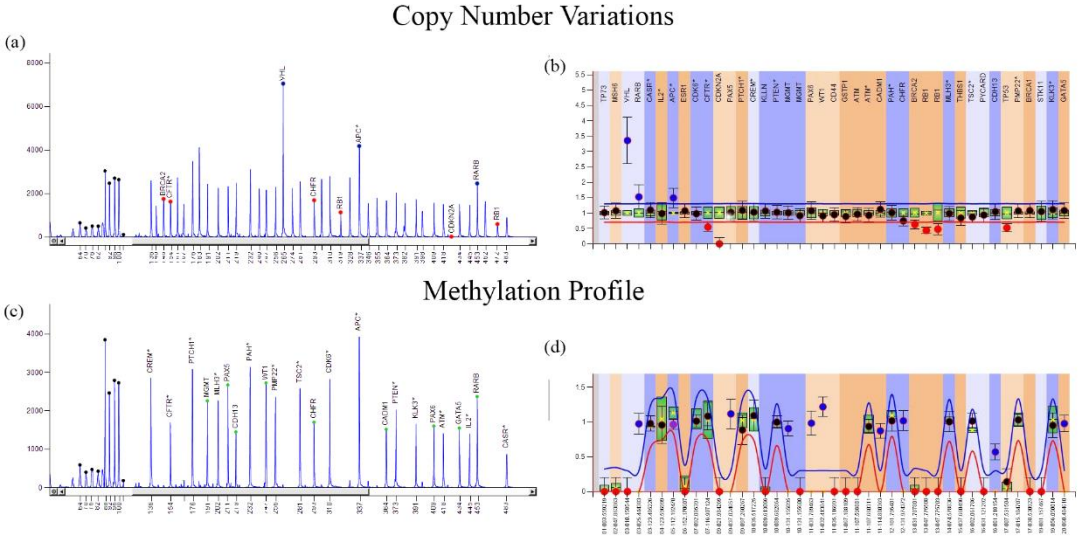

Figure 17 - Electropherograms and ratio chart with the data from copy number variations(a), (b) and methylation profile (c), (d) of BICR-10 cell line. Obtained with the software GeneMapper v4.

4.1.3.1 Methylation Profile

For the analysis of the methylation profile of both cell lines, a cut-off was defined to determine which genes were methylated. So, when the methylation percentage of gene was equal or greater than 20%, the gene was considered to be methylated. This cut off was defined according to literature and previous lab experience in methylation profile analysis of cancer cells.

Applying this cut-off, only nine genes for each cell line were considered to be methylated in this study (Table 7a). Regarding the methylation profile, HSC-3 and BICR-10 cell line have more resemblance than in CNV (Table 7b), since, in nine methylated genes for each cell line, six are the same: *RARβ* (3p24.2), *PAX5* (9p13.2), *PAX6* (11p13), *WT1* (11p13), *CADM1* (11q23.3) and *GATA5* (20q13.33).

Besides the common genes, HSC-3 cell line also had *TP73* (1p36.32), *ESR1* (6q25.1) and *MGMT* (10q26.3) genes methylated and BICR-10 cell line had methylation of *MGMT* (10q26.3), *CHFR* (12q24.33) and *CDH13* (16q23.3) genes. It is important to highlight that, even though both cell lines presented the gene *MGMT* methylated, they correspond to different probes that targeted the gene.

4.1.3.2 Copy Number Variations

Analysing the results regarding CNV from both cell lines (Table 7b), it is possible to observe that HSC-3 and BICR-10 are very different from each other. One of the greater differences is in the number of alterations, HSC-3 cell line has 55,26% (21/38) of the genes with some alteration, whereas BICR-10 only shows ten genes with CNV, which corresponds to 26,32% of all probes.

Comparing the number of genetic gains with genetic losses, BICR-10 cell line has a higher percentage of losses than gains, respectively 60% (6/10) and 40% (4/10). In contrast, HSC-3 cell line has more gains, 63,67% (14/21), than losses, 33,33% (7/21).

The majority of the alterations between both cell lines do not coincide. The only alteration common to both cell lines is located on *CFTR* gene (7q31.2), where both cell lines presented loss of the gene. Five of the ten alterations presented in BICR-10 cell line are losses of *CDKN2A* (9q21.3), *BRCA2* (13q13.1), the two probes for *RB1* (13q14.2) and *TP53* (17p12), all of which had genetic gain in HSC-3 cell line, making these four genes the most interesting.

There are several genes which did not showed any alterations on both cell lines: *ESR1* (6q25.1), *CDK6* (7q21.2), *PTCH1* (9q22.32), *KLLN* (10q23.31), *MGMT* (10q26.3), *PAH* (12q23.2), *CHFR* (12q24.33), *TSC2* (16p13.3), *PYCARD* (16p11.2), *PMP22* (17p13.1), *BRCA1* (17q21.31), *STK11* (19p13.3) and *KLK3* (19q13.33).

It is notorious the presence of CNV in the reference probes (8/14), being the majority altered in the HSC-3 cell line. For BICR-10 cell line, only the reference probes on *APC* (5q22.2) and *CFTR* genes presented alterations.

*Table 7 - Summary of (a) methylation profile (b) and CNV detected by MS-MLPA. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. (■) – Methylated gene. Chr: Chromosome. * - Reference Probe; ** - 319 nt before exon 1; *** - 71 nt before exon 1;*

(a)

Chr	Gene	HSC-3	BICR-10
1p36.32	*TP73*		
2p16.3	*MSH6*		
3p25.3	*VHL*		
3p24.2	*RARB*		
3q21.1	*CASR**		
4q27	*IL2**		
5q22.2	*APC**		
6q25.1	*ESR1*		
7q21.2	*CDK6**		
7q31.2	*CFTR**		
9p21.3	*CDKN2A*		
9p13.2	*PAX5*		
9q22.32	*PTCH1**		
10p11.21	*CREM**		
10q23.31	*KLLN*		
10q23.31	*PTEN**		
10q26.3	*MGMT***		
10q26.3	*MGMT****		
11p13	*PAX6*		
11p13	*WT1*		
11p13	*CD44*		
11q13.2	*GSTP1*		
11q22.3	*ATM*		
11q22.3	*ATM**		
11q23.3	*CADM1*		
12q23.2	*PAH**		
12q24.33	*CHFR*		
13q13.1	*BRCA2*		
13q14.2	*RB1*		
13q14.2	*RB1*		
14q24.3	*MLH3**		
15q14	*THBS1*		
16p13.3	*TSC2**		
16p11.2	*PYCARD*		
16q23.3	*CDH13*		
17p12	*TP53*		
17p13.1	*PMP22**		
17q21.31	*BRCA1*		
19p13.3	*STK11*		
19q13.33	*KLK3**		
20q13.33	*GATA5*		

(b)

Chr	Gene	HSC-3	BICR-10
1p36.32	*TP73*		
2p16.3	*MSH6*		
3p25.3	*VHL*		
3p24.2	*RARB*		
3q21.1	*CASR**		
4q27	*IL2**		
5q22.2	*APC**		
6q25.1	*ESR1*		
7q21.2	*CDK6**		
7q31.2	*CFTR**		
9p21.3	*CDKN2A*		
9p13.2	*PAX5*		
9q22.32	*PTCH1**		
10p11.21	*CREM**		
10q23.31	*KLLN*		
10q23.31	*PTEN**		
10q26.3	*MGMT***		
10q26.3	*MGMT****		
11p13	*PAX6*		
11p13	*WT1*		
11p13	*CD44*		
11q13.2	*GSTP1*		
11q22.3	*ATM*		
11q22.3	*ATM**		
11q23.3	*CADM1*		
12q23.2	*PAH**		
12q24.33	*CHFR*		
13q13.1	*BRCA2*		
13q14.2	*RB1*		
13q14.2	*RB1*		
14q24.3	*MLH3**		
15q14	*THBS1*		
16p13.3	*TSC2**		
16p11.2	*PYCARD*		
16q23.3	*CDH13*		
17p12	*TP53*		
17p13.1	*PMP22**		
17q21.31	*BRCA1*		
19p13.3	*STK11*		
19q13.33	*KLK3**		
20q13.33	*GATA5*		

4.1.4 Array-CGH

Both cell lines were analysed through aCGH and, as expected, both presented various structural rearrangements. With the data obtained two ideograms showing chromosomal gains and losses were built for each cell line.

In BICR-10 cell line, only the chromosome 19, 21 and 22 did not present any copy number variation. Chromosome X e Y were not analysed since the control used was male and this cell line was originated from a female patient. The chromosomes that showed bigger rearrangements were chromosome 5, 7, 8, 13 and 18 and CNV of only one arm are presented at 4p and 7p. (Figure 18).

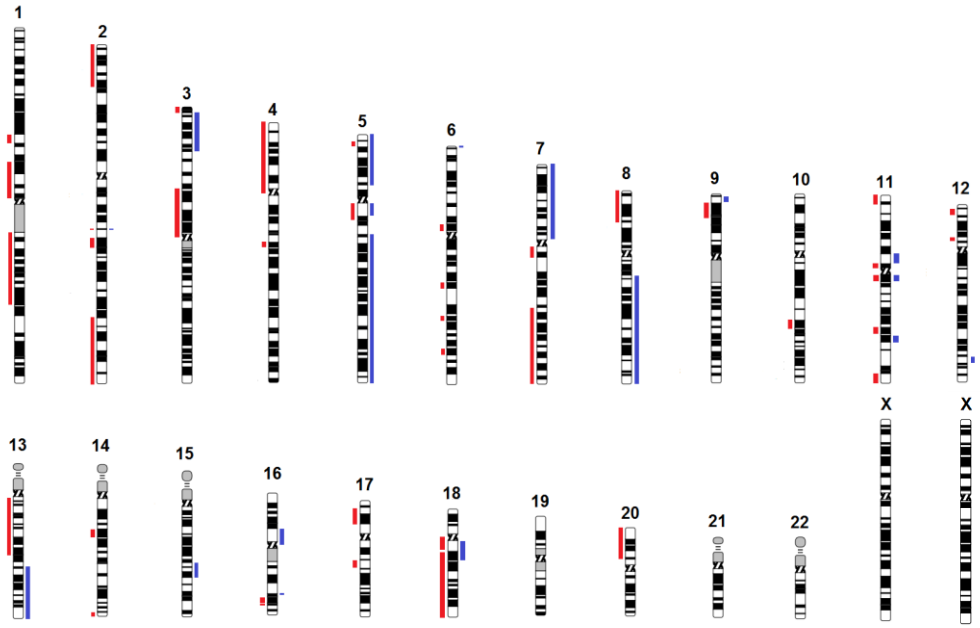

Figure 18- Ideogram showing copy number variations in BICR-10 cell line detected by aCGH (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. Ideogram base obtained from David Adler. Available at: http://www.pathology.washington.edu/research/cytopages/

In HSC-3 cell line, all chromosomes presented alterations. Furthermore, it is important to highlight that the chromosomes 1 , 9, 12, 13, 14, 17 and 20 were completely altered, all of them showing gains of the entire chromosome. The chromosomes less altered are chromosome 2, 16, 19 and 22 and gains of only one arm are observable at 3q, 4p, 5p 7p and 8q (Figure 19). These gains can also be observed when analysing the karyotype for this cell line, with chromosomes in trisomy (example: 9 and 20) and with chromosomes envolving structural translocations, such as i(1)(p10) (Figure 15) and i(7)(p10) , which was observed in other metaphases.

HSC-3 cell line presented more structural rearrangements than BICR-10 cell line. In BICR-10 cell line there is a higher frequency of losses than gains, whereas HSC-3 cell line showed a higher frequency of genetic gains (Figure 19). However, these results were expectable, since BICR-10 only has 41 chromosomes, whereas HSC-3 has 58 chromosomes.

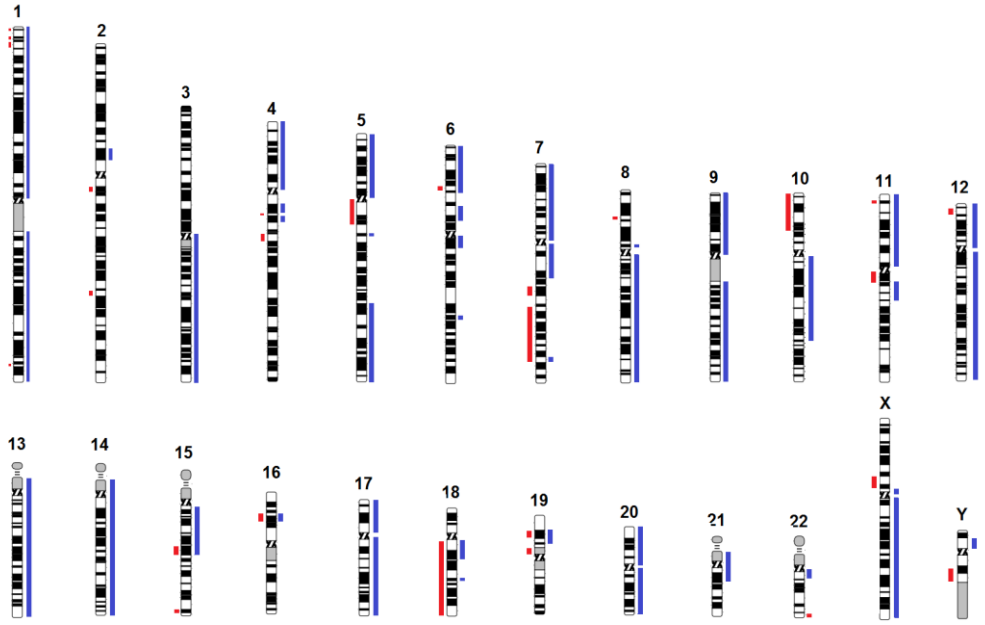

Figure 19 - Ideogram showing copy number variations in HSC-3 cell line detected by aCGH (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. Ideogram base obtained from David Adler. Available at: http://www.pathology.washington.edu/research/cytopages

Overall, there are clear differences between the two cell lines regarding alterations and CNV, namely at chromosome 1p, 1q, 3q, 4p, 9p, 9q, 10p, 10q, 11p, 12p, 12q, 14q, 17p, 17q, 20p and 20q. Moreover, in BICR-10 cell line, the majority of the abnormalities are small, having the smallest 12,3 Kb at 11p15.5 and the biggest 109,2 Mb at 5q13.2-q35.3. In opposite, the majority of the abnormalities presented in HSC-3 cell line are bigger, however they have sizes ranging from 119,7 Mb at 1p36.33-p12 to 145 bp at 1p32.32. Interestingly, there is none amplification or deletion of an entire chromosome in BICR-10, whereas HSC-3 has amplifications of seven entire chromosomes.

In common between the two cell lines are only the gains in chromosome 5p, 7p and 8q and loss in 18q.

4.2 ASSESSMENT OF RADIATION'S EFFECTS

4.2.1 Cell line's Morphology

The morphologic alterations caused by radiation treatment were captured from both cell lines for all the dose spectrum.

For BICR-10 cell line, all the photographs were taken seven days after the radiation treatment. It is possible to observe that, the higher the dose, more different is the appearance of cell morphology when compared with the untreated cell line (Figure 20). The cells lose their typical aspect and appear as tiny lines, being the tiniest when submitted to a dose of 15 Gy. However at a

dose of 5 Gy this differences are also easily observed. Moreover, they are getting branched and some of them have resemblances to astrocytes.

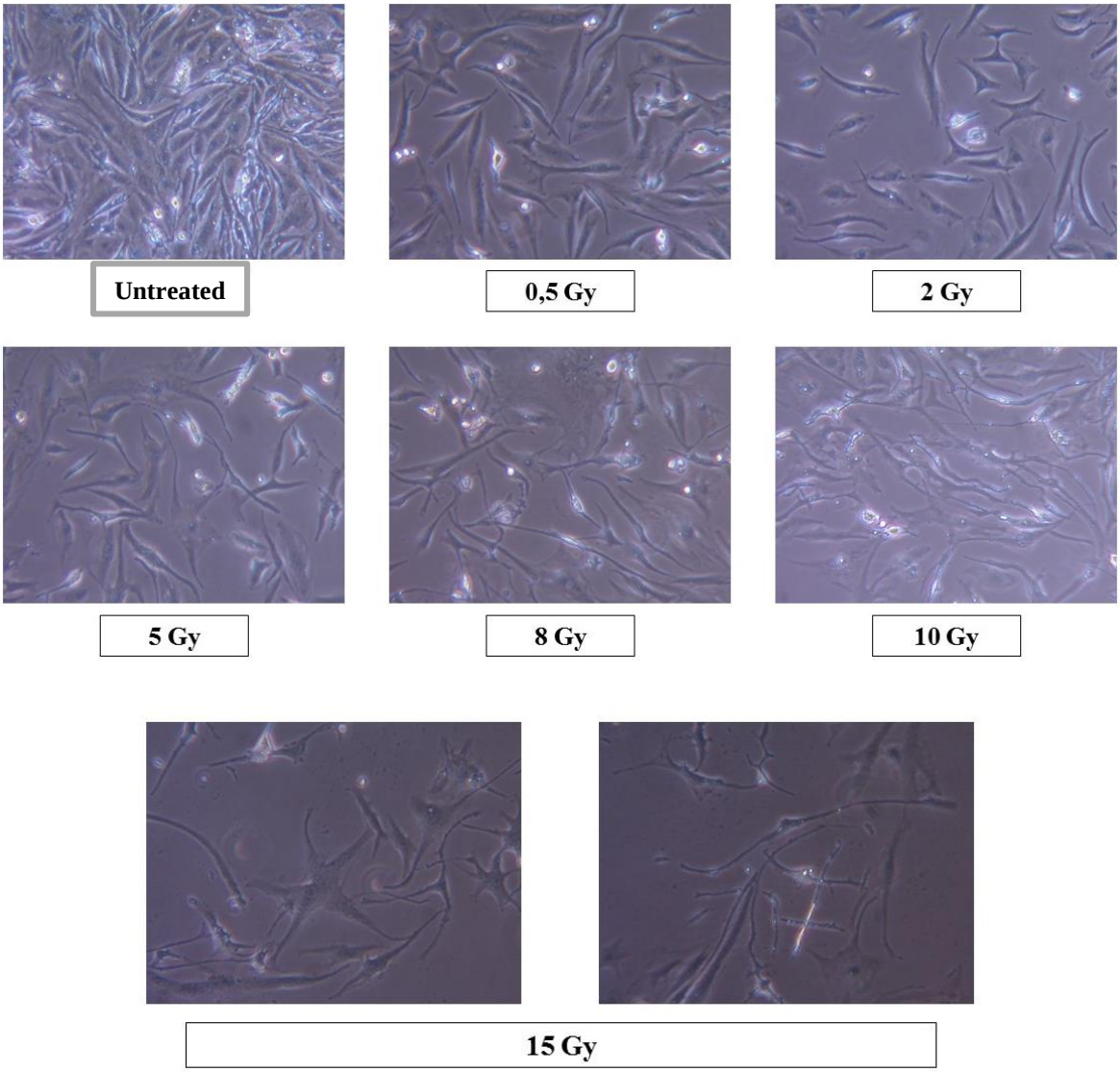

Figure 20 - Photograph's taken by an Axio Cam ERc55 Camera (Zeiss, Germany) of BICR-10 cell line in untreated conditions and submitted to different doses of radiation (0,5; 2; 5; 8, 10 and 50 Gy), all of them after seven days of irradiation treatment.

Regarding HSC-3 cell line (Figure 21), the majority of the photos were taken after twenty days of radiation treatment, except for the cells submitted to 3 Gy, which were taken are after seven days, and the cells submitted to 10 Gy, had photos taken after seven and 20 days after radiation treatment. One of the first things that calls the attention is the different confluences between the different cell cultures submitted to radiation. Excluding the cells submitted to 3 Gy, which photos were taken seven days after radiation treatment, it is possible to denote a big decrease in cell confluence as the radiation dose increases. At the lowest doses the morphologic differences are barely noticeable, however in the higher doses (5, 8 and 10 Gy) it is observable that the cells have an increase

in volume and gain several vacuolar regions. Its morphologic appearance is almost lost, and at 8 and 10 Gy there it is almost impossible to observe cells with a typical histologic appearance. With respect to differences in time between the photos, after seven days the cells still have a healthy appearance, however, after thirteen more days the differences are huge and the typical aspect is lost, the formation of vacuoles and also the enhancement of cell volume is observed.

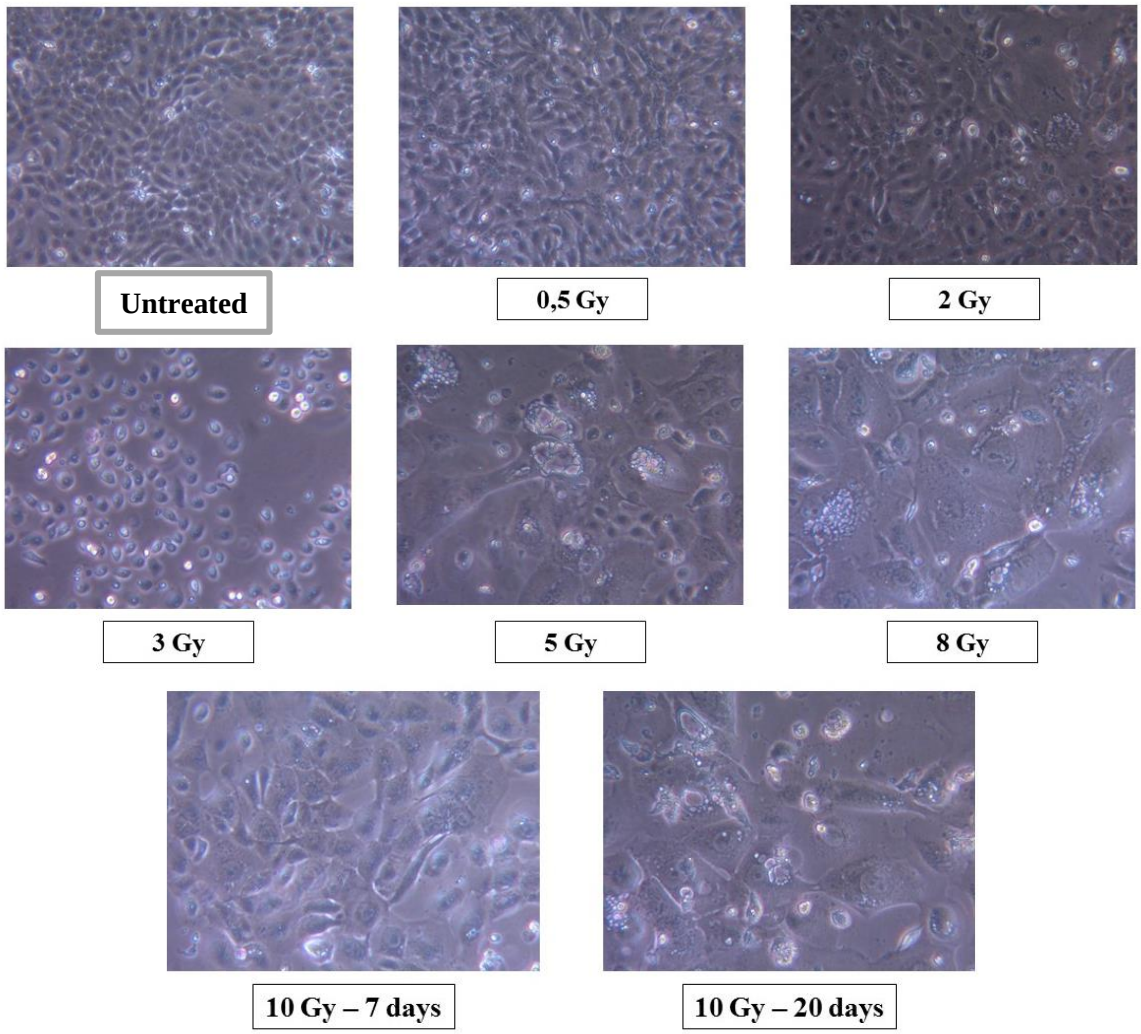

Figure 21 - Photograph's taken by an Axio Cam ERc55 Camera (Zeiss, Germany) of HSC-3 cell line submitted to line in untreated conditions and different doses of radiation (0,5, 2, 3, 5, 8 and 10 Gy) after twenty days of irradiation treatment, except for 3 Gy and the first 10 Gy photograph.

4.2.2 Cell Survival – Clonogenic Assay

For assessing cell survival after irradiation, a clonogenic assay was performed in order to obtain a cell survival curve for both cell lines (Figure 22), allowing the determination of LD50 and the assessment of radiation effect in each one of the cell lines.

For BICR-10 cell line the linear mode was applied, whereas for HSC-3 cell line it was the Linear-Quadratic (LQ) model.

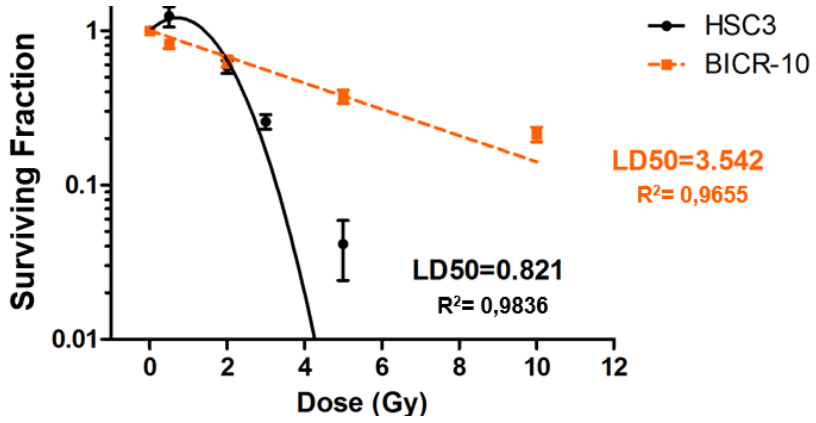

Figure 22 – Cell survival curve for BICR-10 and HSC-3 after irradiation treatment.

In the graphic is possible to observe that, for both cases, the increasing exposure to radiation led to a reduction in cell survival, except for a dose of 0,5 Gy in HSC-3 cell line. Still, the decreased is much more pronounced in HSC-3 cell line than in BICR-10 cell line. This is reflected in the median lethal dose (LD50), which is the dose needed to reduce cell survival by 50%, since for HSC-3 is 0,821 and for BICR-10 is 3,542. For other experiments purposes, the dose applied to HSC-3 to represent the LD50 was 1 Gy, and for BICR-10 cell line was 3 Gy.

In hypoxic conditions we were not able to have adequate results, however the motives are unclear. This needs to be further pursued.

4.2.3 Karyotyping

Karyotyping analysis was performed in HSC-3 cell line after cells were submitted to 0,5 Gy, 1 Gy and 2 Gy, and in BICR-10 cell line after cells were submitted to 0,5 Gy, 2 Gy and 5 Gy. Similar to the analysis performed for the untreated cells, only ten metaphases *per* condition were analysed, except for BICR-10 cell line after submission to 5 Gy, where only 9 metaphases could be analysed.

Regarding differences in the number of chromosomes between untreated and treated cells in both cell lines, no significative differences were observed.

However, in HSC-3 cell line it is possible to notice that the majority of alterations are maintained after irradiation treatment (Table 8). The frequency of monosomy 18 seems to decrease and pass to a disomy at the lowest doses of irradiation, (Figure 23) and, after submission to 0,5 Gy the frequency of del(7)(q?) decreases to half. Additionally, some aberrations first appear after the cells were submitted to irradiation, as for example $11p^-$, der(11;17) and $12p^+$ (Figure 24). Besides, the cells exposed to the lower radiation dose is the one which presents less new chromosomic alterations in comparison to the untreated cells (Table 8).

The differences in BICR-10 cell line between untreated cells and irradiated cells are easy to notice (Table 9). One of the differences is the loss of i(5)(p10). Even though this alteration is not the

most common, only being present in four metaphases in untreated cells, in 28 other metaphases analysed after different doses of irradiation, this aberration is lost. Other alteration that seems to be decreasing the frequency after irradiation is der(8;8) that was highly frequent in the untreated cells. Furthermore, after irradiation treatment, some new aberrations also appear: del(2)(q10), which is particularly common after 0,5 Gy of radiation (Figure 25), $5q^-$ (Figure 26), t(13;17), t(14;17) and the presence of i(7)(p10)del(7)(pter). After a dose of 5 Gy is noticeable that the most common alterations in the untreated cells suffer a decrease in frequency and that there is an enhancement of new aberrations (Figure 27) (Table 9).

Table 8 - Summary table of cytogenetic abnormalities in HSC-3 cell line after irradiation treatment with 0,5, 1 and 2 Gy in comparison with untreated cells.

HSC-3

Condition	Structural											Numeric				Additional
	i(1)(q10)	del(1)(p10)	t(2;15)(q10;q10)	del(2)(q10)	del(5)(q?)	del(7)(q?)	t(11;13)	dup(11q)	der(14)t(14;17)(q10;10)	der(X)t(X;8)(q10;q10)	der(13;13)(q10;q10)	Monosomy 18	Monosomy 10	Trisomy 9	Trisomy 20	
Untreated	10/10	9/10	9/10	9/10	9/10	10/10	8/10	10/10	10/10	9/10	10/10	7/10	9/10	9/10	8/10	4q- (3/10)
0,5 Gy	9/10	10/10	10/10	9/10	7/10	5/10	8/10	9/10	9/10	9/10	8/10	3/10	8/10	9/10	10/10	4q- (1/10)
1 Gy	8/10	9/10	10/10	10/10	7/10	9/10	10/10	10/10	8/10	10/10	8/10	2/10	10/10	9/10	9/10	der(11;17) (2/10) 11p- (1/10) 5p- (1/10)
2 Gy	9/10	9/10	10/10	8/10	9/10	8/10	10/10	10/10	8/10	10/10	10/10	9/10	8/10	9/10	8/10	der(11;17) (1/10) 11p- (2/10) 12p+ (2/10) 9q- (1/10)

Table 9 - Summary table of cytogenetic abnormalities in BICR-10 cell line after irradiation treatment with 0,5, 1 and 2 Gy in comparison with untreated cells.

BICR-10

Condition	Structural											Additional
	der(1)	der(2)t(1;2)	t(3;5)(p10;q10)	i(5)(p10)	i(7)(p10)	der(8)t(5;8)	der(8;8)	del(9)(pter)	der(7)t(7;11)	der(15)t(X;15)(q10;q10)	der(17)t(7;17)	
Untreated	10/10	10/10	10/10	4/10	10/10	8/10	9/10	10/10	10/10	10/10	10/10	2p (1/10)
0,5 Gy	10/10	9/10	10/10	0/10	9/10	8/10	6/10	8/10	10/10	7/10	8/10	2p (8/10) 5q- (3/10)
2 Gy	10/10	9/10	10/10	0/10	9/10	8/10	1/10	7/10	10/10	9/10	9/10	5q- (3/10) 2p (1/10) 17p- (1/10) 21p+ (1/10) i(7)(p10)del(7)(pter) (1/10)
5 Gy	9/9	8/9	6/9	1/9	6/9	8/9	4/9	6/9	8/9	8/9	6/9	5q- (6/9) i(7)(p10)del(7)(pter) (2/9) 21p+ (2/9) 4q- (1/9)

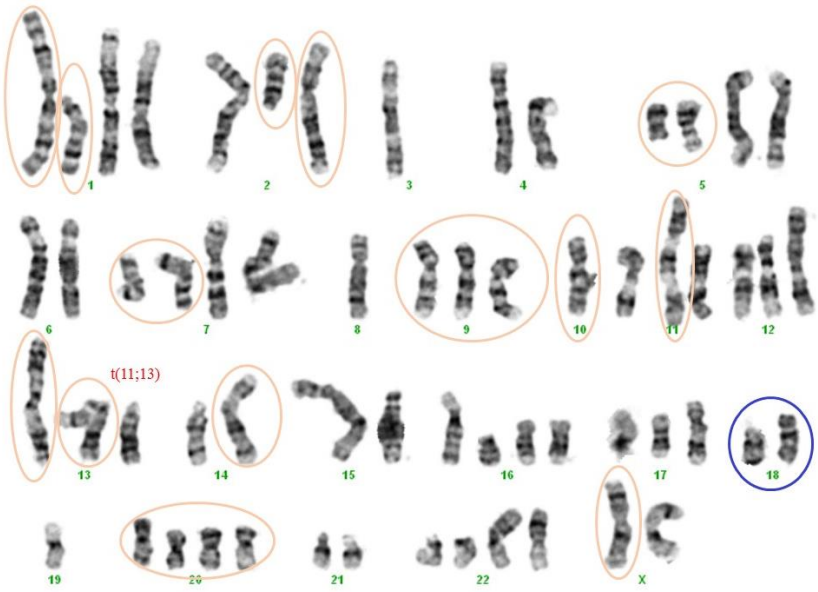

Figure 23 – Karyogram from HSC-3 cell line after a irradiation with a dose of 0,5 Gy. In light Orange are the alterations common to the untreated cells and in blue alterations that happened after irradiation.

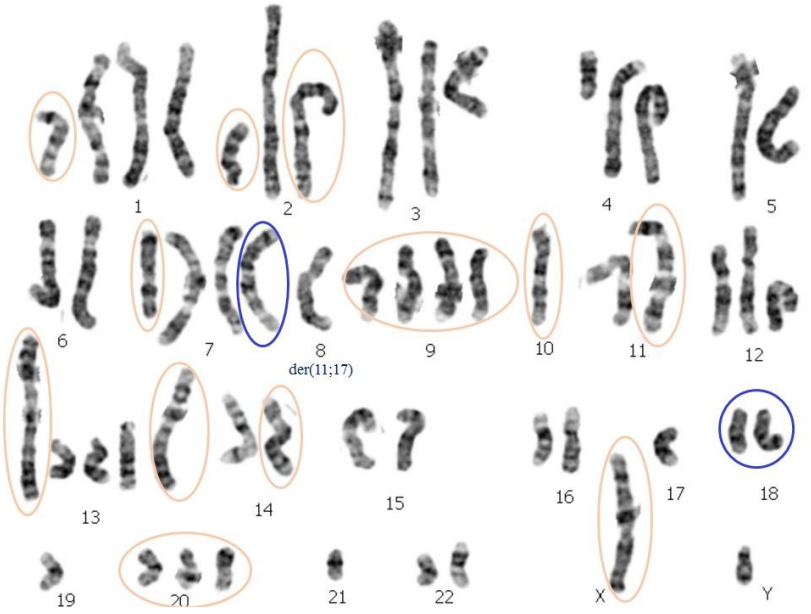

Figure 24 – Karyogram from HSC-3 cell line after a irradiation with a dose of 1 Gy. In light Orange are the alterations common to the untreated cells and in blue are alterations that happened after irradiation.

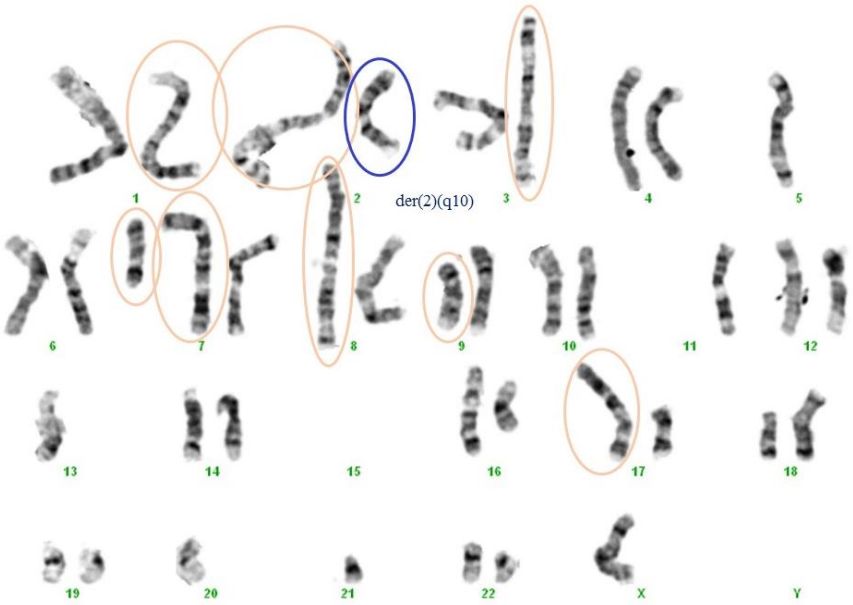

Figure 25 - Karyogram from BICR-10 cell line after a irradiation with a dose of 0,5 Gy. In light Orange are the alterations common to the untreated cells and in blue are alterations that happened after irradiation.

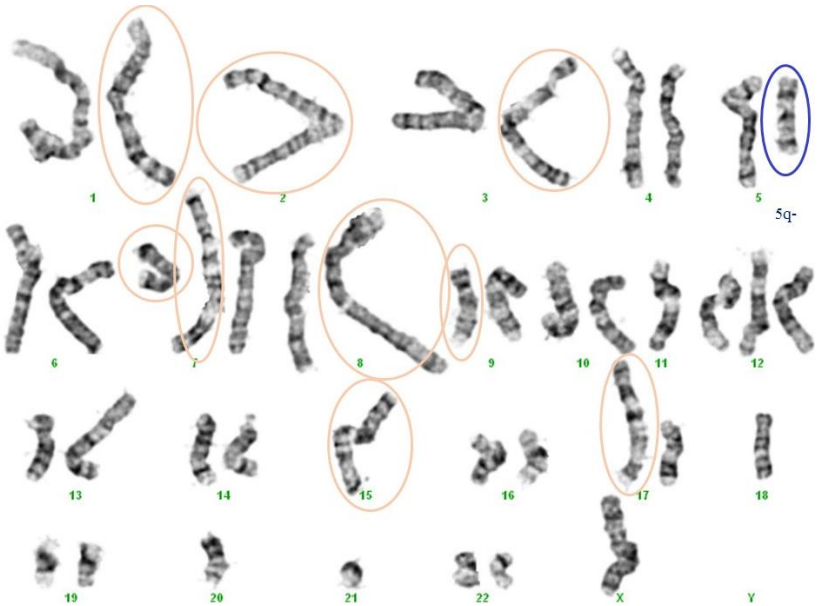

Figure 26 - Karyogram from BICR-10 cell line after a dose of 2 Gy. In light Orange are the alterations common to the untreated cells and in blue are alterations that happened after irradiation.

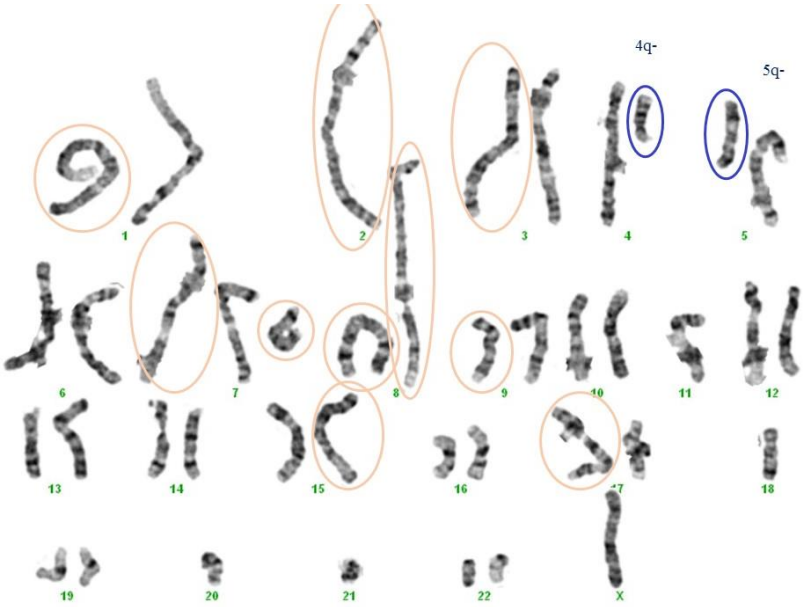

Figure 27 - Karyogram from BICR-10 cell line after a dose of 5 Gy. In light Orange are the alterations common to the untreated cells and in blue are alterations that happened after irradiation.

4.2.4 Copy Number Variations

4.2.4.1 Array-CGH results

After irradiation treatment with 0,5 Gy and 10 Gy, DNA was extracted from both cell lines and it was analysed by aCGH. The differences in CNV between the different irradiations were too small to be presented in an ideogram, so a table was made.

In Table 10 are represented genes and regions that could be further studied in order to understand the possible effects of radiation in a more radioresistant cell line, in a more radiosensitive cell line or even the effects of radiation in general. Chromosome X and Y were not analysed for BICR-10 due to the fact that this cell line is derived from a female patient and the control used for aCGH is from a male patient.

Between the normal cell line (0 Gy) and the cells submitted to radiation (0,5 Gy and 10 Gy) in HSC-3 case, 31 differences in CNV were accounted. Using the same approach of comparison between the untreated (0 Gy) and the radiated cells (0,5 Gy and 10 Gy) there were 30 differences in CNVs observed for BICR-10 cell line. These numbers are similar, especially if we considered that two chromosomes were not analysed in the latter. Furthermore, in HSC-3 case there is an enhancement of gains, being most of the alterations located on chromosome 1. BICR-10 cell line is more heterogeneous.

Regarding genes that showed alterations in CNV between the untreated (0 Gy) and the radiated cells (0,5 Gy and 10 Gy), in both cell lines, an extensive literature search was performed in

order to establish possible genes that could elucidate this question. We seletected 20 genes (Table 10). Only 3 genes showed differences on both cell lines: WD Repeat Containing, Antisense to TP73 (*WRAP73)* (1p36.32), Solute Carrier Family 22, Member 18 *(SLC22A18)* (11p15.4) and Collagen, Type I, Alpha 1 *(COL1A1)* (17q21.33).

The majority of genes altered were on HSC-3 cell line, with 11 noticeable alterations: Arginine-glutamic Acid Dipeptide (RE) Repeats *(RERE)* (1p36.23), Filamin Binding LIM Protein 1 *(FBLIM1)* (1p36.23), *VHL* (3p25), Collagen, Type I, Alpha 2 *(COL1A2)* (7q21.3), Interferon Regulatory Factor 5 (IRF5) (7q32.1), microRNA 183 (MIR183) (7q32.2), T-box 4 (TBX4) (17q23.2), Nascent Polypeptide-associated Complex Alpha Subunit 2 *(NACA2)* (17q23.2), BRCA1 Interacting Protein C-terminal Helicase 1 *(BRIP1)* (17q23.2), Integrator Complex Subunit 2 *(INTS2)* (17q23.2) and Mediator Complex Subunit 13 *(MED13)* (17q23.2).

As for BICR-10 cell line, 6 genes presented alterations: Kinesin Family Member 26B *(KIF26B) (1q44)*, G Protein-coupled Receptor 39 *(GPR39)* (2q21-q22), N-acetylated Alpha-linked Acidic Dipeptidase-like 2 *(NAALADL2)* (3q26.33), Msh Homeobox 1 *(MSX1)* (4p16.2), Heparan Sulfate (glucosamine) 3-O-Sulfotransferase 3A1 *(HS3ST3A1)* (17p12), and TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor *(TAF4B)* (18q11.2).

Table 10 – Table showing CNV in HSC-3 and BICR-10 cell lines after exposure to X-irradiation detected by aCGH. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material; (■) – Absence of CNV; (■) – Not analysed.

Chromosome	Arm	HSC-3			BICR-10			Genes
		0 Gy	0,5 Gy	10 Gy	0 Gy	0,5 Gy	10 Gy	
1	p	p36.32	p36.32	p36.32		p36.32		*WRAP73*
		p36.23	p36.23	p36.23				*RERE, FBLIM1*
		p36.21	p36.21	p36.21				
		p36.13	p36.13	p36.13				
		p36.13	p36.13	p36.13				
		p13.3	p13.3	p13.3			p13.3	
	q	q42.3	q42.3	q42.3				
		q44	q44	q44		q44	q44	*KIF26B*
2	q	q12.21						
			q21.1 - q21.2					
					q21.2			*GPR39*
		q31.1				q31.1		
3	p		p25.3		p25.3	p25.3	p25.3	*VHL*
	q	q11.2	q11.2	q11.2	q11.2			
		q26.33	q26.33	q26.33		q26.33	q26.33	*NAALADL2*
4	p				p16.2	p16.2	p16.2	
		p16.2	p16.2	p16.2			p16.2	*MSX1*
					p11	p11	p11	
		p11	p11	p11		p11		
	q	q31.21		q31.21				
5	p	p12-p11	p12-p11	p12-p11		p12-p11		
6	p	p21.32	p21.32					
7	q					q11.1-q11.21		
		q21.3	q21.3					*COL1A2*
			q22.1					
		q22.3-q34	q22.3-q34	q22.3-q32.1	q22.3-q34	q22.3-q34	q22.3-q34	*IRF5, MIR183*
				q32.3				
				q32.3-q34				
8	p	p21.3			p21.3	p21.3	p21.3	
			p11.21					
			p11.1					
9	p				p24.1	p24.1	p24.1	
		p24.1	p24.1	p24.1	p24.1			
11	p	p15.5	p15.5	p15.5	p15.5	p15.5	p15.5	
		p15.4	p15.4	p15.4	p15.4	p15.4	p15.4	*SLC22A18*
		p11.2-p11.12	p11.2-p11.12	p11.2-p11.12	p11.2-p11.12		p11.2-p11.12	
						p11.2		
	q					q11-q12.1		
		q14.3	q14.3					
		q22.3	q22.3	q22.3		q22.3		
					q25		q25	
12	p	p13.31	p13.31	p13.31	p13.31	p13.31	p13.31	
			p13.31					
13	q	q24.13	q24.13	q24.13	q24.13		q24.13	
		q24.33	q24.33	q24.33			q24.33	
14	q	q32.33	q32.33	q32.33	q32.33		q32.33	
15	q	q21.3	q21.3	q21.3				
		q21.3						
		q26.2				q26.2		
16	p						p13.2	
	q				q22.2	q22.2		
17	p				p12	p12	p12	
		p12	p12	p12			p12	*HS3ST3A1*
	q	q21.33	q21.33	q21.33		q21.33	q21.33	*COL1A1*
		q21.31	q21.31	q21.31	q21.31			
		q23.2	q23.2	q23.2				*TBX4, NACA2, BRIP1, INTS2, MED13*
18	q				q11.1-q11.2	q11.1-q11.2	q11.1-q11.2	
		q11.2	q11.2	q11.2		q11.2		*TAF4B*
		q12.3	q12.3	q12.3	q12.3	q12.3	q12.3	
							q12.3	
19	p	p13.2	p13.2	p13.2				
		p13.2						
X	p		p11.22					
			p11.22					
Y	p	p11.2		p11.2				
	q			q11.1-q11.21				
				q11.23				

4.2.4.2 MS-MLPA results

The cells submitted to 0,5, 1 (in HSC-3 case only), 2, 3 (in BICR-10 cell line only), 5 and 10 Gy were analysed by MS-MLPA. The cells in hypoxic conditions and in hypoxic conditions plus LD50 Gy for each cell line were also analysed. The results are presented in Table 11 for both cell lines

In HSC-3 there are several genes that maintain the alterations in all the conditions: *CASR* (3q21.1), *CFTR* (7q31.2), *CDKN2A* (9p21.3), *CD44* (11p13), *GSTP1* (11q13.2), *ATM* (11q22.3), *BRCA2* (13q13.1), *RB1* (13q14.2), *MLH3* (14q24.3), *CDH13* (16q23.3) and *GATA5* (20q13.33). Moreover, *TP73* (1p36.32), *PAX5* (9p13.2), *PAX6* (11p13) and *TP53* (17p12) have slight alterations, being the ratio closer to the limit of the cut offs. Besides, alterations noteworthy are the loss of *APC* (5q22.2), *VHL* (3p25.3), *WT1* (11p13) and *TSC2* (16p13.3), gain of *PMP22* (17p13.1) all at 1 Gy and hypoxia plus 1 Gy. The loss of *MSH6* (2p16.3) and *ESR1* at low doses of irradiation also seem interesting alterations. Finally, the gain of *CHFR* at cells submitted to 0,5 Gy and loss after they were submitted to 2 Gy of irradiation are also noteworthy.

Furthermore, HSC-3 cell line submitted to 2 Gy is the one who presents more alterations in comparison with all the other conditions. The ones who showed less alterations in comparison to the control are cells in hypoxic conditions and when they were submitted to high doses of radiation (5 and 10 Gy).

In BICR-10, it is noticeable the alteration of several genes when cells are submitted to different doses of radiation. When cells are submitted to 3 Gy, either in normoxic or hypoxic conditions occurred gains of *TP73, PTCH1* (9q22.32) and *CREM* (10p11.21). Comparing the results obtain from normoxic and hypoxic conditions, there are slight differences, as the decrease in copy number of *MSH6,* gain of *PTCH1, CREM, MLH3* and *BRCA1*, and loss of *MGMT*. Furthermore, *BRCA1* gain also happens in hypoxic conditions after a dose of 3 Gy. In untreated cells, the exposure to low dose radiations leads to an enhancement in copy numbers of *PAX5, PTCH1* and *CREM*. In general, the genes that were altered in the untreated cell line are maintained after irradiation, with exception to the enhancement of copy number of *CFTR* at 10 Gy, the loss of *GATA5* in hypoxic conditions and the decrease in copy number for the *MSH6* gene in all conditions.

*Table 11 – Copy Number Variations detected by MS-MLPA in HSC-3 and BICR-10 cell lines after submission to radiation and in hypoxic conditions(■) – Gain of Genetic Material; (■) – Loss of Genetic Material; Chr: Chromosome. * - Reference Probe; ** - 319 nt before exon 1; *** - 71 nt before exon 1;*

		HSC-3								BICR-10							
Chr	**Gene**	**0 Gy**	**0,5 Gy**	**1 Gy**	**2 Gy**	**5 Gy**	**10 Gy**	**Hypoxia**	**Hypoxia + 1 Gy**	**0 Gy**	**0,5 Gy**	**2 Gy**	**3 Gy**	**5 Gy**	**10 Gy**	**Hypoxia**	**Hypoxia + 3 Gy**
1p36.32	*TP73*																
2p16.3	*MSH6*																
3p25.3	*VHL*																
3p24.2	*RARB*																
3q21.1	*CASR**																
4q27	*IL2**																
5q22.2	*APC**																
6q25.1	*ESR1*																
7q21.2	*CDK6**																
7q31.2	*CFTR**																
9p21.3	*CDKN2A*																
9p13.2	*PAX5*																
9q22.32	*PTCH1**																
10p11.21	*CREM**																
10q23.31	*KLLN*																
10q23.31	*PTEN**																
10q26.3	*MGMT***																
10q26.3	*MGMT****																
11p13	*PAX6*																
11p13	*WT1*																
11p13	*CD44*																
11q13.2	*GSTP1*																
11q22.3	*ATM*																
11q22.3	*ATM**																
11q23.3	*CADM1*																
12q23.2	*PAH**																
12q24.33	*CHFR*																
13q13.1	*BRCA2*																
13q14.2	*RB1*																
13q14.2	*RB1*																
14q24.3	*MLH3**																
15q14	*THBS1*																
16p13.3	*TSC2**																
16p11.2	*PYCARD*																
16q23.3	*CDH13*																
17p12	*TP53*																
17p13.1	*PMP22**																
17q21.31	*BRCA1*																
19p13.3	*STK11*																
19q13.33	*KLK3**																
20q13.33	*GATA5*																

4.2.5 Alterations on the methylation pattern

In order to assess differences in the methylation pattern induced by irradiation, MS-MLPA was applied to HSC-3 submitted to 0,5 Gy, 1 Gy, 2 Gy, 5 Gy, 10 Gy of irradiation, HSC-3 in hypoxic conditions and hypoxic conditions submitted to 1 Gy of irradiation. As for BICR-10, cells submitted to 0,5 Gy, 2 Gy, 3 Gy, 5 Gy, 10 Gy of irradiation, hypoxic conditions and hypoxic conditions submitted to 3 Gy of irradiation were analysed for alterations in the methylation profile (Table 12).

In both, the methylation pattern that was observed in the untreated cell line is maintained after irradiation and in hypoxic conditions. In HSC-3, the 2 Gy dose is the one with the most alterations. It showed methylation of both *MSH6* (2p16.3) and *ATM*. The other alteration is observable in the cells submitted to 0,5 Gy, where *MSH6* is methylated. Hypoxic conditions did not induced any methylation alteration besides the methylation pathern of the untreated cells.

As for BICR-10, only one alteration was detected regarding methylation profile. This alteration occurs after the cell line was submitted to a dose of 10 Gy, which leaded to the methylation of *MSH6* gene.

*Table 12 – Methylation alterations induced by different doses of radiation and hypoxic conditions in HSC-3 and BICR-10 cell line accessed by MS-MLPA. (■) – Methylated gene. Chr: Chromosome. * - Reference Probe; ** - 319 nt before exon 1; *** - 71 nt before exon 1;*

		HSC-3								BICR-10							
Chr	**Gene**	**0 Gy**	**0,5 Gy**	**1 Gy**	**2 Gy**	**5 Gy**	**10 Gy**	**Hypoxia**	**Hypoxia + 1 Gy**	**0 Gy**	**0,5 Gy**	**2 Gy**	**3 Gy**	**5 Gy**	**10 Gy**	**Hypoxia**	**Hypoxia + 3 Gy**
1p36.32	*TP73*	■	■	■	■	■	■	■	■								
2p16.3	*MSH6*		■		■										■		
3p25.3	*VHL*																
3p24.2	*RARB*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
3q21.1	*CASR**																
4q27	*IL2**																
5q22.2	*APC**																
6q25.1	*ESR1*	■	■	■	■	■	■	■	■								
7q21.2	*CDK6**																
7q31.2	*CFTR**																
9p21.3	*CDKN2A*																
9p13.2	*PAX5*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
9q22.32	*PTCH1**																
10p11.21	*CREM**																
10q23.31	*KLLN*																
10q23.31	*PTEN**																
10q26.3	*MGMT***									■	■	■	■	■	■	■	■
10q26.3	*MGMT****	■	■	■	■	■	■	■	■								
11p13	*PAX6*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
11p13	*WT1*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
11p13	*CD44*																
11q13.2	*GSTP1*																
11q22.3	*ATM*				■												
11q22.3	*ATM**																
11q23.3	*CADM1*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
12q23.2	*PAH**																
12q24.33	*CHFR*									■	■	■	■	■	■	■	■
13q13.1	*BRCA2*																
13q14.2	*RB1*																
13q14.2	*RB1*																
14q24.3	*MLH3**																
15q14	*THBS1*																
16p13.3	*TSC2**																
16p11.2	*PYCARD*																
16q23.3	*CDH13*									■	■	■	■	■	■	■	■
17p12	*TP53*																
17p13.1	*PMP22**																
17q21.31	*BRCA1*																
19p13.3	*STK11*																
19q13.33	*KLK3**																
20q13.33	*GATA5*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

4.2.6 Comet Assay

The comet assay, a method that allows the measure of DNA strand breaks, was performed in order to discovery possible explanations for the different response to radiation of both cell lines regarding the amount of damages induced by irradiation to the cell lines (Figure 28 and Figure 29). The amout of damages is proportional to the tail length, this is, the longer the tail, the greater the amount of damages. The objective was to analyse the amount of damages induced by irradiation when the cells were in normoxic and hypoxic conditions.

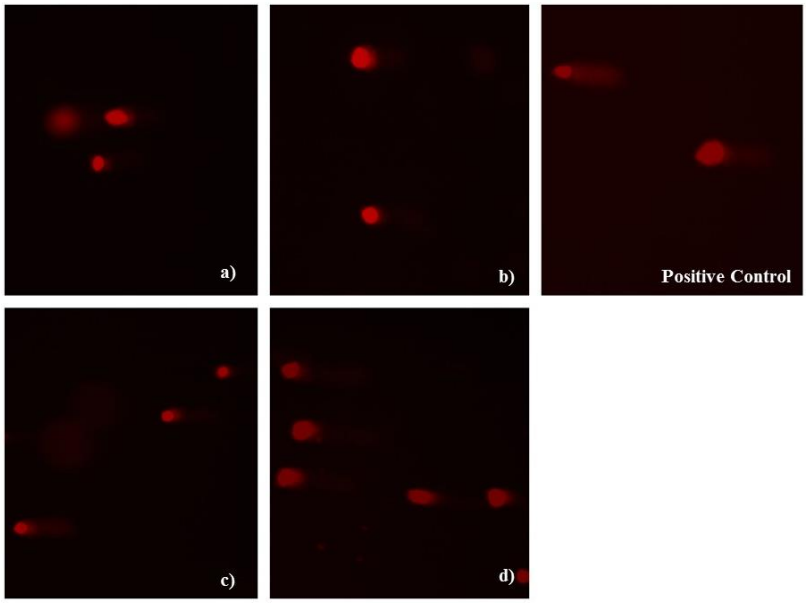

Figure 28 – Comet Assay in the BICR-10 cell line. Results for the (a) untreated cells, (b) cells submitted to a dose of 3 Gy, (c) cells in hypoxic conditions, (d) cells in hypoxic conditions submitted to a dose of 3 Gy, as well as the positive control.

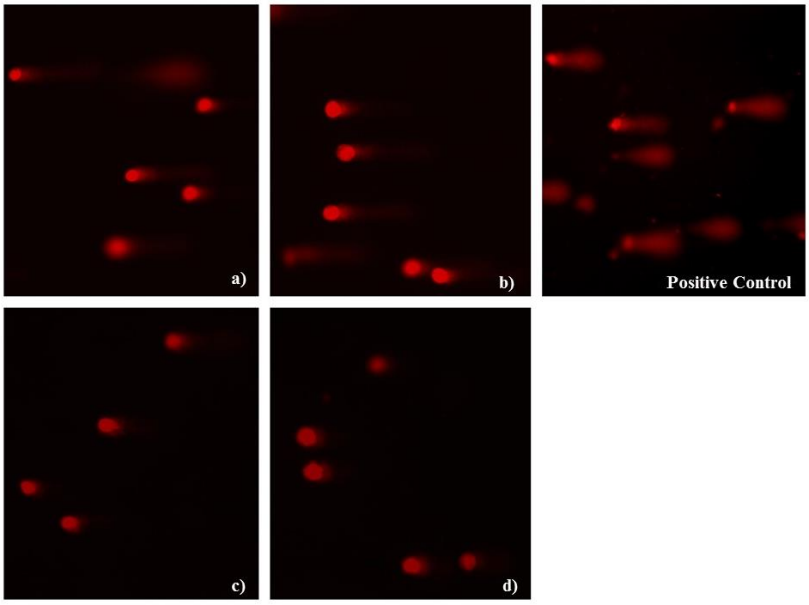

Figure 29 - Comet Assay in the HSC-3 cell line. Results for the (a) untreated cells, (b) cells submitted to a dose of 1 Gy, (c) cells in hypoxic conditions, (d) cells in hypoxic conditions submitted to a dose of 1 Gy, as well as the positive control.

Analysing the comets obtained, the data was organized into percentiles. The results are presented in Table 13 and Table 14. The Kruskal-Wallis was used for comparision between the different conditions and Bonferroni correction was applied (Table 15).

Table 13 – Data obtained from tail moment analysis for BICR-10 cell line in the diferente conditions.

BICR-10	Untreated	3 Gy	Hypoxia	Hypoxia + 3 Gy
Median	16,04	19,83	18,16	11,94
Percentile 90	184,11	179,51	64,89	50,1

Table 14 – Data obtained from tail moment analysis for HSC-3 cell line in the different conditions.

HSC-3	Untreated	1 Gy	Hypoxia	Hypoxia + 1 Gy
Median	46,79	50,56	31,26	29,37
Percentile 90	123,76	128,85	143,74	132,59

Table 15 - Results obtained from statistical analysis of the tail moment for the different conditions in both cell lines. Each row tests the null hypothesis that the different samples distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level considered was 0,05. Statistical significance between the conditions is highlighted in yellow.

Sample Comparison		Adjusted Significance
HSC-3	Untreated - 1 Gy	1,000
	Untreated - Hypoxia	0,012
	Untreated - Hypoxia + 1 Gy	0,000
	Hypoxia - Hypoxia + 1 Gy	0,444
	1 Gy - Hypoxia + 1 Gy	0,000
BICR-10	Untreated - 3 Gy	1,000
	Untreated - Hypoxia	1,000
	Untreated - Hypoxia + 3 Gy	0,000
	Hypoxia - Hypoxia + 3 Gy	0,005
	3 Gy - Hypoxia + 3 Gy	0,000

4.2.7 Flow Cytometry

Flow Cytometry was applied in order to access cell death and cell cycle phases of both cell lines. It was applied at both untreated cell lines and after submission to the dose of radiation calculated for the LD50, hypoxic conditions and cell lines in hypoxic conditions submitted to the LD50 dose.

The data is the median of two independent experiments.The objective of this FC analysis was to compare differences in cell death in a radioresistant and a radiosensitive cellular line after they were submited to irradiation (Figure 30). In order to assess possible explanations for the different response to radiation, the percentage of cells in the different cell cycle phases on both cell lines was also compared (Figure 31).

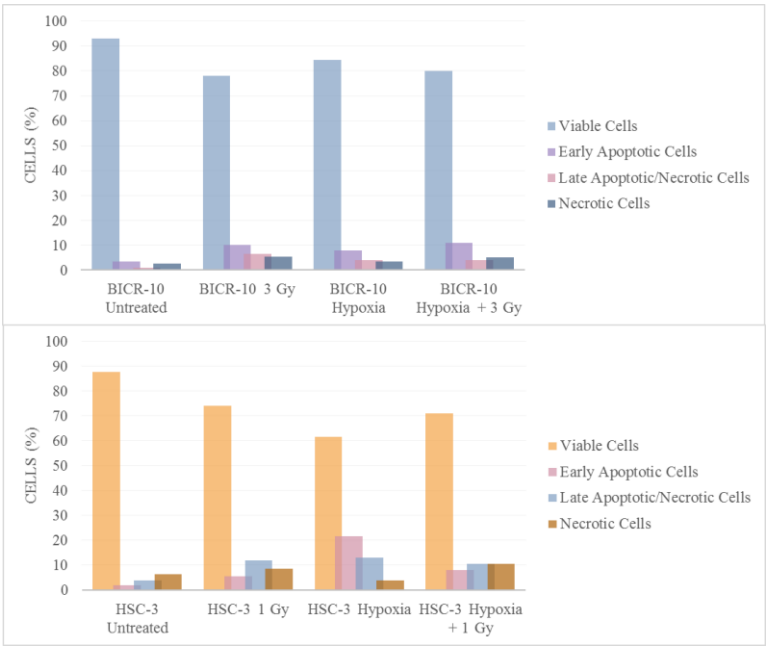

Figure 30 - Preliminary data from flow cytometry cell death analysis of BICR-10 cell line (on the top) and HSC-3 cell line (at the bottom).

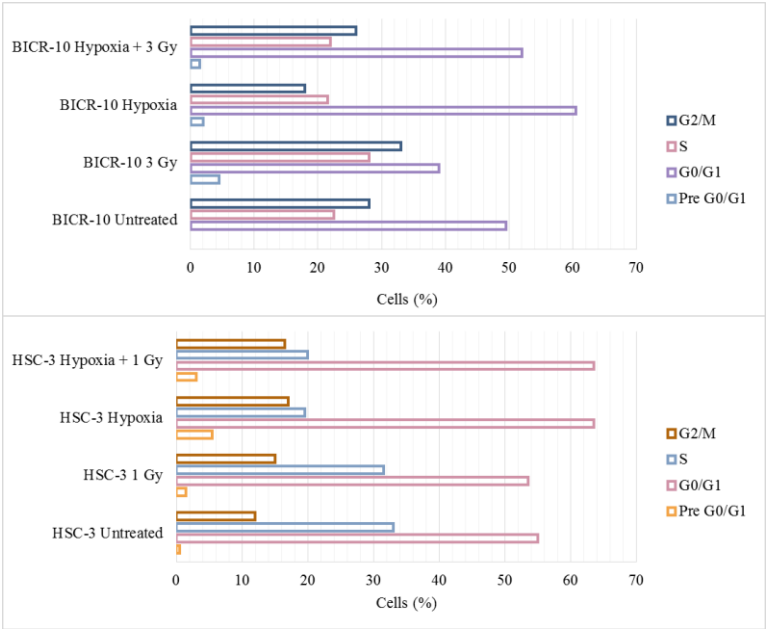

Figure 31 - Preliminary data from flow cytometry cell cycle analysis of BICR-10 cell line (on the top) and HSC-3 cell line (at the bottom).

4.3 PREDICTING RADIOTHERAPY RESPONSE

Analysing the aCGH results and the literature, we schematize the possible regions that could be associated with a worse prognosis and to radiation treatment response in our cell lines.

In BICR-10 cell line (Figure 32) is possible to associate the losses of 4p11-pter and 11q distal to radioresistance. At the short arm of chromosome 8 there is a small band that, when it is loss, is also associated to radioresistance: 8p23.3. The loss in 17p represents the deletion of the *TP53* gene. Gains of 3q26.1 and 7p11.2-12 have also been associated to a more radioresistant phenotype. As for CNV associated to a more radiossensitivity, in HSC-3 cell line (Figure 33). We found losses in 18q and gains of several regions: 8p23.3, 10q11-q22, 14q13, 14q distal, 15q11.2-q21.3, 17q, 18q21.2 and 22q.

Furthermore, several regions with CNV from both cell lines were associated with a worse prognosis: Losses of 5q11, 8p23.3, 8p21-p22, 8p21.2 and 11q distal; Gains of 1q, 3q21-q29, 11q13 and 22q.

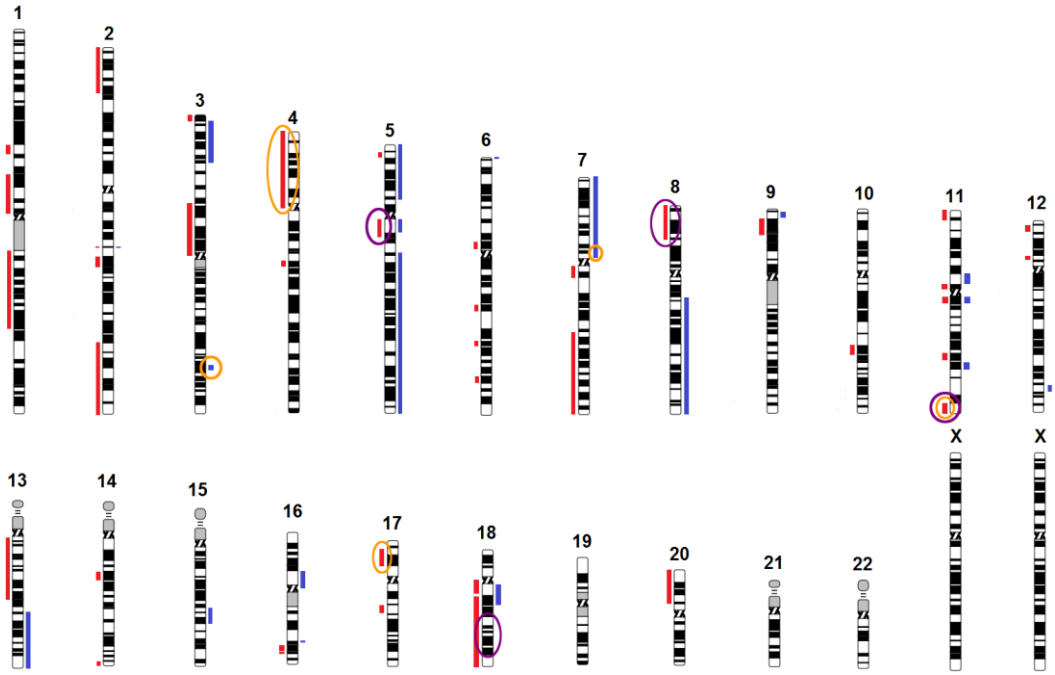

Figure 32 - Ideogram of BICR-10 cell line obtain by aCGH. Gains and Losses are represented with blue and red, respectively. Orange circles: CNV previous associated to radioresistance. Purple circles: CNV previous associated to a worse prognosis.

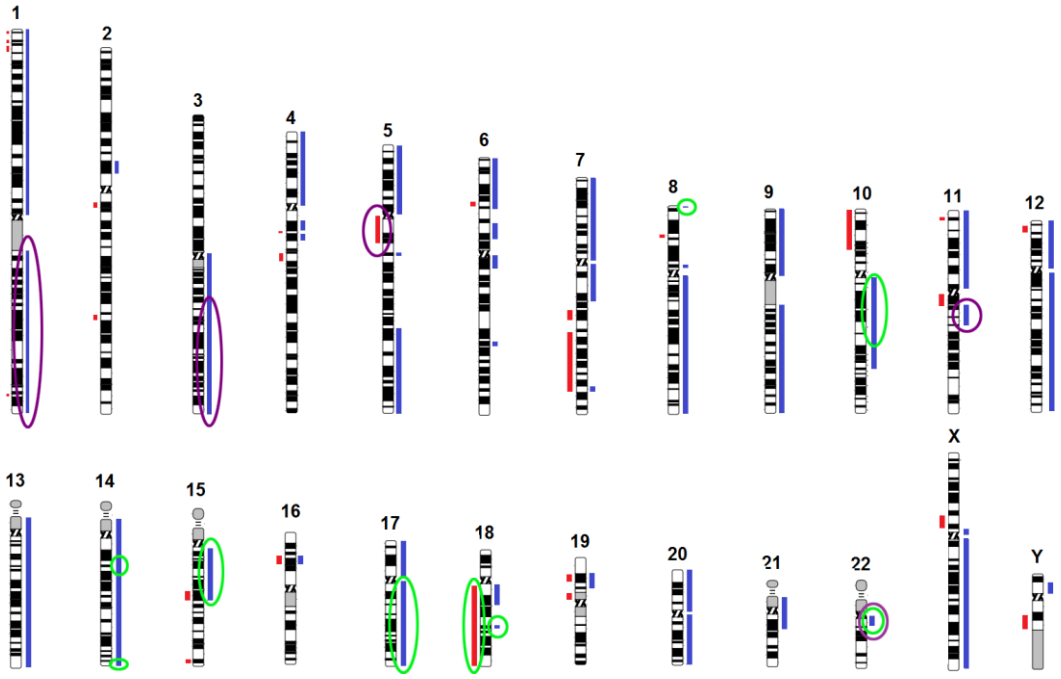

Figure 33- Ideogram of HSC-3 cell line obtain by aCGH. Gains and Losses are represented with blue and red, respectively. Green circles: CNV previous associated to radiossensitivity Purple circles: CNV previous associated to a worse prognosis.

5 DISCUSSION

5.1 CHARACTERIZATION OF HSC-3 AND BICR-10 CELL LINES

As previous referred, in spite the fact that HSC-3 and BICR-10 cell lines were established from different anatomic regions, both are classified as HNSCC commercial cell lines. Moreover, they can both be classified as OSCC, since both, buccal mucosa and tongue, belong to this sub anatomic region. On the other hand, they have different cell types and so, different characteristics. Taking this into consideration, it is important to mention that different anatomic regions have distinct clinical presentations and different clinical outcomes, which results in different treatment approaches. Taking this line of reasoning and the complexity of this disease, it is expected that the two different anatomic regions (buccal mucosa and tongue), from which the cell lines were established, present different genetic and epigenetic characteristics (Mao *et al.*, 2004). Finally, it is important to highlight that HSC-3 was established from a patient with lymph node metastasis whereas BICR-10 cell line was established from a patient without metastasis, however, its clinical stage is unknown.

The characterization of commercial cell lines has obvious benefits, since they are one of the most used model in biomedical studies. As such, genetic and epigenetic characterization are necessities in order to have as much information as possible about the cell lines, especially if they are used for translational research (White *et al.*, 2007).

Some scientists argue that cell cultures bring biased results, since specific cell populations are selected and those are the ones that evolve in culture conditions. However, it is already been proved that cell lines are similar to the tumours from which they arise and long-term HNSCC cell cultures present aberrations from the tissue which they were derived (Martin *et al.*, 2008). So, since cell lines are representative from the tumours from they were derived, they remain helpful models in clinical research, enabling the correlation between their results and with patients' results.

For the genetic and epigenetic characterization of both cell lines, cytogenetic and genomic techniques were applied: aCGH, karyotyping and MS-MLPA.

5.1.1 Methylation Profile

Since DNA methylation is the most common epigenetic modification in cancer (Mascolo *et al.*, 2012), the methylation profile of both cell lines was assessed through MS-MLPA. MS-MLPA is a target technique, allowing only the analysis of some genes. The probe panel used was not specific for HNSCC, but instead for cancer in general.

For this study a cut-off of 20% was defined, meaning that a gene was considered to be methylated when the ratio of methylation was over 20%. This cut off was established based on laboratory experience, as well as in scientific papers (Yalniz *et al.*, 2011; Chmelarova *et al.*, 2012). Concerning the reference samples used, none of them presented any genes with a methylation frequency above 20%, meaning that all the genes were unmethylated. However, it is important to increase the number of controls to define a more precisely cut-off, since three controls is considered to be a small number.

Both cell lines presented *RARβ, PAX5*, *PAX6*, *WT1*, *CADM1*, *GATA5* genes methylated. Even with some differences, both of them also had the *MGMT* gene methylated.

Regarding *RARβ* gene, it encodes the retinoic acid receptor that belongs to a superfamily of nuclear transcriptional regulators, and it is associated with cell growth and differentiation. RARβ loss in cancer frequently results from promotor hypermethylation, rather than genetic aberrations (Noorlag *et al.*, 2014). Since methylation of the promoter is associated with gene silencing, it is probable that an analysis of mRNA expression showed lack of expression of *RARβ* on both cell lines. This lack of *RARβ* expression is associated to an enhanced mitotic activity and loss of differentiation (Marzese *et al.*, 2012). In studies of cervical cancer, the decrease of the gene and protein expression were correlated with late response to therapy (Kim *et al.*, 2011), whereas in breast cancer studies, it was correlated with poor prognostic (Marzese *et al.*, 2012). In HNSCC, the lack of expression was verified in the majority of oral potential malignant lesions in comparison to normal tissue samples in a study performed by Lotan *et al.* (1995), indicating the involvement of *RARβ* methylation in the early stages of HNSCC carcinogenesis. Given that both our cell lines showed methylation of this gene, this hypothesis cannot be discarded, since HSC-3 cell line may have kept this alteration since the first steps of the carcinogenesis process and BICR-10 cell line could represent a early stage tumour. This hypothesis is also supported by the association of *RARβ* expression loss with the evolution from mortality to immortality in HNSCC (McGregor *et al.*, 1997), making this epigenetic alteration an important characteristic in HNSCC development.

Both *PAX5* and *PAX6* genes belong to a family with nine developmentally regulated genes that code for the PAX proteins, nuclear transcription factors that play roles in cellular differentiation, proliferation and migration. This family is thought to be a target for disruption during the oncogenesis process due to the fact that they are string transcriptional regulators. Besides, they are located at specific chromosomic regions that frequently suffer translocations in cancer, as the example of t(9;14)(p13;q32) in non-Hodgkin's lymphoma, which harbours *PAX5* gene (9p13) (Barr, 1997).

Concerning *PAX5*, although its methylation has already been described in HNSCC, its role in cancer development is controversial. Whereas Liu *et al.* (2011) reported downregulation of this gene associated with its methylation status in Hepatocellular carcinoma and Deng *et al.* (2014)

highlighted its potential function of tumour suppressor in gastric cancer through direct regulation of the p53 signalling pathway, Norhany *et al.* (2006) and Kanteti *et al.* (2009) showed that in OSCC and small-cell lung cancer (SCLC), respectively, *PAX5* gene was overexpressed, suggesting an oncogenic role. Even in HNSCC, the function of this gene is strongly controversial. Although Norhany *et al.* (2006) reported overexpression, a study performed by Guerrero-Preston *et al.* (2014) showed that *PAX5* was methylated and downregulated in HNSCC. Additionally, Kanteti *et al.* (2009) also examined HNSCC cell lines and observed absence of expression. The possibility of tissue-specificity interactions can be placed, however, since different studies with HNSCC cell lines showed opposite results, this hypothesis is unlikely. Other hypothesis is that this gene could play a double role in cancer, and could regulate different effectors during carcinogenesis, nonetheless this possibility lacks confirmation. In the present study, our results showed promotor methylation on both cell lines, suggesting the silencing of the gene, supporting the tumour suppressor role for *PAX5* in HNSCC. In HSC-3 cell line, MS-MLPA also detected gain in copy number for this gene. Since the methylation ratio was not very high (24%), it is possible that this gene is able to have a normal expression or overexpression. Supporting this hypothesis, Norhany *et al.* (2006) realized gene expression studies on HSC-3 cell line, the metastatic cell line, and demonstrated overexpression of the gene. These results may be explained with the fact that methylation is a reversible process and the cell line may showed differences at the time of the analysis in both studies. On the other hand, recent investigations have correlated DNA methylation with both a decrease and an increase in gene expression and the further study of this gene could prove this theory (Wagner *et al.*, 2014; Wan *et al.*, 2015). Kanteti *et al.* (2009) putted the possibility of the overexpression of this gene being associated with a more metastatic and aggressive stage in small cell lung cancer cells due to the interaction between PAX5 and c-Met. Proving the two latter theories right, the expression of this gene could be an important biomarker in cancer progression. In BICR-10 cell line case, to our knowledge, no similar study was previous done, so gene expression analysis must be performed in order to highlight this possibility and take more accurate conclusions.

Unlike *PAX5*, the *PAX6* gene does not have this controversy associated and its tumour suppressor role is well accepted. It is though that its contribution to oncogenesis is due to the silencing of the gene, abolishing this way, tumour suppression (Robson *et al.*, 2006). It was found to be methylated in breast and lung squamous cell carcinoma (Moelans *et al.*, 2011; Rauch *et al.*, 2012). It is not a gene well studied in HNSCC, but Gasche *et al.* (2011) found it to be methylated due to IL-6 inflammation. Our results show that methylation of *PAX6* gene could be a characteristic of HNSCC, since both cell lines were found to be methylated.

The *WT1* gene is other example of controversy. Just like *PAX5*, *WT1* is considered to have a tumour suppressor and an oncogenic role in carcinogenesis. It was considered to be a tumour

suppressor involved in kidneys Wilms' tumours, however, its overexpression in several tumours, such as carcinomas of the lung, colon, pancreas, breast and brain tumours, suggest an oncogenic role (Hartkamp and Roberts, 2008). Oji *et al.* (2003) showed an overexpression of this gene in HNSCC, leading to the conclusion that this gene may have an oncogenic role in this disease. However in Mikami *et al.* (2013) and in our study, the results showed promotor methylation of *WT1* gene. Regarding gene expression, Mikami *et al.* (2013) showed that HSC-3 cell line did not had mRNA or protein expression. This result is interesting if we have in consideration that, accordingly to MS-MLPA and aCGH, in HSC-3, besides the methylation of this gene, a gain was also detected. However, methylation ratio was closed to 100%. For BICR-10 cell line, as far as we know, no study was performed regarding *WT1* gene expression. Our results suggest a tumour suppressor role for this gene in HNSCC pathogenesis. Recently, it has been proposed that downregulation of this gene was associated with a decrease in mRNA and protein levels of both Bcl-2 and Cyclin D1 and overexpression is thought to be associated with apoptosis, a quiescent state, and increase or a block in differentiation depending on the differentiation status of the affected cell. Rather than categorise the gene, Huff (2011) put the hypothesis that the developmental stage of the cell was an important aspect to determine the effect of *WT1* gene expression alteration, suggesting a model where the alteration of this gene had a direct effect on the differentiation status of the cell, which would result in an alteration in cell viability and/or proliferative status (Hartkamp and Roberts, 2008; Huff, 2011).

Other gene with promotor methylation on both cell lines was *CADM1* gene. This gene was originally called Tumour Suppressor in Lung Cancer and it codes for a transmembrane protein from the immunoglobulin superfamily that plays a role in cell-cell interactions between epithelial cells (van den Berg *et al.*, 2011). It was found methylated or downregulated in various cancers, including HNSCC (Steenbergen *et al.*, 2004; Worsham *et al.*, 2006; van den Berg *et al.*, 2011). Methylation of this gene was associated with tobacco and worst prognosis in lung cancer in a study conducted by Kikuchi *et al*. (2006). Our results are in accordance with the literature, showing that this gene could be important in cancer development.

Finally, we have *GATA5* that was methylated in both HSC-3 and BICR-10 cell lines. The GATA family members are transcription factors that have essential roles in cellular growth and differentiation during embryogenesis and early development (Patient and McGhee, 2002). This gene is considered a tumour suppressor gene (TSG), since its silencing can prevent normal differentiation programs and contribute to tumour development (Fu *et al.*, 2007). *GATA5* is described to be methylated in a few cancers, like carcinomas of the lung, pancreas and renal (Guo *et al.*, 2004; Fu *et al.*, 2007; Peters *et al.*, 2014). In the latter, it was associated with tumour progression and worse prognosis (Peters *et al.*, 2014). Applying this to our results is possible that this association could also happen in HNSCC, since HSC-3 is a metastatic cell line and BICR-10 is a radioresistant cell line.

However studies for this gene in HNSCC are not common, Gasche *et al*. (2011) also found this gene to be methylated in HNSCC due to IL-6 inflammation, just like *PAX6* gene.

It is possible that these six genes, *RARβ, PAX5, PAX6, WT1, CADM1, GATA5,* could be important biomarkers for HNSCC, since two cell lines so different from each other, with molecular, histologic and anatomic differences, showed methylation on the gene's promoters. However, studies regarding their expression and protein analysis are imperative in order to establish their roles in HNSCC tumorigenesis. Furthermore, an analysis on HNSCC patients could be helpful to understand their importance and if they could be reliable biomarkers.

In the case of *MGMT* gene, the results obtained showed that both cell lines were methylated in this gene, however with some differences, since the probe panel had two different probes for the gene, one located 319 nt after the exon 1 and the other 72 nt before the exon 1. BICR-10 cell line had the first probe methylated, whereas HSC-3 had the second one methylated. Accordingly to the manufacturer's instructions, this probemix is not the most adequate for the assessment of *MGMT* methylation. This happens because *MGMT* has two methylation hot spots near the transcription start site that are associated to the gene silencing and, between these two hot spots there is a region that has a much lower frequency of methylation (Qian and Brent, 1997). Both probes of this probemix are located in these two hot spots. For the assessment of MGMT methylation, MRC-Holland recommends the use of the ME011 probemix that has six probes for the gene. Taking this line of reasoning, it was decided in this work not take in consideration the results for the *MGMT* gene.

Regarding the differences between the two cell lines, the genes *TP73* and *ESR1* were methylated in the metastatic cell line, HSC-3, and the genes *CHFR* and *CDH13* were methylated in the non-metastatic cell line, BICR-10.

TP73 gene is involved in cell cycle regulation and induction of apoptosis. The biggest problem of accessing the function of this gene in tumorigenesis is the fact that *TP73* locus encodes two isoforms with opposite effects: TAp73, which is able to activate p53-responsive genes, inducing cell cycle arrest and apoptosis (tumour suppressor function); and ΔNp73, that is an inhibitor of both TAp73 function and p53-induced apoptosis (oncogenic function) (Melino *et al.*, 2002). This gene was found to be methylated in HNSCC (Worsham *et al.*, 2006; Stephen *et al.*, 2010; Worsham *et al.*, 2014) and in other types of cancer (Stephen *et al.*, 2009; Marzese *et al.*, 2012). Based in our results, it is hypothesized that the methylation of this gene could be associated to lymph node metastasis. Furthermore, in invasive ductal breast tumours, Marzese *et al*. (2012) concluded that *TP73* methylation was independently associated with high-grade tumours and co-methylation of *TP73* and *RARβ* could be associated to a higher histologic grade, an increased proliferation ration and tumour size, and to lymph node metastasis. Since our metastatic cell line, HSC-3 showed methylation of both genes, whereas the non-metastatic cell line only showed methylation of one gene (*RARβ)*, and

has a higher proliferation rate, it is possible that this conclusions could also be applied to HNSCC, however, further studies should be performed in order to confirm this result.

Regarding *ESR1* gene, it encodes an estrogen receptor, which is a ligand-activated transcription factor that is important for hormone binding, DNA binding and activation of transcription [NCBI-Gene: 2099]. Stephen *et al.* (2010) considered that this gene had a role as a TSG by suppressing metastasis development, which is in agreement with our results, since this gene is only methylated in the metastatic cell line and the most common result of gene methylation is gene silencing, thereby not contributing to metastasis suppression. Stephen *et al.* (2010) also considered *ESR1* as a good predictor of advanced stage in laryngeal carcinoma and poorer survival outcomes. This gene was also found to be methylated in colorectal (Molinari *et al.*, 2013), breast cancer (Gaudet *et al.*, 2009) and in osteosarcoma (Sonaglio *et al.*, 2013).

According to the literature and our results, it is possible that the co-methylation of this two genes, *TP73* and *ESR1*, could be a predictor of metastasis development, particularly with the envolvement of the lymph nodes. Regarding the association with *RARβ* methylation, it is more unlikely, since the non-metastatic cell line also presented this epigenetic alteration and other studies have shown this to be associated with early events in HNSCC (Lotan *et al.*, 1995; Worsham *et al.*, 2014).

BICR-10 cell line showed methylation of other two genes: *CHFR* and *CDH13*.

CHFR is a checkpoint gene that delays metaphase entry in response to mitotic stress (Scolnick and Halazonetis, 2000). Baba *et al.* (2009) showed that the methylation of this gene was more frequent in buccal mucosa rather than in tongue. In our results, *CHFR* was methylated in BICR-10 cell line. BICR-10 cell line is derived from a tumour in the buccal mucosa, whereas HSC-3 cell line is derived from the tongue. This goes in agreement with the results above mentioned. The results from Baba *et al.* (2009) also identified this epigenetic abnormality as a good distinguisher between primary tumours and normal adjacent mucosa, since the latter had a lower frequency of *CHFR* methylation. This gene was considered to have a high methylation frequency in HNSCC (Stephen *et al.*, 2010; Demokan and Dalay, 2011; Noorlag *et al.*, 2014; Worsham *et al.*, 2014). Additionally, a study was performed with HSC-3 cell line, showing that *CHFR* was not methylated (Toyota *et al.*, 2003), which agrees to our results.

The *CDH13* gene encodes an intracellular adhesion molecule with a role on cellular proliferation regulation, invasion, and intracellular signalling during cancer progression. It works as an inhibitor for the Ras/PI3K/AKT pathway (Conacci-Sorrell *et al.*, 2002; Adachi *et al.*, 2010). It has a tumour suppressor role, maintaining cell adhesion integrity. Aberrant methylation for this gene was found in several cancers, like colorectal, lung and breast. It was considered to be an early event in HNSCC cell lines (Worsham *et al.*, 2006; Stephen *et al.*, 2010). Only one of the cell lines showed

methylation of this gene, the BICR-10 cell line and, since the methylation of this gene is thought to be an early event in HNSCC cell lines, it is possible that BICR-10 is derived from a tumour in an early stage, or kept this alteration during its development through the tumorigenesis process.

Moreover, in the epigenetic model proposed by Worsham *et al.* (2014), the promotor methylation of four genes was considered to be specific for primary tumours. This four genes are *CDH13, CHFR, CADM1* and *RARβ*. According to our results this is an unlikely possibility since the metastatic cell line, that is derived from a primary tumour did not presented the methylation of the four genes, whereas BICR-10 cell line, which was derived from a recurrent tumour, showed promoter methylation of this four genes.

5.1.2 Karyotype

The analysis of genetic abnormalities in both cell lines was accessed through conventional karyotyping, in order to provide a full picture of genetic alterations present in HSC-3 and BICR-10.

Chromosome banding analysis allows the examination of individual tumour cells, which is particularly interesting in HNSCC since a high percentage shows intratumoral heterogeneity (Jin *et al.*, 2005). This heterogeneity can be due to cytoskeletal alterations that originate chromosomal segregation problems, leading to differences between the progeny after mitosis. Cytogenetic aberrations have already been classified as good biomarkers for diagnostic, prognostic and treatment response (Gollin, 2014) and conventional cytogenetics is able to give a full image of the genome and detect structural aberrations, such as balanced translocations, that are not detected by other molecular cytogenetic techniques, as aCGH. However, a problem with karyotyping analysis concerns the fact that it is only possible in limited cases, since it can be difficult to prepare metaphase spreads (Uchida *et al.*, 2006). Regarding our cell lines, only ten metaphases were analysed mostly due to staining difficulties. However, this number is rather small and, at least, more 20 metaphases have to be analysed for more accurate results.

Although, in our cell lines was noticeable some aberrations that are in accordance with literature results previously published (Jin *et al.*, 2000; Uchida *et al.*, 2006; Martin *et al.*, 2008; Gollin, 2014). Concerning numeric aberrations, monosomy 18 and loss of Y chromosome were noticeable in HSC-3 cell line and are highly common in patients with HNSCC (Jin *et al.*, 2005; Jin *et al.*, 2006).

Regarding structural aberrations, in both cell lines, there are various isochromosomes. In HSC-3 we have two isochromosomes: i(1)(q10) and der(13;13)(q10;q10), whereas in BICR-10 we have two other: i(5)(p10) and i(7)(p10). Regarding whole-arm translocations we have t(3;5)(p10;q10) and der(15)t(X;15)(q10;q10) in BICR-10 cell line and der(14)t(14;17)(q10;q10) and der(X)t(X;8)(q10;q10) in HSC-3 cell line. Other aberrations are derivative chromosomes that are

formed due to translocations, as der(1), der(7)t(7;11), der(8;8), der(8)t(5;8) and der(17)t(7;17) in BICR-10 cell line and in HSC-3 cell line. Some breaks through centromere were observed as well, especially in HSC-3 case, for example, del(1)(p10) and del(2)(q10).

These results suggest that centromeric breaks are common events in HNSCC, since the majority of the alterations observed were aberrations involving whole arm chromosomes. This hypothesis has already been suggested and, moreover, it is though that centromere cleavage is the mechanism behind these alterations (Jin *et al.*, 2000; Uchida *et al.*, 2006).

Of particular interest is BICR-10 cell regarding i(5)(p10) and der(8;8). This derivative of chromosome 8 is very similar to an isochromosome since it shows a duplication of the q arm of the chromosome, however it has also some genetic material that we were not able to identify. Nevertheless, the presence of these two isochromosomes in HNSCC is associated with genetic gain of 5p and 8q, which occurs in this cell line, and it is hypothesized that these two aberrations are important in OSCC, remaining to know if they are causes or effects in this type of cancer (Uchida *et al.*, 2006).

As for HSC-3 cell line, a previous study (Uchida *et al.*, 2006) has already described some cytogenetic alterations, however, they were assessed by fluorescence *in situ* hybridization (FISH) - spectral karyotyping (SKY). Among the alterations that they described, four of them were also found in our study: der(X)t(X;8)(q10;q10), i(1)(q10), der(13;13)(q10;q10) and monosomy 18. However, some alterations detected in our study are not referred in this paper and even the average number of chromosomes is dissimilar due to the cell lines instability. To our knowledge, no previous study has been performed in order to characterize BICR-10 cell line, being this the first one.

Furthermore, it is important to highlight the small number of aberrations identified in this study. HSC-3 and BICR-10, especially HSC-3, have much more aberrations than the ones mentioned above, however, by classical cytogenetic analysis they were not possible to identify. For this reason, a study with FISH, in particular SKY, is going to be helpful in order to make a more profound and accurate karyotype of both cell lines.

5.1.3 Genetic Characterization

In regard to the particular case of CNV, aCGH was used to obtain results from the whole genome, and MS-MLPA was applied for a more targeting analysis. Besides the previous mentioned problems with respect to the histological and anatomic characteristics of the two cell lines, the different stage in the disease progression was found to be a huge problem in the analysis of the cell lines.

In our aCGH and MS-MLPA analysis is possible to verify that both cell lines are extremely different from each other. aCGH, being a technique that allows the analysis of all the genome,

demonstrated that HSC-3 has a higher number of gains, whereas BICR-10 presented a higher number of losses. Additionally, the metastatic cell line has a higher frequency of abnormalities. One possible explanation for the higher chromosomal instability (CIN) in HSC-3 is regarding its advanced stage of development. As it is known, cancer is characterized for genetic instability, once HSC-3 is in a higher stage of development, it is considered normal that it presents more aneuploidies and a higher CIN. However the differences could also be associated to the different sub anatomic regions from which each cell line was established. Other factors that may influence the results are the aggressiveness and response to treatment of each patient from who the cell line was established. The fact that the outcome of none of the patients is known makes even harder the analysis of the results obtained.

One important thing to highlight is the fact that, even so different, both cell lines present abnormalities previous described as common events in HNSCC patients.

5.1.3.1 Characteristics commonly present in HNSCC patients

Chromosome 3 is commonly altered in HNSCC patients, both the long and the short arm (Gollin, 2014). In HSC-3 cell line there is a gain of the long arm of chromosome 3. This could induce the idea of a isochromosome 3q, which is very common (Gollin, 2014), however, our karyotyping results does not showed the presence of an isochromosome 3q. The gain of the distal part is also common and is associated to a worse outcome, especially the dup(3)(q21q29) (Freier *et al.*, 2010). As the gain of this arm is common, there are suggestions regarding the presence of an important oncogene. The most considered gene is the *ATR* (3q22-q24), which is responsible for the activation of the checkpoint when DNA damages, due to stresses, are present on the cell (Gollin, 2014) This hypothesis is sustained in HSC-3 cell line, since the amplification of this gene is observed in aCGH. The *PIK3CA* is also an important oncogene in this region and it is involved in cell survival in HNSCC (Leemans *et al.*, 2011). HSC-3 also presented amplification of this gene. It was also associated to an advanced stage, which is concordant to our results, once BICR-10 cell line did not presented any alteration of the gene.

Regarding the short arm of chromosome 3, in HNSCC, it frequently showed losses, as in the case of BICR-10 cell line. Since they are associated to early events (Gollin, 2001; 2014) it is difficult to claim that BICR-10 cell line was established from a tumour in early stages, because it is possible that the tumour had suffered these losses and has kept them to the passage through later stages. Two TSG in this regions are the *FHIT* and *RARβ* (Lotan *et al.*, 1995; Gollin, 2001). The first one is deleted on BICR-10 cell line and the deletion of *FHIT* combined with mutation on *TP53* was associated to a worse prognosis in HNC cell lines (Raju *et al.*, 2015). Even though we did not analyse the *TP53* gene for mutations, BICR-10 cell line is described to have this gene mutated. In this case, this could indicate that BICR-10, even though the stage of development is unknown, could represent a model

for an aggressive HNSCC. However, the reason for the worse outcome it is not clear: could the tumour be resistant to therapy or evolve to metastasis? Regarding the *RARβ* gene, MS-MLPA analysis showed it to be amplified, however, as previous described, it was also methylated (98%), meaning that it was probably silenced. In MS-MLPA analysis, BICR-10 cell line presented a gain in *VHL* gene. This gene, encoded at 3p25.3, is a known tumour suppressor and it is involved in the regulation of various cellular processes, being its interaction with HIF-1α the most studied. When the VHL protein is absent, HIF-1α is not degraded, leading to the transcription activation of several target genes involved in processes like angiogenesis and glycolysis (Gossage *et al.*, 2015). To our knowledge, there are no reports of gains associated to *VHL* so the hypothesis of this gene also had an oncogene role is discarded. The most probably explanation is that, even though *VHL* is amplified, it is non-functional due to other processes and its amplification is a contribution to genetic instability, characteristic of cancer cells. Nevertheless, further studies are required to assess the *VHL* function in this cell line.

Other common aberration in HNSCC is 7p gain and both our cell lines showed this gain, as observed by aCGH and conventional cytogenetics. Furthermore, Bockmühl & Peterson (2002) have associated gain of 7p to earlier stages in HNSCC carcinogenesis. Since both of our cell lines presented this gain, it is possible that this alteration has occurred in the early stages and had been maintained. This could mean that 7p gain is highly important in order to keep the malignant phenotype once it happens in the beginning of the process and remains, even in the metastatic stage. One of the most important genes known to be associated with cancer is mapped at 7p12.3-p12.1, *EGFR*. The *EGFR* gene is highly involved in the development of HNSCC, as in other types of cancer, since it plays an important role in growth, survival, invasion, metastasis and angiogenesis. *EGFR* gain is correlated with poor prognosis and treatment resistance (Gollin, 2014). HSC-3 and BICR-10 showed gain of this gene. The gain in the cell lines allows to drawn the possibility of both tumours have had worse prognosis: HSC-3 cell line is metastatic and this, by itself, has a worse prognosis. In BICR-10 cell line, it is possible other explanations: the tumour could had characteristics that made it prone to the development of metastasis or resistance to treatment could had happen.

Both HSC-3 and BICR-10 cell line showed 8q gains. This gain is also a common alteration present in HNSCC tumours. One important oncogene in this region is *MYC*, which is associated to poorer survival rates (Ribeiro *et al.*, 2014b). Results from both cell lines showed a gain of *MYC* gene, leading to the same hypothesis made previously for *EGFR* gene. *PTK2* (8q24-qter) is other gene in this region, which gain has been associated to a higher invasiveness stage (Ribeiro *et al.*, 2014a). This gain is only seen in HSC-3 cell line, which is concordant to the metastatic profile. BICR-10 did not showed this alteration, which is in accordance with the fact that it is not derived from a patient

with metastasis. Moreover, this also supports the hypothesis that this cell line was derived from a tumour in early stages of development.

Losses on chromosome 9 are very common in HNSCC, especially at the short arm (Gollin, 2014). This loss is associated to an early event in this type of cancer and LOH at 9p21-p22 appears in a high frequency. As mentioned, in this region are mapped genes that encode CDKs, which are very important in cell cycle regulation (Scully *et al.*, 2000; Gollin, 2014). Even though BICR-10 cell line did not showed loss of the entire arm, this band is lost. Moreover, karyotpe analysis of this cell line detected del(9)(pter). As for HSC-3, it has a gain of all chromosome 9. Regarding the 9p, it is possible that, in earlier stages of development, one allele was lost, however, due to the high genomic instability and the progression in carcinogenesis, it is possible that, the allele that was not lost, had suffered exponential amplification in a way that the loss of the allele is not accounted for anymore. As showed by MS-MLPA results, BICR-10 has loss of one gene that it is commonly deleted in HNSCC: *CDKN2A*. This gene is a CDK inhibitor, which blocks cell cycle progression, leading to an unregulated cellular proliferation (Martin *et al.*, 2008). Interestingly, HSC-3 shows genetic gain of this gene. A recent study, have associated the expression of this gene to a positive response to treatment (Tehrany *et al.*, 2015).

Amplification of 11q13 band is very common and was observed in HSC-3 cell line. This is a band with several genes, the most of all are overexpressed in HNSCC. Furthermore, this alteration is thought to be involved in the transition from moderate to severe dysplasia (Gollin, 2014). Since BICR-10 cell line did not showed this alteration, it is possible to hypothesize that the stage of this cell line was before the transition to severe dysplasia, meaning that it would represent a hyperplasia or a dysplasia. However, despite being common, this amplification does not happen in all HNSCC cases, so BICR-10 cell could also be an example of a case where this amplification did not occur. Nevertheless, BICR-10 showed 11q distal loss without 11q13 band amplification which has been associated to a stage between dysplasia and carcinoma *in situ* (Gollin, 2014). Accordingly to the literature, is possible that BICR-10 cell line represents a tumour in the dysplasia stage.

Finally, loss of 18q is also a cytogenetic aberration that occurs frequently in HNSCC and both our cell lines showed this alteration (Scully *et al.*, 2000). However the possible tumour suppressor gene (TSG) in this region was not determined yet, and, since the MS-MLPA panel used did not had any probe for any gene in chromosome 18 and aCGH gives an elevated number of altered genes, there are no conditions to draw any hypothesis without more results.

5.1.3.2 Comparison between a metastatic and a non-metastatic cell line

Both cell lines are much different from each other. Analysing the aCGH and the MS-MLPA results, the cell lines showed big differences.

The biggest similarities between the lines were described above. Even though, according to MS-MLPA, they have one more similar genetic alteration was the loss of *CFTR*, mapped at 7q31.2. The CFTR protein is a chloride channel, required for transepithelial salt and water transport. This gene is primarily expressed in epithelial cells, such as those of airways, gut, pancreas and reproductive tract. The major disease associated to alterations in *CFTR* is cystic fibrosis, however, its role has been studied in different types of cancer, including lung cancer (Zhang *et al.*, 2013; Li *et al.*, 2015; Mall and Galietta, 2015). It is suggested that the role of this gene in carcinogenesis depends upon the type of tumour. To our knowledge, no study was performed in order to analyse this gene in HNSCC carcinogenesis. Li *et al.* (2015) analyse *CFTR* in non-small-cell lung carcinoma (NSCLC) and concluded that it had a tumour suppressor role in this type of cancer, being its downregulation associated to an advanced stage, lymph node metastasis and poorer prognosis. Taking this into consideration and the fact that both cell lines showed a decrease in copy number for this gene, it is possible that *CFTR* also plays a part in HNSCC tumorigenesis, probably as a TSG. It is possible, however, that this gene is not associated to lymph node metastasis, since the non-metastatic cell line also had loss of the gene. Likewise, the association with an advanced stage is also unlikely, since BICR-10 cell line is hypothesized to be a dysplasia. Nevertheless, further studies are crucial in order to understand the role of *CFTR* in HNSCC development.

Regarding differences between both cell lines, chromosome 1 is a good example. Whereas in HSC-3 chromosome 1 has a total gain, BICR-10 shows only losses. One interesting alteration observed in HSC-3 karyotyping was the presence of isochromosome 1q, already reported for this chromosome (Jin *et al.*, 2001). Moreover, gains of several regions, such as 1q21-q24 and 1q32-q41 have been associated with the presence of metastasis (Bockmuhl and Petersen, 2002). Our data is in accordance with these assumptions. Furthermore, 1q gain was associated to a metastatic phenotype in SCCs of the lung and it is one of the most frequent alterations in brain metastasis (Bockmuhl and Petersen, 2002). Together, the literature and our data suggest a strong relation between the presence of metastasis and 1q gains. Then, 1q gain could possibly be biomarker for metastasis development.

Regarding chromosome 4p, in HSC-3 we are presented with all arm gain, whereas in BICR-10 cell line we have 4p loss. This 4p loss is thought to be a relatively common alteration in HNSCC cell lines (Martin *et al.*, 2008; Freier *et al.*, 2010). Moreover, this loss was also associated to lymph node metastasis in oral cancer (Gollin, 2014). Although our results are contradictory, 4p gain in the metastatic line could be one case whereas a random amplification could have occurred, contributing for a higher genomic instability. This hypothesis is sustained by the fact that are no references to 4p gains in HNSCC. For BICR-10 cell line, it is probably that this alteration could indicate that this tumour would had a propensity to metastasis. It is important to highlight that this association with lymph node metastasis is not exclusively to oral cancer (Petersen *et al.*, 2000; Yen *et al.*, 2001).

Chromosome 10 appears to have big differences between both cell lines. BICR-10 shows a deletion in 10q23.1-q23.2, whereas HSC-3 has a loss in 10p15.3-p12.4 and a 10q11.21-q24.2 gain. It could be possible that aberrations on this chromosome allowed the distinction between metastatic and non-metastatic stages. However, since none of this alterations have been described in HNSCC, a more likely hypothesis is that the alterations seen in HSC-3 were due to randomly alterations usually seen in cancer cells. Furthermore, chromosome 10 is not usually mention as a common characteristic in the majority of cancers. In spite that, a few genes that are located on this chromosome are frequently studied, as the case of *PTEN* (10q23.31) (Scully *et al.*, 2000; Mriouah *et al.*, 2014). Still, only HSC-3 showed CNV of *PTEN*, detected by aCGH. Nevertheless, a more specific analysis of this chromosome on HNSCC could help understanding if it has a role in carcinogenesis.

Looking at the results obtained, alterations on chromosome 12 are more characteristic of the metastatic cell line, HSC-3. BICR-10 cell line barely shows any alteration and HSC-3 as a gain of all the chromosome. These aberrations on chromosome 12 are not common in HNSCC (Scully *et al.*, 2000). In germ cell tumours, however, it is described that 12p gains are common and are associated to the progression to a more invasive stage (Looijenga *et al.*, 2011). In chronic lymphocytic leukemia trisomy 12 is common and it is considered to be a clonal driver mutation implicated in the formation of other genetic abnormalities. Even though it is common, no set of candidate genes was possible to define since, usually, all the chromosome appears altered. However, genes such as cyclin-dependent kinase inhibitor 1B (*CDKN1B1*), *CDK4*, huntingtin interacting protein 1 related *(HIP1R)*, myogenic factor 6 *(MYF6)* and MDM2 proto-oncogene (*MDM2)* have been proposed (Puiggros *et al.*, 2014). Recently it was also suggested that trisomy 12 could have a role in mammary neuroendocrine carcinoma (Xiang *et al.*, 2014). Taking all this into consideration, it is important to analyse in more detail the role of chromosome 12 in HNSCC. In spite the fact that reports of alterations are not common, some important genes could be mapped at chromosome 12, and it could be as in the case of germ cell tumours and become a biomarker for invasiveness. The role of a clonal driver mutation is, however, unlikely, since these alterations occur early in carcinogenesis and BICR-10 cell line did not present much alterations on chromosome 12.

Analysing the data obtain by MS-MLPA the greater differences between the two cell lines are in chromosome 13, since *BRCA2* (13q13.1) and *RB1* (13q14.2) have genetic gain in HSC-3 cell line and in BICR-10 cell line loss. These results are in agreement with aCGH data and cytogenetics. It is possible that chromosome 13 could be pivotal in distinguish the metastatic from the non-metastatic cell line. *BRCA2* codes for the nuclear protein BRCA2, which binds to BRCA1 and to RAD51. The interaction with the latter, a recombination and repair molecule, reveals a role for BRCA2 in DNA repair, particularly in RAD51-dependent pathways for homologous recombination. Data have showed that cells deficient in BRCA2 accumulate aberrations, leading to the assumption

that this gene also plays an important role in genetic stability (Venkitaraman, 2001; Cornejo-Moreno *et al.*, 2014). Although it is known the involvement of this gene in several types of cancer (Cornejo-Moreno *et al.*, 2014), there is a lack of evidence for the involvement of this gene in HNSCC pathogenesis (Nawroz-Danish *et al.*, 1998). Regarding *RB1*, it is a critical tumour suppressor gene (TSG) with three different roles in cells. The most studied is the checkpoint function. Rb protein blocks the passage from G1 phase to S phase of the cell cycle when DNA is damage. The second is the inducing of apoptosis, since Rb is capable of activate apoptosis regulator BAX, a protein that binds to the apoptotic repressor Bcl-2, leading to the release of cytochrome c from the mitochondria. This release is a known apoptosis trigger. Finally, RB1 is thought to cause telomeric attrition and spontaneous CIN (Gollin, 2014). However, just like *BRCA2*, the frequency of alterations in *RB1* in HNSCC is considered to be low (Yoo *et al.*, 1994). Curiously, HSC-3 has a greater chromosomal instability (CIN) than BICR-10, which supports the function of *RB1* in inducing CIN. Even so, deletions on 13q are very common in patients with HNSCC (Gollin, 2014). Sabbir *et al*. (2006) studied the chromosome 13 on an Indian population and found four regions with high frequencies of LOH: 13q13.1, where *BRCA2* gene is mapped, 13q14.2 (*RB1* gene), 13q21.2-22.1 (breast cancer 3 *(BRCA3)* gene) and 13q31.1. They concluded that this four regions were highly associated with an advanced stage and poor patient outcome, having some cumulative effect in the progression of this type of tumour. Furthermore, this four regions are hypothesized to be needed for the development of later stages of HNSCC. BICR-10 cell line showed loss of *BRCA2* and *RB1* genes but not showed loss of *BRCA3* or in the 13q31.1 region. One possible explanation is that loss of *BRCA2* and *RB1* are deletions that occur first in the development, being followed by the deletion of the other two regions, and, as the four regions are being deleted, the tumour is progressing (Sabbir *et al.*, 2006). As for HSC-3 cell line, it shows a gain of all 13q, including gain of *BRCA2* and *RB1*. This could be explained by the fact that this tumour already reached the last stage in the development, once it already metastasized, so these deletions could had occurred in the past but for some reason, possibly only one of the chromosomes was lost in previously stages, remaining one chromosome that was highly amplified, enhancing the genetic instability of the tumour.

Deletions on chromosome 17p are relatively common and mutations on the *TP53* gene are associated to early events in tumorigenesis (Scully *et al.*, 2000). It is interesting in the case of BICR-10 cell line, since it is described has having mutations on this gene and aCGH and MS-MLPA studies showed copy loss, meaning that it is highly probably that this line do not have any p53, which is frequent to be associated to a worse prognosis (Scully *et al.*, 2000). For the HSC-3 cell line, all chromosome is amplified, including *TP53* gene. This could also be the case of an alteration that could predict metastasis, since gains of 17q are often seen in invasive and metastatic tumours (Bockmuhl and Petersen, 2002).

Chromosome 20 is not one of the most mentioned chromosomes involved in HNSCC pathogenesis. However, 20q gains have been described (Bockmuhl *et al.*, 1998; Smeets *et al.*, 2006) and have been associated with metastasis in several cancers, such as colorectal, and with poor patient survival in breast cancer and even in HNC (Ashman *et al.*, 2003; Bergamaschi *et al.*, 2006; Bruin *et al.*, 2011). Recently, a study in patients with hepatocellular carcinoma concluded that the specific gain of 20q13.12-13.33 was a prognostic marker of metastasis and death and suggested that the genes DEAD (Asp-Glu-Ala-Asp) box polypeptide 27 (*DDX27),* UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5 *(B4GALT5),* ring finger protein 114 *(RNF114),* ZFP64 zinc finger protein *(ZFP64)* and prefoldin subunit 4 (*PFDN4)* could be oncogenes involved in the unfavourable outcome of these patients (Wang *et al.*, 2015). BICR-10 cell shows loss of 20p, whereas HSC-3 has a gain of the entire chromosome, which is evident in karyotype, with the presence of trisomy 20 or even tetrasomy in most of the metaphases. The 20q gain in HSC-3 cell line could indicate that this arm could also have a role in HNSCC carcinogenesis, like in other types of cancer and even be associated to metastasis development. Further studies are required to analyse the particular involvement of this arm, and even of the 5 genes proposed by Wang *et al.* (2015), in HNSCC carcinogenesis.

Chromosome 3, 9 and 11 are other examples of differences between the metastatic and the non-metastatic cell line, however, they were described previously.

5.1.3.3 BICR-10 and HSC-3 stages of development

HSC-3 is a metastatic cell line and our study confirmed that it presented alterations that were associated with metastasis in different stages, such as gains of 1q21-q24, 1q32-q41, 1q21-q22, 1q42-q43, 1q32, 3q25-q27, 5p, 7p, 7q11.2, 10q21 and 12q13 and loss of 5q11 (Bockmuhl and Petersen, 2002; Wreesmann *et al.*, 2004; Gollin, 2014). Additionally, it has the amplification of 11q13 band, very common in HNSCC tumours that is associated to a poor prognosis (Bockmuhl and Petersen, 2002).

Regarding BICR-10 cell line, its stage is unknown. Nonetheless, this study revealed that the majority of alterations in BICR-10 cell lines were associated to earlier events, such as 3p, 9p21-p22, 13q losses, 7p gain, 11q distal loss without 11q13 amplification and *TP53* mutations (Scully *et al.*, 2000; Gollin, 2001; Martin *et al.*, 2008; Gollin, 2014). So, according to several progression models for HNSCC, this line shows more alterations of the mild dysplasia stage, however due to 13q and 11q distal deletions, it is possible that it was progressing towards severe dysplasia (Argiris *et al.*, 2008; Haddad and Shin, 2008; Pai and Westra, 2009). Although, this line also presented alterations associated to metastasis and invasive carcinoma, this could be due to an enhancement of genetic instability or the tumour from which BICR-10 was obtain could had a propensity to develop metastasis.

5.2 ASSESSMENT OF RADIATION'S EFFECTS

Radiotherapy is one of the most common treatments for cancer cells, and, although it has been used for many years, little is known about its effects on tissues. Anyhow, it is known that its effect differs depending on cell type, cell characteristics, cell surrounding, and even the dose applied translates in differences. Nevertheless, the mechanisms and genetic alterations that lead to different responses to radiation are not fully elucidated. Radiation-induced genomic instability (RIGI) is defined as the enhancement of *de novo* mutation events in the progeny of irradiated cells. Among the manifestations of RIGI are: delayed reproductive death, increased apoptosis, gene expression changes, micronuclei formation, chromosomal abnormalities and DNA methylation alterations (Ilnytskyy and Kovalchuk, 2011).

In this study, we analyzed the effect of different doses in two different cell lines regarding copy number variations, alterations in the methylation profile and on the karyotype.

Accordingly to the clonogenic assay performed, the two cell lines have big differences regarding their radiation response. The clonogenic assay measurements are based on the presence of the so called clonogenic cells. It is known that carcinomas have stem cells, which are cells with capacity to maintain their numbers while giving rise to cells that are able to differentiate and proliferate in order to replace the other functional cellular population. Some tumours regrow after treatment, namely after radiotherapy. This happens because some of the neoplastic stem cells were not killed during treatment, which means that the key in radiobiology is figuring out a way to kill all the stem cells of a tumour, preventing it from regrowing. Then, the efficacy of the radiation treatment is measured regarding the quantity of stem cells that are still in the tumour and have, therefore, the ability to form colonies – the clonogenic cells. A cell is considered to be clonogenic if it leads to the formation of a colony with, at least, 50 cells. The number 50 is used because it represents five to six generations of proliferation and the objective is to exclude two types of cells: (1) the ones that have entered on differentiation stage and have a limited growth potential and (2) the cells that suffered sublethal damages by therapeutic treatments. It is important to highlight that, after irradiation, the damaged cells do not die right after and some of them are able to originate descendants before they died. Taking this into consideration, the important factor of radiation response is not the cell killing, but rather the loss of reproductive integrity, which happens some hours after treatment due to genome damages, leading to metabolic and cell death processes (Joiner and van der Kogel, 2009). Thus, the cell survival curves that were obtained after clonogenic assays analysis measure reproductive cell death.

Nowadays, there are three mathematical models that are used for the shape of the survival curve: the single-target/single-hit model, the multitarget model and the LQ model. All of them are

based on the idea that there is a target in the cell, which may be specific regions that are important for the maintenance of the reproductive integrity (Joiner and van der Kogel, 2009; Kelsey *et al.*, 2013). For BICR-10 cell line, the single-target/single-hit model was the one that was more adequate accordingly to the results obtained, whereas for HSC-3, the LQ model was the most adequate. The most common model used for survival cell curves of mammalian cells is the LQ model. However, this can vary between different cells.

As expected, there is a decrease in cell survival with an enhancement of dose applied, except for HSC-3, which shows and enhancement of cell survival at 0,5 Gy. This is an example of an adaptive response, previously described. Furthermore, BICR-10 is a much more radioresistant cell line than HSC-3, which is noticeable not only by the cell survival curve but also for the LD50. HSC-3 has a LD50 of 1 Gy and BICR-10 a LD50 of about 3 Gy. Even though they appear similar, in radiobiology they are very different, particularly if we take into consideration the fact that HNSCC is treated with 2 Gy daily doses of radiation. These changes in radiosensitivity are of big concern, since tumours with high levels of response to radiation can be treated with much higher doses than what it is needed, which leads to the submission of normal tissues to damages that could be avoided and are unnecessary for the accurate treatment of the tumour.

5.2.1 Genomic Instability assessed by Karyotyping

The major alterations associated with radiation-induced instability are chromosomal changes. Chromosomal gaps, breaks, duplications and partial trisomies are the ones that are most described. Bearing this in mind, the most supported hypothesis is that radiation exposure is capable of destabilizing the genome, leading to the formation of new genomic events in progeny of the irradiated cell, as, for example, chromosomal aberrations (Morgan, 2003).

First it is important to highlight the fact that it was not possible to analyse all conditions by karyotype due to the fact that, for the highest doses the cells were not in division or the chromosome G banding showed a pattern that was not possible to analyse. In HSC-3 cell line this problem occurred with cells submitted to 5 Gy, 8 Gy and 10 Gy. In BICR-10 cell line, the cells submitted to 8 Gy, 10 Gy and 15 Gy presented this problem.

Concerning HSC-3 cell line, there are small diferences in the karyotype analysis for the different radiations. The cells submitted to a dose of 2 Gy are the ones that showed a frequency of the common aberrations more similar to the untreated cells. However, it is also the one which shows more new chromosomal aberrations as: $9q^-$, $11p^-$, $12p^+$ and der(11;17). Some of these alterations are also observable after the cells were irradiated with 1 Gy, particularly der(11;17). An aberration that appeared with some frequency in untreated cells was $4q^-$ and, after irradiation it is only seen in one metaphase.

Regarding BICR-10 cell line the diffrences are more notorious. There is a decrease in some common alterations from BICR-10 cell line as the radiation dose is enhancing, as del(9)(pter) and der(7)t(7;11). One alteration that appears very frequently in cells submitted to 0,5 Gy of irradiation is der(2)(q10), that is observable in 8 of the 10 metaphases analysed. Two very interesting alterations between untreated and treated cells are the loss of der(8;8) and i(5)(p10) after irradiation treatment. der(8;8) is still observable, but with less frequency, as opposed to i(5)(p10), which only appears in one metaphase after irradiation treatment. This der(8;8), which resembles an isochromosome 8q and i(5)(p10) are two abberations that appear commonly in HNSCC patients (Uchida *et al.*, 2006), so it is interesting to verify that alterations associated to this disease are being lost after radiation treatment. These losses could be results of the radiation treatment.

Another alteration new in some metaphases after irradiation was $5q^-$, which frequency is enhancing with the enhancement of radiation dose. Furthermore, the highest dose of radiation, 5 Gy, is the one with less frequency of the most common alterations in untreated cells and the one where there is a higher frequency of new aberrations.

The most common *de novo* alterations after radiation treatment are deletions. Since it is known that radiation produces double strand breaks (DSBs) in DNA (Joiner and van der Kogel, 2009), it is possible that these losses are direct consequences of breaks on the chromosomes induced by irradiation.

To our knowledge, there is no study in HNSCC characterizing the karyotype after radiotherapy. These results suggest that the aberrations that occur in the progeny of irradiated cells are not random and their significance must be assessed with further studies. However, it is important, in the first place, to analyse more metaphases *per* condition, since some alterations that seem to be important in this analysis may only be due to the small number of metaphases analysed.

5.2.2 Copy Number Variations

There is a great number of CNV between the untreated cells and the cells submitted to radiation treatment on both cell lines. Since the amount of CNV between the cell lines after irradiation does not change a lot, it is possible that the difference in alterations induced by irradiation are not the cause for the radiosensitive differences observed between both cell lines. Nevertheless, some CNV in specific genes could help understand the mechanisms that lead to this radiation response difference.

5.2.2.1 CNV assessed by aCGH

Most of the genes that presented some CNV between the different doses at which cells were submitted were noticeable in HSC-3, the most radiosensitive cell line. *RERE* (1p36.23) subexpression triggers apoptosis. It is able to recruit BAX and consequently activate the caspase

cascade, leading to apoptosis (Waerner *et al.*, 2001). Taking into consideration its function it is easy to understand the fact that lead to a gain of this gene after radiation treatment in the more radiosensitive cell line, regardless the fact that this gene was lost in the control cells. Also in this region it is mapped the *FBLIM1* gene, commonly called *migfilin*. The encoded protein is a component of actin-cytoskeleton membrane junctions and it is postulated to regulate cellular processes such as shape modulation, motility and differentiation (Wu, 2005) Similar to *RERE*, this gene is lost in HSC-3 cel line untreated and suffers a genetic gain after radiation treatment. This could mean that the tumour was losing its malignant phenotype, since the cells were losing the capacity to invade other tissues.

The results for *VHL* (3p25.3), previously described, are interesting. BICR-10 cell has a gain of this gene, that it is maintained after the cells were submitted to radiation treatment. However, HSC-3 cell line had no CNV for this gene, except after irradiation with 0,5 Gy, which gives a proliferative advantage to this cell line. It is considered to be a TSG and there is no data regarding gain or overexpression of this gene in any type of cancer. This gene could have an important role in radioresistance, or could be associated to worse prognosis.

COL1A2 (7q21.3) [NCBI-Gene: 1278] has been suggested to be a tumour suppressor gene in HNSCC pathogenesis and its hypermethylation associated to a higher tumour stage and metastasis (Misawa *et al.*, 2011). This gene coded for the chain α2 of type I collagen, the most abundant collagen molecule. In the metastatic cell line, HSC-3, there is a genetic loss of this gene, that is maintained after cells were submitted to 0,5 Gy, which enhances cell survival. After 10 Gy, this gene suffers a gain and its copy number is considered to be normal. However, the non-metastatic cell line, BICR-10, is normal in all conditions. The CNV in HSC-3 cell line could be representative of the loss of the the tumour malignant phenotype. However, since BICR-10 cell line was not in a metastatic stage, this gene had not suffered alterations yet.

IRF5 (7q32.1) and *MIR183* (7q32.2) are two genes that are lost in both cell lines, in all conditions except for HSC-3 cell line submitted to a dose of irradiation of 10 Gy. *IRF5* [NCBI-Gene 3663] codes for a protein that is a member of the interferon regulatory factor family. Proteins of this family have pleiotropic biological effects and are involved in several cellular processes, including differentiation and apoptosis. This gene plays as mediator for Interferon (IFN) and DNA damage-induced signalling, which can lead to apoptosis and/or cell death (Hu *et al.*, 2005). As for *MIR183*, to our knowledge, there are no studies regarding its involvement in HNSCC pathogenesis, and its role in carcinogenesis remains controversy. miR-183 is able to regulate the expression genes that have functions related to migration and invasion. In osteosarcoma and lung cancer, downregulation of miR-183 promotes migration and invasion of cancer cells (Wang *et al.*, 2008; Zhu *et al.*, 2012).

By inducing cell death or inhibiting the enhancement of the malignant phenotype, these genes could be examples of the efficacy of radiation treatment in the radiosensitive cell line.

At 17q23.2, five genes showed genetic imbalances in the radiosensitive cell line: *TBX4, NACA2, BRIP1, INTS2* and *MED13.* All five genes presented genetic gains in untreated cells, loss after submission to 0,5 Gy, and gain after submission to 10 Gy. These five genes could be important for the understanding of the mechanisms associated to one of the effects of radiation in tissues: the adaptive response, in our study represented in the HSC-3 cells submitted to 0,5 Gy of irradiation. As for their functions, *TBX4* [NCBI-Gene: 9496] belongs to the T-box family gene, and codes for transcription factors involved in the regulation of developmental processes. The function of *NACA2* remains to be elucidated. However, it is though that it encodes a protein that prevents inappropriate targeting of non-secretory polypeptides to the endoplasmic reticulum [UniProt: Q9H009]. The protein encoded by *BRIP1* [NCBI-Gene: 83990] interacts with BRCA1 for the normal repair of double-strand breaks. As for *INTS2* [NCBI-Gene: 57508], it encodes a subunit of the Integrator complex, which mediates 3-prime end processing of small nuclear RNAs U1 and U2. Finally, *MED13* [NCBI-Gene: 9969] codes for a protein that forms the mediator complex, which is responsible for the expression of almost all genes.

As for BICR-10 cell line, the number of genes that suffered alterations that could lead to the radiation response is lower. The genes are *KIF26B, GPR39, NAALADL2, MSX1* and *HS3ST3A1*.

On the q arm of chromosome 1 there is one gene that after submission to radiation is lost. *KIF26B* (1q44) encodes a protein of the kinesin superfamily proteins, which are microtube-dependent molecular motor proteins with several important cellular functions, such as vesicle transport, mitotic spindle formation, cytokinesis completion and chromosome segregation. Even though this family of proteins has already been associated with development and cancer progression, KIF26B is not well studied, with exception for a study in breast cancer, where its overexpression was associated to higher tumour size, grade and lymph node metastasis (Rath and Kozielski, 2012; Wang *et al.*, 2013). First of all, this gene has not been studied for HNSCC yet. However, our results also suggest that it could be associated to lymph node metastasis due to gain of the gene in HSC-3 cell line. In the radioresistant cell line, its loss could be a representation of the decrease in the malignant phenotype and it is possible that this alteration could be related to the stage of tumour progression. This could mean that the tumours response to radiation could be different regarding their stage. Further studies are imperative for this gene.

On chromosome 2, our results indicate other gene that could be involved in tumours response to radiation: *GPR39* (2q21.2). It is a member of G protein-coupled receptors family, which is a family highly involved in cell signalling. Dittmer *et al.* (2008) suggested that overexpression of GPR39 could inhibit cell death induced by different stresses, including oxidative stress (Dittmer *et al.*, 2008).

Ionizing radiation can induce oxidative stress, and the fact that this gene loses copies in comparison to untreated BICR-10 (where it had genetic gain) could indicate that, in cells after irradiation there is less stimuli to the inhibition of oxidative stress-induced cell death induced by radiation, which eventually leads to a better elimination of cancer cells. HSC-3 cell line never presented gain of this gene. This could also be associated to the reason behind the differences in radiation response of both cell lines, since the radioresistant has alterations that inhibit cell death induced by damages that are caused by irradiation. Proving this theory right, *GPR39* could be an important therapeutic target to a better result in cancer treatment.

NAALADL2 (3q26.33) is another example of alteration after radiation that is only seen in the radioresistant cell line. There was no copy number alteration in the cells whitout radiation, and after being submitted to radiation treatment, both after 0,5 and 10 Gy, there is a gain of this gene. This gene is the least characterized gene that belongs to the N-acetyl-L-aspartyl-L-glutamate peptidase I family, which members act as M28 membrane metalloproteases and catalyse the hydrolysis of N-acetylaspartylglutamate to glutamate and N-acetylaspartate. Whitaker *et al*. (2014) analysed the expression of this gene in several types of cancer, including in several regions of HNSCC and concluded that it was highly expressed and had an oncogenic role. They focused their study on prostate cancer, acknowledging that this overexpression was associated to a more aggressive disease, with more likelihood to metastasize. HSC-3 already showed gain of this gene, indicating that this assumption could also be true for HNSCC. However, BICR-10 cell line only had a gain after irradiation, possibly meaning that the radioresistant tumour was trying to counterbalance the loss of some of its cells, by trying to maintain the malignant phenotype. In addition, this protein family is interesting in a therapeutic point of view, since they act as matrix metalloproteases and are able to alter tumour microenvironment. Taking this into consideration, *NAALDL2* needs further studies, not only in HNSCC but in cancer in general, and it is possible that this gene could eventually become a therapeutic target to maximize the therapeutic effects of radiation in radioresistant tumours.

MSX1 [NCBI-Gene: 4487], encoded at 4p16.2, belongs to the muscle segment homeobox gene family. It codes for a protein that is a transcription repressor during embryogenesis. Moreover, this gene interacts with p53, inhibiting tumour growth by inducing apoptosis (Park *et al.*, 2005). *MSX1* did not show any copy number variations in BICR-10 cells and not even after irradiation with 0,5 Gy. However, when submitted to 10 Gy, these cells showed a gain of this gene, indicating that it could represent a mechanism to cell death by apoptosis after radiation treatment. HSC-3 cell line showed gain of this gene which is maintained after irradiation treatment. This gene, due to its functions, may be related to the highest response to radiation of HSC-3 cell line when compared to BICR-10 cell line.

HS3ST3A1 (17p12) [NCBI-Gene:9955] encodes the enzyme 3-O-sulfotransferase, which catalyzes the biosynthesis of a specific subtype of heparan sulfate, 3-O-sulfated heparan sulfate. To our knowledge, the function of this gene remains unknown and because of its lack of study, there is no information regarding its involvement in carcinogenesis, with the exception of a paper characterizing a metastatic lung cancer cell line, where they postulated that the overexpression of this protein was associated to a higher propensity of a tumour to develop metastasis (Nakano *et al.*, 2012). However, for obvious reasons, this association lacks verification. This gene has a genetic gain in all conditions, except for BICR-10 in untreated conditions and after submission to 0,5 Gy, where no CNV was detected. It is possible that this gene is associated to higher sensitivity, since HSC-3 is a sensitive cell line, and after irradiation with 10 Gy the cells were dying, even in BICR-10 cell line.

At last, *TAF14B* (18q11.2) [NCBI-Gene: 6875] participates in the TFIID protein complex and it is involved in the activation of several anti-apoptotic genes. Even though HSC-3 showed loss of the gene in all conditions, it is interesting to compare with BICR-10 cell line. In BICR-10 the only alteration is after submission to the lowest dose, where this gene has copy gains, which probably leads to an enhancement of apoptosis inhibition, in an attempt of the tumour to maintain its cells. In HSC-3 cell line, since this gene is lost, the balance between anti-apoptotic and pro-apoptotic genes is deregulated in favour of apoptosis. Thus, this gene could help provide a more radiosensitive response. To the best of our knowledge, there is no study involving this gene in irradiation-response.

In both cell lines, only three genes have shown differences that could help understand the differences of the radiation's effects: *WRAP73, SLC22A18* and *COL1A1*.

In 1p32.32 we have the *WRAP73* gene, it encodes for a protein that is a member of the WD repeat protein family. These family members have a wide spectrum of functions, being involved in cell cycle progression, signal transduction, apoptosis and gene regulation (Li and Roberts, 2001). HSC-3 cell line shows loss of this gene. However, when submitted to a dose of 0,5 Gy, which enhances cell survival, there is a gain of this gene. When submitted to a dose of 10 Gy, the loss remains. This could mean that this gene could have a role in the promotion of cell proliferation or inhibition of cell death. Moreover, in BICR-10 cell line, this gene only shows CNV in cells submitted to a dose of 0,5 Gy. It is possible, however, contradictory to the assumptions made for HSC-3, that this gene inhibits cell proliferation. In this cell line, it would balance the high proliferation rate of the tumour, leading to a more slowly proliferation, this would turn this cell line more radioresistant, since more proliferative cells are more sensible to radiation. However, when submitted to radiation, the cell, by deletion of this gene, reduces its expression, leading to an enhancement in cell proliferation, in order to counterbalance the death produced by radiation treatment. In cells submitted to 10 Gy, there is a higher frequency of cell death and it could be impossible for the tumour to activate this type of response, or it could have other mechanisms that would act faster. It is also possible that this

gene could have totally different responses due to the different stages of development of the cell lines. Since HSC-3 already showed metastasis, the effects of this gene regarding proliferation inhibition could not be relevant to its aggressive phenotype, contrary to BICR-10, which is still in a progression tumour phase.

It has been postulated that *SLC22A18* (11p15.4) [NCBI-Gene: 5002] functions as a TSG in tumorigenesis and, although it has not been well studied in HNSCC, our results support this function, since both our cell lines showed loss of this gene in untreated conditions. Furthermore, a study with glioma cell lines showed that the upregulation of SLC22A18 enhanced radiosenstivity, irradiation-induced apoptosis and a delay in repair of DSBs, due to radiation treatment (Chu *et al.*, 2014). These results are particularly interesting since, in HSC-3 cell line after irradiation, this gene passes from a loss to a gain in both conditions. This could indicate that alterations of *SLC22A18* gene could be a mechanism of cell death after irradiation and consequently a good therapeutic target for enhancing radiation treatment response. In the most radioresistant cell line, a gain of this gene is also observed. However, it was the case only for cells submitted to 0,5 Gy. A possible explanation for the absence of copy gain of this gene could be that this is not the primary mechanism leading to irradiation-induced cell death at 10 Gy, another mechanism of cell death could be more frequent.

At 17q21.33 is mapped the gene that codes for pro-α chains of type I collagen, *COL1A1* [NCBI-Gene: 1277]. This gene was previously described as being overexpressed in tumour cells and associated to invasiveness of the tumour (Ryu *et al.*, 2001). According to this paper, it is possible that this gene could also be associated to invasiveness, since HSC-3 cell line shows gain of the gene, whereas BICR-10 is normal. After irradiation, the gain is maintained in the cells submitted to 0,5 Gy in HSC-3. However, in 10 Gy and in BICR-10 cell line, there is loss of the gene. Then, alteration in this gene could be associated to a reverse of the malignant phenotype, caused by irradiation.

5.2.2.2 CNV assessed by MS-MLPA

MS-MLPA analysis showed differences in copy number in TSG after irradiation treatment. However, the alterations are very different when comparing one cell line to the other.

In HSC-3, for example, regarding cells after submission to low O_2 concentration, the majority of the differences are not relevant, since the ratios were close to the cut-offs. However, in BICR-10 cell line, the microenvironment change leads to some interesting alterations regarding CNV. Beyond that, some alterations detected by MS-MLPA were not detected by aCGH. This difference can be due to differences on probes and due to resolution differences between the two techniques, even though this topic is still controversial (Homig-Holzel and Savola, 2012; Evangelidou *et al.*, 2013).

The majority of the alterations are maintained in the different conditions. In HSC-3 *CASR, CDKN2A, CD44, GSTP1, BRCA2, RB1, MLH3* and *GATA5* gains and *CFTR, ATM* and *CDH13*

losses remain unaltered. In BICR-10 the gain of *APC* and the losses of *CDKN2A, BRCA2, RB1* and *TP53* also did not show any alteration in all the conditions. Moreover, in HSC-3 cell line, the alterations on *TP73, PAX5, PAX6* and *TP53* have slight differences in some conditions. However, the ratios were closed to the cut-offs, so it is highly possible that HSC-3 cell line also maintained this alterations in all conditions. The same is not applicable to BICR-10 cell line. Bearing this in mind, some alterations presented in the Table 11 need further studies to be considered relevant.

Taking into account the most radiosensitive cell line, HSC-3 cell line, there are eight genes with alterations that are worthy to be mentioned: *VHL, APC, WT1, TSC2, PMP22, MSH6, ESR1* and *CHFR.*

VHL, *APC*, *WT1, TSC2* and *PMP22* presented alterations only in the cells submitted to the LD50 dose of irradiation, 1 Gy in both normoxic and hypoxic conditions. *VHL* has already been described in this study regarding its function and genetic gain. In HSC-3 cells submitted to 1 Gy in both environmental conditions this gene suffers loss and, interestingly, in aCGH results, this cell line suffers a genetic gain after a dose of 0,5 Gy (adaptive response). These alterations are both contradictory with the literature results for this gene (Gossage *et al.*, 2015). If, on the one hand we have a more proliferative stage and a gain of *VHL*, in the LD50 dose we have a loss. Then, this gene needs further studies in order to elucidate its role in HNSCC development, since by these results, it appears to have an oncogenic role, rather than the tumour suppressor. *APC* is deleted after a dose of 1 Gy of irradiation. The same happens with *TSC2* and *WT1* passes from gain to a normal number of copies. *PMP22* is the only that suffers a gain after irradiation. *APC* [NCBI-Gene: 324] encodes a known tumour suppressor protein that functions as an antagonist of the Wnt signalling pathway and it is involved in cellular processes, including migration and cell adhesion, apoptosis and transcriptional activation. The activation of the canonical pathway of Wnt signalling leads to several neoplastic characteristics common in cancer such as evasion of apoptosis, insensitivity to growth inhibitors and tissue invasion and metastasis (Ilyas, 2005). It is possible that 1 Gy can also induce an adaptive response in HSC-3 cell lines, since the loss of *APC* decreases the inhibition of Wnt signalling. This is also observed in normoxic and hypoxic conditions. The clonogenic assay applied to this dose can highlight this issue. Regarding *TSC2*, it codes for the tumour suppressor protein tuberin [UniProt: P49815], which forms a complex with hamartin and negatively regulates Mammalian Target of Rapamycin complex 1 (mTORC1) signalling. This function allows this complex to exert translational control of protein synthesis and cell growth. Cells lacking this complex show increased proliferation. Moreover, it is also involved in cell adhesion (Nobukini and Thomas, 2004). These alterations suggest a possible adaptive response when cells are submitted to 1 Gy in normoxic and hypoxic conditions, since these alterations favour the malignant phenotype of the tumour.

PMP22 role in carcinogenesis is controversy. In a study by Wilson *et al*. (2002), the enhancement of expression of this gene induced apoptosis. However, a more recent study indicated that the overexpression of this gene was associated, in breast cancer, to a more metastatic phenotype, indicating an oncogenic role for this gene (Tong *et al.*, 2010). Due to the amplification of this gene when cells are submitted to 1 Gy of radiation, we want to believe that it will induce an enhancement of apoptosis. However, putting the hypothesis of this dose inducing an adaptive response, the oncogene role for this gene can also be taken into consideration. To our knowledge, no studies of *PMP22* were performed in HNSCC, hampering any conclusions. *WT1* has already been discussed and it is unlikely that the deletion of this gene in these two conditions would alter any cell characteristics, due to promoter methylation analysis, that revealed that it remains highly methylated (near 100%).

ESR1 is lost after HSC-3 cell line was submitted to 0,5 and 1 Gy (in normoxic and hypoxic conditions). This gene was already mentioned as being a TSG that could suppress metastasis development (Stephen *et al.*, 2010). Besides being methylated in all conditions, particularly, in these three conditions, it also is deleted. This supports the hypothesis that 1 Gy can also induce an adaptive response.

Regarding *MSH6,* it shows loss after small doses of radiation in HSC-3 cell line and there is a loss of copy numbers in BICR-10 cell line, the radioresistant one, in all conditions, except the untreated cells. *MSH6* [NCBI-Gene: 2956] encodes a member of the DNA mismatch repair MutS family. MSH6 [UniProt: P52701] is involved in the recognition of mismatched nucleotides. Thereby, it is involved in the initiation of DNA repair. Loss of this gene makes the cell advance to later stages of the cell cycle without repairing DNA damage. This advance can lead to an enhancement in the cell death by mitotic catastrophe once the DNA is not repaired after irradiation. In the radiosensitive cell line, HSC-3, this alteration is observable after low doses of treatment, which suggests that the low doses could induce an enhancement in the cell death by mitotic catastrophe after irradiation. At higher doses there is no alteration, probably because they induce other types of signalling that lead to cell death. However, it is also possible that the loss of this gene could lead to an enhancement of the malignant phenotype, once the damages induced by low doses of irradiation could not be sufficient to lead to cell death and, by advancing in cell cycle without repairing the damages, the tumour is enhancing its genomic instability. Regarding the hypoxic conditions, the fact that it does not show any alterations could be related to an enhancement in the radioresistance associated to those conditions. As for BICR-10 cell line, it had genetic gain of the gene. However, this gain is lost after the cells are submitted to the different doses and to hypoxic conditions. Being *MSH6* a TSG, its loss contributes to carcinogenesis, meaning that in the radioresistant cell line, the irradiation probably

leads to a decrease in DNA repair capacity, making the tumour enhance its malignant phenotype rather than leading to cell death.

CHFR gene suffers a gain after HSC-3 cells were submitted to 0,5 Gy of irradiation. As mentioned, this gene is responsible for a delay in entry to metaphase after mitotic stress (Scolnick and Halazonetis, 2000). The gain of this gene could help explain the adaptive response after irradiation treatment since, by inducing a delay in metaphase entry, it may allow the repair of the damages induced by irradiation. The fact that this gene is lost after treatment with 2 Gy can lead to the assumption that this checkpoint would not be active, enhancing the probability of the finally outcome of these cells would be mitotic catastrophe, due to the accumulation of irreparable damages to the DNA. Another hypothesis relates to the fact that *CHFR* gene is methylated in the radioresistant cell line, BICR-10, and the absence of this checkpoint makes the cells able to proliferate, avoiding apoptosis by the detection of damages. However, to our knowledge, a study of this gene in the context of radiation biology has not been performed. Our results suggest that this gene could be used as a future target for an enhancement of radiation treatment efficacy.

Moreover, these alterations bring the hypothesis that, at least for a radiosensitive cell line, the alterations on gene copy number could be dependent on the dose that they were submitted, rather than the environment differences since between normoxic and hypoxic conditions, there are no relevant alterations and even when the cells are submitted to the same dose in different environment conditions, the alterations present remain almost the same. Further analysis, however, is required, since it is only possible to compare the results obtained for conditions without submission to radiation and after 1 Gy of irradiation. In HSC-3 cell line, this could explain the major differences towards cells submitted to 2 Gy. 5 Gy and 10 Gy remain very similar to the untreated cells, probably because almost all cells died shortly after treatment.

Analysing BICR-10 cell line, gain of *TP73* is an example that happens both in normoxic and hypoxic conditions after treatment with a dose of 3 Gy of irradiation. Even though there are some alterations that are not consistent with the hypothesis that alterations are dependent on the dose, the majority are, so the hypothesis remains valid. However, it needs to be further studied and evaluated. Additionally, the data from the different doses regarding CNV shows variations in all conditions, which further supports this hypothesis.

Regarding comparison between normoxic and hypoxic conditions, there are some alterations, as the gain of *PTCH1, CREM, MLH3* and *BRCA1* and losses of *MGMT* and *GATA5* in hypoxic conditions. The first two alterations also occurred when cells were submitted to 3 Gy in both environmental conditions and to the other low radiation doses. *PTCH1* [NCBI-Gene: 5727] codes from a member of the patched gene family and functions as a receptor for sonic hedgehog. Hedgehog signalling, in a study developed by Gan *et al.* (2014), was found to be associated to radioresistance,

being involved in tumour repopulation after radiation. The gain of this gene at the lowest radiations could be associated to an enhancement of tumour repopulation, helping explain the radioresistant phenotype of this cell line. At the highest doses, the gain did not happen probably because the damages to the cells were too big for the cancer cells to be able to recover. As for *CREM* [NCBI-Gene: 1390], it encodes a transcription factor that is able to bind to cyclic adenosine monophosphate (cAMP) responsive elements. It already has many alternatively spliced transcripts variants described, which encodes different isoforms that may act as activators and repressors of transcription. Due to the high frequency of cAMP responsive elements in promoters and the different isoforms coded by this gene it is impossible to suggest a relation between radiation response using only this data. Further studies requiring proteomic analysis are needed.

As for *BRCA1* gain, this is an alteration that only appears in cells on hypoxic conditions in BICR-10 cell line. *BRCA1* [NCBI-Gene: 672] codes for a protein that plays a role in maintaining genomic stability and it is described as a tumour suppressor. Furthermore, it is known that it has an important role in DNA repair of DSBs. As it was already mentioned, one of the most deadly lesions produced by irradiation is DSBs. The enhancement of expression of this gene had already been associated to a radioresistant phenotype in breast cancer (Abbott *et al.*, 1999) and, since the lack of O_2 is a known contributor to radioresistance, it is highly possibly that BICR-10 cell line (radioresistant) had a decrease in radiation response on hypoxic conditions, since the amplification of this gene is able to repair the DSB provoked by radiation to the cells, allowing them to avoid apoptosis and progress in the cell cycle.

PAX5 is a gene that suffers gain at the low doses of radiation, only in normoxic conditions. This gene has already been extensively discussed in this study and since there is a lack of concordant results in the literature, accessing the possible role of this gain in radiation response is hampered. In order to take any conclusions, it is necessary to access the role of this gene in HNSCC development. However, the fact that it suffers an amplification at low doses in the radioresistant cell line and it suffers loss at low doses in the radiosensitive cell line may suggest that this gene could be involved in response to radiation, for example, by preventing cell death or enhancing cell proliferation. Nevertheless, these suggestions need to be available after a thorough study of the gene.

A further study of these genes and their functions is imperative, to validate any of the results present in this study. Expression and proteomic analysis are required and, for further validation, the comparison with samples from patients. All together , these data suggest that there are differences in the cell lines submitted to radiation that can help explain not only the effects that radiation has on them, but also why their behaviour differs so much in regarding to radiation treatment. A more focused study can help understand if these genes are really involved and elucidate the possible mechanisms behind it, having in mind that it is highly possible that, even under the same conditions

and after being submitted to different radiation doses, cells could favour CNV of other genes than the ones described here in order to try and maintain their malignant phenotype or alterations that could lead to cell death.

Regarding all the other CNV that this study demonstrated, it is possible that, when irradiation produced DNA DSBs randomly in the cells, some errors had happened that were amplified during the following mitotic divisions of the cells, leading to totally random CNV on both cell lines.

5.2.3 Methylation alterations

It was already proven that irradiation exposure can affect the DNA methylation pattern, even though little is known about how DNA methylation is able to originate alterations in cancer cells after irradiation (Ilnytskyy and Kovalchuk, 2011; Kim *et al.*, 2013). So, it was expected that the results obtained with MS-MLPA showed alterations on both cell lines after radiation treatment. However, there are no great alterations in the methylation pattern of the cell lines after treatment with radiotherapy. Besides, epigenetic alterations originated by changes in the oxygen levels of the cells were also expected (Smits *et al.*, 2014) and, similar to what happened after irradiation, none alteration is noticeable in our study. One of the possible reasons behind these results relates with the fact that MS-MLPA is a target technique, meaning that it is possible that irradiation, and even hypoxia could induce methylation changes on the cells. However, the genes affected were not on this MS-MLPA panel probe. An epigenomic technique would be more appropriate to access this hypothesis. Another possible explanation relates to reports that show that one of the epigenetic events that occurs more frequently after irradiation is hypomethylation, rather than hypermethylation, which is not accessed by MS-MLPA (Kim *et al.*, 2013).

Even so, HSC-3 cell line presented alteration in two genes after irradiation treatment. When submitted to a dose of 0,5 Gy, the response of this cell line falls in the category of adaptive response and a higher proliferation rate is observed. Under these conditions, an unmethylated gene becomes methylated, *MSH6*. It was found to be methylated in cancer (Moelans *et al.*, 2011) and it is possible that its methylation after irradiation is related with a high proliferative ratio. Since it acts in G1 and early S phase, its lack of activity will not induce cell cycle arrest for DNA reparation, thereby leading to a quick advance to the last phases of cell cycle. This gene was also methylated after the HSC-3 cell line was submitted to 2 Gy. This alteration can also induce mitotic catastrophe, because it is possible that the non-repaired damages in the DNA would be too high for the correct finalization of cellular division. This last hypothesis is more likely since the radioresistant cell line only showed one alteration after radiation treatment in all conditions: methylation of *MSH6* in cells submitted to 10 Gy. Since this is a high dose and the cells end up dying, the proliferation enhancement theory is unlikely. On the contrary, inducing cell death by mitotic catastrophe, is a possible explanation for

the results obtained. It is then possible that methylation of *MSH6* induced different outcomes in the cells due to the amount of damages produced by the irradiation dose, also depending on the genetic radiosensitive/radioresistance intrinsic characteristics of cells irradiated.

After a dose of 2 Gy, in HSC-3 cell line, *ATM* gene was also methylated. As previously stated, the encoded protein of *ATM* gene is essential for the survival of cells after irradiation (Sankunny *et al.*, 2014). Due to its methylated state, the cells submitted to 2 Gy would not be able to survive, therefore enhancing treatment efficiency. As previously noticed, cells submitted to a dose of 2 Gy of radiation are the ones which present more alterations, so, a further comprehensive study about the effect of this radiation must be performed.

Further studies on radiation-induced epigenetic alterations in cancer are required and may help develop targeting therapies, improving radiation outcome.

5.2.4 Uncovering possible explanations for the radioresponse differences

There are several pathways that can be involved in the radiation response of the tumours and that could be helpful to further understand the differences of outcome in these cell lines. The explanation for these differences can have a genetic association and, cell death and DNA damage repair differences can also be involved. Ionizing radiation is able to induce a complex response in cells, making it very likely that the answer for this major problem – radioresponse differences in cancer patients – can only be assessed after integrating all the pathways that can influence the cells outcome. After a genetic and epigenetic approach, we analyse the differences in DNA damages between the cell lines in normoxic and hypoxic conditons. Factors that we also intend to take in consideration are the differences regarding the type of cell death chosen by the cells after radiation treatment and the cell cycle phase of the cells, since studies show that the proliferation rate is correlated with radiotherapy response (Joiner and van der Kogel, 2009).

For the analysis of the results obtained with the comet assay, the parameter choosen was tail moment. Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Since this parameter had many zeros due to the comets that did not present tails, the distribution was heavy-tailed and a normal distribution could not be assumed. Taking this into consideration, a non-parametric approach, the Kruskal-Wallis test, for testing the different conditions was applied. Even though three independent experiments were performed for each condition, the results obtained are still preliminary. However, these results suggest that the cell lines exposed to radiation in hypoxic conditions are more resistant to irradiation treatment, since the amount of damages present in the cells on this conditions are significantly smaller. This is in accordance with the literature, whereas lack of oxygen is a known contributor to radioresistance (Kelsey *et al.*, 2013). There is a necessity

to performe more independent experiments, enhancing the *n* of this assay. This will help uncovering possible explanations for the preliminary results obtained with comet assay.

For the analysis of cell death and cell cycle phase, FC was perfomed. However, since only two independent experiments were made and due to the difference in the results, no statistical analysis was performed. It is imperative to perform at least three more times, since the data obtained was too different, to reach any conclusions.

5.3 PREDICTING RADIOTHERAPY RESPONSE

Due to the differences in radiation response, one of our cell lines is much more radioresistant than the other. After obtaining this information, we analyse our data in order to find genetic differences that could, somehow, predict tumour radiosensitivity. This particular aspect has been the focus of radiobiologists, once every tumour has its own particular characteristics that can lead to a more sensitivity or resistance to radiation treatments. The capacity to predict tumour radiossensitivity through the development of an assay or through the discovery of biomarkers, is a big step to personalized medicine, making it possible to develop personalized treatment options in radiobiology, which would lead to fewer side effects, quicken patient recovery and improve cure rates. Since our limitations were high, once we only had two cell lines, which had many differences regarding molecular characteristics and anatomic region, we try to verify some results that were described in literature as possible predictors of radiation response.

Regarding CNV associated to radioresistance, we have losses of 4p11-pter, 8p23.3 and 11q distal and 3q26.1 and 7p11.2-12 gains (Parikh *et al.*, 2007; van den Broek *et al.*, 2007; Gollin, 2014). At 8p23.3 two genes are mapped that could explain the higher radioresistance of the tumours: *MIR596* and Rho Guanine Nucleotide Exchange Factor 10 (*ARHGEF10).* Regarding *MIR596* a study developed by *Endo et al.* (2013) reported that miR-596 was a tumour suppressor miRNA and a potential therapeutic target in OSCC (Endo *et al.*, 2013). They notice that the enhancement of miR-586 and *LGALS3P* knockdown (a target of miR-586) lead to a lower proliferation rate and induced apoptosis (Endo *et al.*, 2013). Then, *MIR596* loss would lead to an enhancement of *LGALS3P* and, consequently, to a high proliferation rate and apoptosis inhibition. *ARHGEF10* has also been postulated to be a strong candidate for a tumour suppressor, since it is responsible for RhoB activation, which is needed for DNA damage-apoptosis. The deletion of this gene could, then, diminish apoptosis in the cells (Cooke *et al.*, 2008). Both alterations can lead to a reduction in apoptosis, which could, then, contribute to the radioresistance observed in BICR-10 cell line. As for the other regions, distal 11q loss and 3q26.1 did not show any alterations in genes, whereas 4p11-pter and 7p11.2-12 are regions too large for this type of analysis.

Furthermore, BICR-10 is described to have *TP53* mutations and, the deletion of *FHIT* gene alongside with *TP53* mutations is associated with a more radioresistant phenotype.(Raju *et al.*, 2015) Moreover, a study also concluded that mutations on *TP53* and *PAX5* methylation were associated to a decrease in radiosensitivity (Guerrero-Preston *et al.*, 2014). BICR-10, the radioresistant cell line, besides *TP53* mutations, also has *FHIT* deletion and *PAX5* methylation.

On the contrary, 8p23.3, 10q11-22, 14q13, 14q distal, 15q11.2-q21.3, 17q, 18q21.2 and 22q gains and 18q losses were associated to a higher radiation sensitivity (Cowan *et al.*, 1993; Singh *et al.*, 2000; van den Broek *et al.*, 2007). At 18q21.2 there are two genes that can help explain the good radiation response observable on HSC-3 cell line: Dynactin Associated Protein (*DYNAP*) and DCC netrin 1 receptor (*DCC*). *DYNAP* codes for dynAP protein, which is thought to activate Akt, leading to high rates of proliferation in cancer cells. As it is known, a high proliferation rate is associated to a higher radiosensitivity (Kunoh *et al.*, 2010). Concerning *DCC* gene, it is TSG that codes for the DCC protein [Uniprot: P43146], which is involved in apoptosis induction. This copy gain could, therefore, be favourable to the induction of apoptosis after irradiation.

In our study, there were four particular regions that showed CNV with some interest and that could help unveil biomarkers for radiation response: 4p11-pter, 8p23.3, 14qter and 18q21.2. These four regions were particularly interesting because 4p11-pter loss seems to be associated to radioresistance and 8p23.3, 14qter and 18q21.2 gains seem to be associated to a more radiosensitive phenotype. BICR-10 shows loss of all the regions, whereas in HSC-3 there is gain. As mentioned, these four regions have already been associated to radiation treatment. However, due to the extension of the alteration, it is not possible to associate to any specific genes, except for the two genes, *DCC* and *DYNAP* at 18q21.2.

Another interesting gene is not located in any of the regions mentioned above, however, its expression has already been correlated to a better response to radiation treatment in HNSCC (Tehrany *et al.*, 2015). *CDKN2A* gene is amplified in the radiosensitive cell line and deleted in the radioresistant one. In this aspect, both aCGH and MS-MLPA results are concordant and, due to the ratios obtained from both techniques, it is highly improbably that this gene has any copy in BICR-10 cell line. More interestingly, this is an alteration that is maintained in all the conditions that both cell lines were submitted, highlighting the hypothesis that this gene could be important to determine the radiation response of the tumour. Due to its role in cell cycle, it makes perfect sense that it could be responsible for a more radioresistant/radiosensitive phenotype of the tumour. Our study supports this theory. However, a gene expression profile must be performed.

Nonetheless, both cell lines present CNV associated to worse prognosis in HNSCC (Bockmuhl and Petersen, 2002; Martin *et al.*, 2008; Gollin, 2014). So, it is imperative to associate these alterations with a more specific cause for the worst prognosis, since HSC-3 is metastatic, but it

has a high response to radiation treatment and BICR-10 is not metastatic but does not respond well to radiotherapy. Furthermore, response to chemotherapy could also be a reason for the association with a worse prognosis. Altogether, our data highlight the need to define the reason behind the association of some alterations to a poor prognosis in order to define better treatment approaches and give the patients a better quality of life.

It is interesting, however, to notice, that, by our study, the increasing genetic complexity was correlated with a more radiosensitive phenotype, which was also observed in another study (Singh *et al.*, 2000). Moreover, a recent paper (Shaukat *et al.*, 2015) concluded that CIN caused sensitivity to metabolic stress. As known, CIN is commonly observable in tumours and is defined as the continuous propensity of a cell to suffer gains and losses of chromosomes at each cell division. This is known to be associated to metastasis and low survival rates. In the paper, they hypothesize that the fact that CIN causes stress to the cells in order to give them the ability to tolerate high-stress conditions, also pushes them close to their tolerance threshold. They showed that the induction of CIN made the cells of the tumour very sensitive to oxidative stress, with high levels of DNA damage and apoptosis as a response to metabolic stress, in particular, since these alterations were not enough to damage normal cells. Then, they concluded that cells with high CIN were sensitive to alterations in cellular metabolism, such as redox status (Shaukat *et al.*, 2015). Since radiation causes damages to the cell mainly by the formation of ROS, which induce oxidative stress (Kelsey *et al.*, 2013), one can hypothesize that CIN cells will be more sensitive to radiation. This could help explain why the tumour with higher radiosensitivity was HSC-3 cell line in our study, since the number of chromosomal alterations in that line is much higher than BICR-10 cell line. However, this theory needs further developments and studies.

5.4 COMPARISON BETWEEN ACGH, KARYOTYPING AND MS-MLPA

In general, the results between aCGH and MS-MLPA regarding CNV are concordant. Furthermore, the results obtained with the realization of karyotyping are complementary to both techniques referred. MS-MLPA has also the advantage to analyse the methylation profile of the samples stydied.

MS-MLPA has been compared to other methods for methylation detection, like methylation-specific PCR (MSP) and it has a big advantage, since it does not need sodium bisulfite conversion of unmethylated cytosine residues, which is hard to optimize. MS-MLPA is able to analyse samples with mixed populations of cells. In addition, MS-MLPA is able to analyse up to 50 probes in one reaction and can be combined with copy number and point mutation analysis simultaneously (Cabello *et al.*, 2011; Homig-Holzel and Savola, 2012). This makes MS-MLPA a valuable technique to use in

clinical and daily analysis in order to profile the patients tumours, helping in a more accurate diagnostic, prognostic and choice of treatment.

However, some results between MS-MLPA and aCGH were not concordant. In some cases there were losses/gains in MS-MLPA that were considered normal in aCGH and genes normal in MS-MLPA that presented CNV in aCGH results. This is explained by different possible reasons: (1) there is a possibility of a mutation or polymorphism in or close to the probe-homologous sequence, which can lead to decreases in the peak, bringing the necessity of confirmation of MS-MLPA results by other techniques; (2) MS-MLPA probes are target to a specific exon, meaning that the totality of the gene may not be altered, just that specific exon; (3) aCGH needs at least three probes for the region in order to validate a result, and it is possible that aCGH did not have them for some genes/regions; (4) Existence of different subclones in the samples, since the different sensitivities of the techniques prevent the identification of some alterations that only happen in some subclones.

Although aCGH is not able to detect alterations in a single exon, it provides the advantage of detecting chromosomal imbalances through all the genome, making it possible to have a full picture of all the CNV presented in the tumour cells. It is very useful for the discovery of genetic/chromosomic alterations that could be used as biomarkers in cancer. However, it is a very expensive technique to perform on daily basis. One possibility is precisely the use of aCGH as a first approach in a study, for example to determine biomarkers for radiation response, and then create a MS-MLPA probe panel with the biomarkers discovered, since the latter technique is a much cheaper and faster technique.

Even so, both aCGH and MS-MLPA are not able to detect polyploidy, balanced aberrations or marker chromosomes. For accessing this problem, karyotyping is a possible solution. Despite the lower resolution (5-10 Mb) (Bickmore, 2001), it is a good complementary technique. However, it is important to highlight that solid tumour cells normally have low mitotic index and a low quality of metaphases, making difficult to analyse them. Furthermore, cancer is characterized for a high chromosomal instability and this can hamper the karyotype analysis. The best way to address this problem is by Spectral Karyotyping (SKY), since it “paints” each pair of chromosomes with a different fluorescent colour, allowing the visualization of all chromosomes at one time.

Finally, there is another problem with MS-MLPA, particularly, when using it to study cancer samples. MS-MLPA is only able to establish CNV/methylation alterations based on the results from references probes, which are used to normalize the values obtained from the target genes. However, cancer is a very heterogeneous disease with high genomic instability and these characteristics hamper the choice of adequate reference probes. In our study, several reference probes presented CNV, indicating that those reference probes are not adequate for analysis of tumour samples of HNSCC.

Overall, the results obtained with the three techniques were rather concordant.

6 CONCLUSIONS

Taken all the data together, the goals proposed for this study were accomplished. The experiments allowed several conclusions and assumptions regarding genetic alterations and its association with radiation treatment in HNSCC. Additionally, the results between the karyotyping, MS-MLPA and aCGH were rather concordant, being the three techniques helpful for genetic and epigenetic characterizations and their results are complementary. The following conclusions were drawn from this study:

- The genetic and genomic characterizations obtain from this study are considered to be pivotal for the use of these cell lines in following studies;
- The results obtained with MS-MLPA, aCGH and karyotyping allowed the confirmation of HSC-3 as a metastatic cell line and hypothesized BICR-10 as a cell line in a phase of dysplasia;
- In general, HSC-3 and BICR-10 are two commercial cell lines well suited to studies regarding HNSCC, since both presented alterations that were associated to the carcinogenesis model suggested to HNSCC;
- Besides the alterations associated to different stages of development, these two cell lines are very dissimilar from one another, as the example of the number of chromosomes and the ratio between copy gains and copy losses;
- Co-methylation of *TP73* and *ESR1* could be a predictor of the development of metastasis, especially when the lymph nodes are involved;
- It is possible that these six genes, *RARβ, PAX5, PAX6, WT1, CADM1, GATA5*, could be important biomarkers for HNSCC, since two cell lines so different from each other showed methylation on the gene's promoters
- According to the clonogenic assay, the BICR-10 cell line (LD50 = 3,542) is more radioresistant than the HSC-3 cell line (LD50 = 0,821);
- Chromosomal aberrations after radiotherapy do not appear to be random and conventional cytogenetics demonstrates that there is a higher frequency of deletions in some chromosomes after irradiation treatment, has del(2)(q10) and $5q^-$;
- aCGH allowed the identification of several genes that could be involved in tumours response to radiation, highlighting *WRAP73*, *SLC22A18* and *COL1A1*, that showed alterations on both cell lines;
- MS-MLPA also suggested possible alterations on CNV and methylation of TSG in response to radiotherapy, as *MSH6* and *CHFR*;

- This study suggested, however, that the alterations on the tumour cells were dependent of the dose of irradiation to which they were subjected;
- This study emphasized the possible role of four regions in predicting radiotherapy response: 4p11-pter, 8p23.2, 14qter and 18q21.2. In the latter, possible important genes are *DCC* and *DYNAP*. In 8p23.2, *MIR596* and *ARHGEF10* also seem good biomarkers for radiotherapy response;
- Other important gene that could be helpful in predicting radiotherapy outcome is *CDKN2A*;
- This study showed that, even though both cell lines presented very different responses to radiotherapy, both had CNV associated to worse prognosis, being important to understand why some regions are associated to worse prognosis.

Overall our data suggests genetic, epigenetic and chromosomic alterations associated to radiation response and radioresistance, which could allow, in the future, the prediction of patients' outcome, better choice of treatment approaches and a better quality of life for HNSCC patients. This study demonstrates to be pivotal for a comprehensive knowledge of radiation effects in HNSCC patients and a pilot for further analysis in the radiation biology area applied to HNSCC, in particular.

7 FUTURE PERSPECTIVES

In spite all technological advances, cancer remains one of the major causes of dead worldwide, and the incidence of HNSCC is alarming. Since this number is still enhancing, an extended knowledge about HNSCC carcinogenesis and the need to find better biomarkers, either for prognostic, early diagnostic and treatment outcome, are imperative. Taking this into consideration, researchers worldwide develop their studies with commercial cell lines and, in order to fully understand the results obtained, a comprehensive study of the cell line must be performed. In here, we elucidate some genetic and epigenetic characteristics of HSC-3 and BICR-10 cell line, however, their study is not concluded, especially regarding karyotyping. Only 10 metaphases were analysed *per* condition, and so, in order to achieve a more robust conclusion, more cells have to be analyzed. Furthermore, SKY analysis is one of the next tasks in order to perform a more accurate evaluation of the karyotype. The continuing characterization of these cell lines should provide and increasingly useful resource to further studies, especially if they are used for translational research.

Moreover, the five-year survival rate remains low in this type of cancer, being one of the major problems the late diagnostic of the disease. These patients are treated with a multidisciplinary approach with high toxicity rates. One of the options is radiotherapy, which is associated to several problems in patients quality of life and it is now well accepted that it also causes late toxicities in tissues distant from the irradiated region. Besides, patients often respond different to this approach, some of them developing radioresistance. With this study we proposed some genetic alterations that could be important to understand and predict patients' different responses to treatment, however, gene expression and protein analysis are required for the validation of these results. In the future, a study comparing the results obtain with the two commercial cell lines and HNSCC patients may also contribute for this validation and even introduce other possible biomarkers not mentioned here.

Finally, the enhancing of independent events regarding flow cytometry and comet assay need to be looked into eith more detail in order to draw some conclusions regarding the different responses induced by radiation in cell lines, particularly the differences in a radioresistant and a radiosensitive cell line. This may elucidate the mechanisms behind the worse response of the first one, contributing to the development of target therapies for improving treatment outcome in patients that do not respond well to radiotherapy.

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APPENDICES

Appendix I - Abstract of an oral presentation in the VIII Conferences of Genetic and Biotechnology, 26-28th March 2015. Vila Real, Portugal

Comparison between a radiosensitive and a radioresistant cell line – could the difference be in copy number variations? A preliminary study.

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Keywords: HNSCC; aCGH; Radioresistance; Radiotherapy;

Introduction:

Head and Neck Cancers (HNC) are a group of tumours located in the upper aero-digestive tract. Head and Neck Squamous Cell Carcinoma (HNSCC) represent about 90% of all HNC cases. It has been considered the sixth most malignant tumour worldwide and, despite clinical and technological advances, the overall 50% five-year survival rate has not improved much in the last years.

Nowadays, HNSCC is well established as a heterogeneous disease and its development is due to accumulation of genetic events, which play major roles in key functional pathways.

Apart from the majority of the patients being diagnosed in an advanced stage, HNSCC is also a disease with poor therapeutic outcome. One of the therapeutic approaches is radiotherapy. However, this approach has different drawbacks like the radioresistance acquired by some tumour cells, leading to a worse prognosis.

Identification of genetic markers associated to radiotherapy response in patients is an essential step towards an improved diagnosis, higher survival rate and a better life quality for the patients.

Methods:

HSC-3 and BICR-10 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1% of penicillin and streptomycin. In the case of BICR-10 cell line, 1% of hydrocortisone was also added. Both lines were exposed through different doses of RX, ranging from 0,5 to 15 Gy. Cell viability was accessed using the clonogenic assay. The genetic characterization of the two cell lines was performed by Array Comparative Genomic Hybridization.

Results:

Comparing the preliminary results from the clonogenic assay for the two cell lines, HSC-3 cell line appears to be more radiosensitive than BICR-10 cell line since, for the same irradiation, HSC-3 has a higher decrease in colonies formation.

The genetic characterization showed huge differences between the two cell lines, regarding the number of alterations and copy number variations, namely at 1p, 1q, 3q, 4p, 5q, 9p, 9q, 10p, 10q, 11p, 12p, 12q, 14q, 17p, 17q, 20p and 20q.

Conclusion:

Taken together our preliminary data suggests chromosomal alterations that could be related to the radioresistance acquired by tumour cells after radiation treatment, allowing, in the future, the prediction of patients' outcome and a better choice of treatment approaches.

Appendix II - Abstract of a poster presentation in XXXIX Portuguese Genetics Conference, 25-27th May. Braga, Portugal

Genetic Characterization of Two Head and Neck Squamous Cell Carcinoma Cell Lines by Karyotyping and Array CGH

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Head and Neck Cancers represent tumours located in the upper aero-digestive tract, being about 90% Head and Neck Squamous Cell Carcinoma (HNSCC). It was considered the sixth most malignant tumour worldwide and it is estimated the occurrence of 600 000 new cases per year. Despite clinical and technological advances, the five-year survival rate has not improved much in the last years. The characterization of commercial cell lines has obvious benefits, since they are one of the most used model in biomedical studies. As such, genetic characterization is a necessity to have as much information as possible about the cell lines, especially if they are used for translational research.

HSC-3 (metastatic) and BICR-10 (non-metastatic) cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1% of penicillin and streptomycin. For BICR-10, 1% of hydrocortisone was also added. The genetic characterization was performed by karyotyping and array Comparative Genomic Hybridization (aCGH).

Our results showed the presence of copy number variations (CNV) on BICR-10 that were associated with early events and progression from a dysplasia to a carcinoma *in situ* stage, as the case of 11q distal loss. The HSC-3 cell line presented regions associated with a metastatic stage, such as 1q and 3q gains. Both exhibited regions associated to a worse prognosis. The non-metastatic line showed less CNV than the metastatic one and the latter presented alterations at nearly every chromosome. The BICR-10 cell line showed on average 40 chromosomes and HSC-3 56. Our results between aCGH and karyotyping were concordant.

We conclude that both aCGH and karyotyping are helpful for genetic characterizations and their results are complementary. Furthermore, both lines presented CNV that were associated to the carcinogenesis model suggested to HNSCC. All sum up, these two cell lines represent an important resource for further investigation into HNSCC's development.

Appendix III - Abstract of a poster presentation in XXXIX Portuguese Genetics Conference, 25-27th May. Braga, Portugal

Predicting Radiotherapy Response: Could the answer be in Copy Number Variations?

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Head and Neck Cancers (HNC) are a group of tumours located in the upper aero-digestive tract. Head and Neck Squamous Cell Carcinoma (HNSCC) represent about 90% of all HNC cases. It has been considered the sixth most malignant tumour worldwide and, despite clinical and technological advances, the five-year survival rate has not improved much in the last years Nowadays, HNSCC is well established as a heterogeneous disease and its development is due to accumulation of genetic events.

Apart from the majority of the patients being diagnosed in an advanced stage, HNSCC is also a disease with poor therapeutic outcome. One of the therapeutic approaches is radiotherapy. However, this approach has different drawbacks like the radioresistance acquired by some tumour cells, leading to a worse prognosis.

Identification of genetic markers associated to radiotherapy response in patients is an essential step towards an improved diagnosis, higher survival rate and a better life quality.

HSC-3 and BICR-10 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1% of penicillin and streptomycin. For BICR-10 cell line, 1% of hydrocortisone was also added. Both lines were exposed through different doses of RX, ranging from 0,5 to 15 Gy. Cell viability was accessed using the clonogenic assay. The genetic characterization was performed by Array Comparative Genomic Hybridization.

Comparing the results from the clonogenic assay, HSC-3 cell line appears to be more radiosensitive than BICR-10 cell line since, for the same irradiation, HSC-3 has a higher decrease in colonies formation.

The genetic analysis showed four regions that could be associated to radiotherapy response: 4p11-pter, 8p23.3, 14q distal and 18q21.2.

Taken together all of our data suggests that its chromosomal alterations could be related to the radioresistance, allowing, in the future, the prediction of patients' outcome and a better choice of treatment approaches.