Faculdade de Medicina de São José do Rio Preto
Programa de Pós-Graduação em Ciências da Saúde

GLAUCIA MARIA DE MENDONÇA FERNANDES

IDENTIFICAÇÃO E EXPRESSÃO GÊNICA DE CÉLULAS TRONCO TUMORAIS NO CÂNCER DE CABEÇA E PESCOÇO E A RESPOSTA À QUIMIOTERÁPICOS

São José do Rio Preto
2019

Glaucia Maria de Mendonça Fernandes

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Orientadora: Profa. Dra. Eny Maria Goloni Bertollo

Co-orientadora: Profa. Dra. Ana Lívia Silva Galbiatti Dias

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4º Examinador: Juliana Garcia de Oliveira-Cucolo

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2º Suplente: Tiago Henrique

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Dedicatória

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Epígrafe

"Não interessa quem disse isso ou aquilo, a natureza não dá a menor bola para a autoridade. O único modo de aprender algo sobre os fenômenos naturais é mediantes experimentos cuidadosos."

Galileu Galilei

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LISTA DE ABREVIATURAS E SÍMBOLOS

ADP	Difosfato de Adenosina
AKT	Proteína Proto-Oncogênica akt
ALDH	Aldeído Desidrogenase
ATP	Trifosfato de Adenosina
BNDF	Fator Neurotrófico Derivado de Cérebro
CCP	Câncer de cabeça e pescoço
CD117	Proteínas Proto-Oncogênicas c-kit
CD133	Prominina (Antígeno AC133)
CD44	Receptores de Hialuronatos (Antígeno CD44)
CEC	Carcinoma Espinocelular
CNPq	Centro Nacional de Desenvolvimento Científico e Tecnológico
CTTs	Células tronco tumorais
DNA	Ácido desoxirribonucléico *(Desoxirribonucleic acid)*
EGF	Fator de Crescimento Epidérmico
EGFR	Receptor do Fator de Crescimento Epidérmico
ERbB	Receptor epidérmico de proteína-tirosina quinase
ERK	MAP Quinases Reguladas por Sinal Extracelular
FAMERP	Faculdade de Medicina de São José do Rio Preto (*São José do Rio Preto Medical School*)
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo (*São Paulo State Research Foundation*)
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto

GAP	Proteínas de ativação da GTPase
GAPs	GTPase de ativação
GDP	Guanosina difosfato
GEFs	Fatores de troca de nucleótidos de guanina
GTP	Guanosina trifosfato
IgG1	Proteína de Fusão LFA-3 IgG(1) (Alefacept)
KRAS	Kirsten Ras Oncogene Homólogo
MAbs	Anticorpos monoclonais
MEK	Proteína cinase quinase ativada por mitogênio
mTOR	Serina-Treonina Quinases TOR
PCR	Reação em Cadeia da Polimerase (*Polimerase Chain Reaction*)
PI3K	Fosfoinositida 3-cinases
RAF	Proteínas Proto-Oncogênicas A-raf
RasGEFs	Fatores ras de Troca de Nucleotídeo Guaninafatores de troca de nucleotídeos guanina
TNM	Classificação dos Tumores Malignos *(TNM classification)*
TrkB	Receptor para Fator Neurotrófico Derivado de Cérebro
UPGEM	Unidade de Pesquisa em Genética e Biologia Molecular (*Genetics and Molecular Biology Research Unit*)

Resumo

Introdução: O câncer de cabeça e pescoço (CCP) é o quinto tipo de câncer mais comum no Brasil e baixa taxa de sobrevivência. O tratamento apresenta resultados pouco favoráveis devido à resistência tumoral. Possivelmente por uma pequena subpopulação de células, denominadas células-tronco tumorais (CTTs) que apresentam capacidade de autorenovação e iniciação tumoral, identificadas por meio de biomarcadores de superfície, tais como CD44, CD117, CD133 e ALDH. Além disso, tem sido evidenciada a alta expressão de genes da progressão tumoral como o Receptor do Fator de Crescimento Epidérmico (EGFR) e o Receptor Tirosina Kinase (TrkB) que ativam a cascata de sinalização por meio do gene Kirsten Ras Oncogene Homólogo (*KRAS*). **Objetivos**: Identificar e separar as células tronco tumorais por meio de biomarcadores moleculares; avaliar o potencial tumorigênico das CTTs bem como a eficácia do tratamento e analisar a expressão dos genes *CD44*, *TrkB*, *EGFR* e *KRAS* em CTTs de CCP. **Materiais e Métodos**: Para a identificação e separação das CTTs foi utilizado equipamento FACSAria Fusion. Após a separação, as células foram testadas paras os ensaios de migração, invasão e formação de esferas. Também foram submetidas ao tratamento com 0.37 mg/mL de 5-fluorouracil, 2.0 mg/mL de cisplatina, 0,06mg/ml de cetuximabe e 0,05mg/ml de paclitaxel por 24h, e analisada a viabilidade celular por meio do ensaio de MTS. A expressão gênica de *CD44*, *TrkB*, *EGFR* e *KRAS* foi avaliada pelo método de PCR quantitativo, utilizando-se as não-CTTs como controle relativo da reação. **Resultados**: CTTs foram identificadas e separadas por meio dos biomarcadores CD44, CD117 e CD133 em combinação e do ALDH1 isolado. A avaliação do potencial tumorigênico das CTTs separadas por meio dos biomarcadores mostrou maior migração, potencial de invasão e formação de esferas quando

comparadas as não-CTTs (p<0,0001, p=0,0324 e p=0,0013). A eficácia dos tratamentos avaliados não apresentou diferenças estatísticas entre CTTs e não-CTTs. A subpopulação de CTTs apresentaram alta expressão gênica de *CD44* (RQ=102,775) e baixa expressão de *EGFR* (RQ=0,741) na linhagem celular de câncer oral-HN13 e baixa expressão de CD44 (RQ=0,658), alta expressão de *EGFR* (RQ=7,559) e *KRAS* (RQ=1,482) e não houve expressão de *TRKB* na linhagem celular de câncer de laringe-HEP2. Nos tumores primários os genes *EGFR* (média de RQ=7,081) e KRAS (média de RQ=1,568) foram encontrados altamente expressos nas CTTs, porém, não houve diferença estatística entre as duas subpopulações (p=0,5625 e p=0,5296, respectivamente). **Conclusão**: As marcações com CD44/CD117/CD133 em combinação e ALDH isolado são eficientes para separar subpopulações de CTTs em CPP. As CTTs apresentam potencial tumorigênico mais agressivo e relativamente mais resistente aos tratamentos estudados. Em linhagem de câncer oral o gene *EGFR* foi encontrado subexpresso e o gene *CD44* superexpresso, entretanto, em linhagem celular de câncer de laringe foi encontrado o inverso além da superexpressão do *KRAS* e expressa tardia do gene *TrkB*. O que corrobora com os achados em tumores primários expressando altamente os genes *EGFR* e *KRAS* nas CTTs. Este estudo contribui na compreensão dos mecanismos de proliferação celular, resistência e recidiva ao tratamento do CCP por meio da caracterização das CTTs.

Palavras Chave: Células Tronco Tumorais, Câncer de Cabeça e Pescoço, Proliferação Celular.

Abstract

Introduction: Head and neck cancer (HNC) is the fifth most common cancer and presents low survival rate. The treatment has showed unfavorable results due to the tumor resistance. This is due a small subpopulation of cells, called tumor stem cells (CSCs) that have self-innovation and tumor initiation capabilities, identified by surface biomarkers such as CD44, CD117, CD133 and ALDH. In addition, the high expression of tumor progression genes such as Epidermal Growth Factor Receptor (EGFR) and tyrosine kinase receptor (TrkB) that activate the signaling cascade through the homologous Kirsten Ras Oncogene (KRAS) gene has been evidenced in cancer development and treatment. **Objectives**: To identify and separate tumor stem cells by molecular biomarkers; To evaluate the tumorigenic potential of CSCs as well as the efficacy of HNC treatment in CSCs and to analyze the expression of *CD44*, *TrkB*, *EGFR* and *KRAS* genes in HNC CSCs. **Materials and Methods**: For identification and separation of CSCs, FACSAria Fusion equipment was used. After separation, the cells were tested for migration, invasion and colony formation assays. Cells were treated with 0.37mg/mL of 5-fluorouracil, 2.0 mg/mL of cisplatin, 0.06 mg/mL of Cetuximab and 0.05 mg/mL of Paclitaxel for 24h and the cell viability assay was analyzed by MTS. The gene expression of the *CD44*, *TrkB*, *EGFR* and *KRAS* genes was used the quantitative PCR method, using non-CSCs as relative reaction control. **Results**: CSCs were identified and separated by the CD44, CD117 and CD133 biomarkers in combination and ALDH1 alone. The evaluation of tumorigenic potential of CSCs showed higher migration, invasion potential and colony formation when compared to non-CSCs ($p < 0.0001$, $p = 0.0324$ and $p = 0.0013$). The efficacy of the evaluated treatments did not show statistical differences between CSCs and non-CSCs. The

subpopulation of CSCs showed high *CD44* gene expression (RQ = 102.775) and low *EGFR* gene expression (RQ = 0.741) in the oral cancer cell line and low CD44 gene expression (RQ = 0.658), high *EGFR* gene expression (RQ = 7.559) and *KRAS* (RQ = 1.482) and there was no expression of the *TrkB* gene in the laryngeal cancer cell line. In primary tumors, EGFR (mean RQ = 7.081) and *KRAS* (mean RQ = 1.568) genes were found to be highly expressed in CSCs, but there was no statistical difference between the two subpopulations (p = 0.5625 and p = 0.5296, respectively). **Conclusion**: CD44/CD117/CD133 labeling in combination and ALDH alone are efficient for separating subpopulations of CSCs in HNC. CSCs present a more aggressive tumorigenic potential and relatively more resistant to the studied treatments. In oral cancer lineage the *EGFR* gene was found underexpressed and *CD44* gene overexpressed, however, in laryngeal cancer cell line the opposite was found in addition to *KRAS* overexpression and did not express the *TrkB* gene. The results corroborate withfindings in primary tumors expressing highly the *EGFR* and *KRAS* genes in CSCs. The present study contributes to the understanding of the mechanisms of cell proliferation, resistance and recidive to HNC treatment through the characterization of CSCs.

Key words: Cancer Stem Cell, Head and Neck Cancer, Cell Proliferation.

1.INTRODUÇÃO

1. Introdução

O câncer de cabeça e pescoço (CCP) compreende um grupo heterogêneo de tumores que englobam o lábio e cavidade oral (2,0%), hipofaringe (0.4%), orofaringe (0.5%), nasofaringe (0.7%), e laringe (1.0%) [1]. É o sexto tipo de câncer mais frequente no mundo[1, 2] com incidência global de 830 mil novos casos diagnosticados anualmente e mais de 430 mil mortes por ano[1]. No Brasil, é o quinto tipo de câncer mais comum e, para o ano de 2019, foi estimado 11.200 casos novos em homens e 3.500 em mulheres com câncer de cavidade oral e 6.390 homens e 1.280 mulheres com câncer de laringe[3].

O planejamento terapêutico baseia-se principalmente em parâmetros clínicos e histopatológicos, os quais consistem no local do tumor primário e no sistema de estadiamento TNM, ou seja, no tamanho do tumor, na presença de metástase em linfonodos cervicais e de metástase a distância. As opções de tratamento para esta doença são cirurgia, radioterapia e quimioterapia, que podem ser utilizados de forma isolada ou combinada, dependendo do grau e do tipo tumoral[3-6].

Estudos mostram que, apesar do bom planejamento terapêutico, os resultados podem ser insatisfatórios e apresentarem recidiva loco-regional, metástase e desenvolvimento de segundo tumor primário, com baixa taxa de sobrevida global e também quimioresistência[1, 7-11]. Por muito tempo, o tratamento do câncer baseou-se na premissa de que as células cancerosas são homogêneas, mas distintas das células normais. Porém, recentemente, os pesquisadores têm determinado, por meio das bases celulares, moleculares e dos significados clínicos, que as células tumorais são heterogêneas. Assim, algumas destas células, se assemelham às células normais, apresentando a capacidade de autorenovação e diferenciação, chamadas de células tronco tumorais (CTTs)[2, 12-15].

Uma das razões pelas quais a terapêutica do câncer, com frequência, não é bem sucedida reside na existência das CTTs, pois são células cancerosas quiescentes e resistentes a apoptose, as quais não são sensíveis à quimioterapia convencional, com capacidade ilimitada de auto renovação, iniciação, diferenciação e tumorigênese[16, 17]. A biologia e a patogênese das CTTs em CCP não foram bem caracterizadas, portanto, estudos com CTTs podem oferecer novos caminhos sobre o crescimento do tumor primário e progressão metastática, bem como podem levar a terapias mais eficazes para reduzir o potencial metastático em CCP[2, 12, 16-19].

CTTs são geralmente reconhecidas pela presença ou ausência de marcadores de superfície, moléculas envolvidas no metabolismo ou vias de sinalizações específicas. Os principais biomarcadores para identificação de CTTs são CD44, CD133, CD117 e ALDH[2, 10, 12, 14, 20-23]. Além da identificação de CTTs por meio de biomarcadores, há a necessidade de estudos de outros biomarcadores envolvidos na progressão tumoral, tais como *EGFR* e *KRAS*[24-26].

Como as CTTs estão relacionadas com a progressão do tumor, alguns dos principais genes envolvidos nesse processo é o Receptor do Fator de Crescimento Epidérmico (EGFR), um receptor transmembrana da tirosina quinase da família ErbB que faz ligação com Fator de Crescimento Epidérmico (EGF)[25] e o receptor de tropomiosina kinase B (TrkB) que faz ligação com o fator neurotrófico derivado do cérebro (BDNF) [27, 28]. Estas ligações levam à autofosforilação e a ativação de múltiplas vias de sinalização para o núcleo da célula por meio de proteínas efetoras, como as PI3K/Akt, Ras/Raf/MEK/ERK como representado na Figura 1[24, 26, 28-30]. A ativação da cascata de sinalização EGFR-Ras/Raf/MEK/ERK é uma via responsável pelo

desenvolvimento do câncer e é considerada uma via fundamental para a ação de quimioterápicos[20, 21, 31, 32].

Um importante gene nessa via de sinalização é o Kirsten Ras Oncogene Homólogo (*KRAS*) da família de genes RAS de mamífero, o qual codifica uma proteína que é membro da superfamília de aproximadamente 150 pequenas GTPases[29, 33, 34].

A proteína KRAS existe na forma ativa (KRAS-ATP) e inativa (KRAS-ADP), ambas são rigorosamente controlados pelos fatores de troca de nucleótidos de guanina (GEFs) e proteínas de ativação da GTPase (GAP). KRAS é uma proteína intracelular chave que ativa múltiplas vias, incluindo as vias RAF/MEK e PI3K. A Figura 1 mostra como essa via de sinalização funciona e influencia no câncer[29].

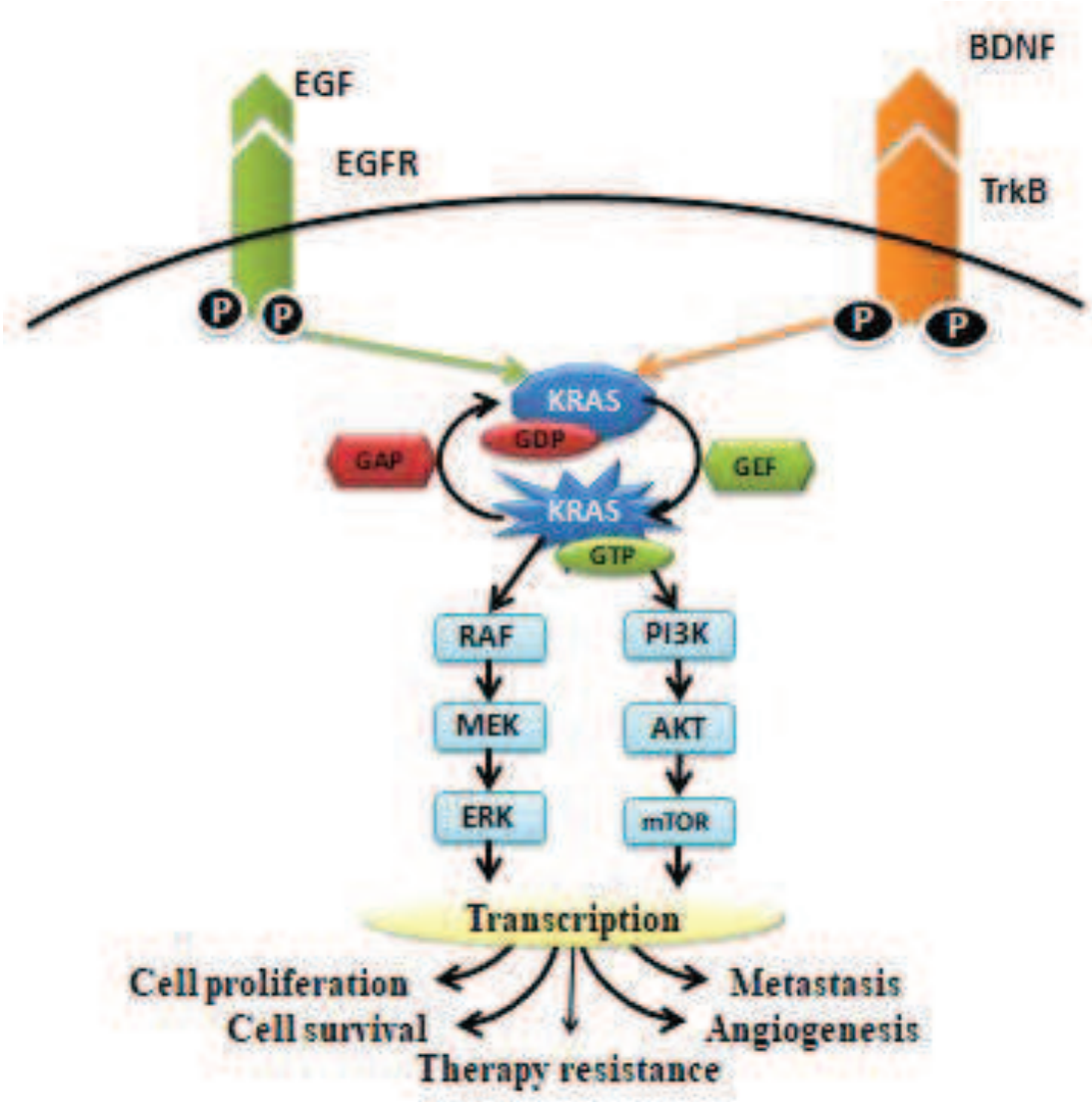

Figura 1: Cascata de proteínas Ras/Raf/MEK/ERK é ativada por meio da autofosforilação acionada pelo receptor EGFR quando ligado ao EGF (Adaptado de Leite et al., 2014[26]) ou pelo receptor TrkB quando ligado ao BNDF[28]. Proteínas Ras funcionam como interruptores que se ligam e desligam (guanosina difosfato (GDP)/guanosina trifosfato (GTP) – binários), onde o ciclo PIB/GTP é regulado de fatores de troca de nucleotídeos guanina (RasGEFs) que promovem a formação da forma ativa RAS-GTP, e as proteínas GTPase de ativação (GAPs) estimulam a hidrólise GTP e formação da forma inativa RAS-PIB. Em células quiescentes normais ela se apresenta na forma inativa RAS-PIB até receber estímulos extracelulares que causam a formação transitória da forma ativa quando ligada RAS-GTP. Tanto o tipo selvagem e mutante, quando ativados, RAS-GTP liga-se a um espectro de vias efetoras para o núcleo. Proteínas Ras mutantes não sofrem ação sobre a proteínas GAPs tornando as proteínas RAS-GTP continuamente ativas, o que leva ao estímulo-independente, persistente ativação de efetores do núcleo, incluindo RAF → ativada por proteína mitógeno quinase quinase (MEK) → ERK e fosfatidilinositol 3-quinase (PI3K) → AKT → alvo da rapamicina em mamíferos (mTOR) para promover a proliferação celular,

sobrevivência e metástases (Adaptado de Fernandes e Colaboradores[16] com Samatar e colaboradores[27]).

Ao ocorrer alterações nas proteínas EGF, e EGFR, há transmissão de sinais para os núcleos sinalizando células cancerosas a proliferar, sobreviver, diferenciar, aderir, migrar, regular a angiogênese e formar a metástase[26, 31], e KRAS é uma dessas moléculas de sinalização que contribuem para o desenvolvimento e progressão do câncer[29, 30].

As terapias anti-EGFR interrompem a cascata de sinalização de disparo de câncer, no entanto, se o gene *KRAS* estiver mutado, a proteína KRAS estará bloqueada em conformação ativa, inibindo que a sinalização seja desligada[35], independentemente de o EGFR estar terapeuticamente bloqueado[31].

Assim, EGFR tem sido extensivamente estudado como alvo terapêutico bem como biomarcador de prognóstico, pois apresenta superexpressão na maioria dos casos de CCP, e os níveis aumentados de EGFR está associado com mau prognóstico da doença e diminuição da sobrevivência[24, 36]. A resistência à inibição de EGFR pode ser relacionada com anormalidades no receptor redundante ou em moléculas de sinalização do núcleo da célula[36].

A superexpressão de *KRAS* tem sido associada com os tipos mais agressivos de câncer [37-39]. Entretanto, poucos estudos têm avaliado a associação do *KRAS* com a diminuição da sobrevivência e a resistência à quimioterápicos no CCP[40].

O entendimento da função da ligação entre o EGF/EGFR e as vias de sinalização que desencadeiam a carcinogênese do CCP tem propiciado o desenvolvimento de terapias com mecanismo de ação no EGFR e suas vias de sinalização, tanto no domínio extracelular e intracelular, bem como na fase de transição

da sinalização[30, 40]. A expressão do *EGF* e de seu receptor tem sido correlacionada com pior prognóstico e como responsável pela regulação pós-traducional levando ao aumento na expressão de genes relacionados à presença de CTTs[20-22, 32, 41, 42].

Estas drogas incluem anticorpos monoclonais (MAbs), que bloqueiam a ligação do EGF com seu principal receptor EGFR, inibidores de tirosina-quinase (tirfostinas) que podem interferir com as vias de sinalização intracelulares, e o EGF conjugado com toxinas específicas que atuam como inibidores potentes da síntese de proteínas citoplasmáticas das células cancerosas[43]. A resposta à terapia de inibidores de EGFR que depende de mutação no gene *KRAS* está bem estabelecida em câncer colorretal[7, 44], porém em CCP ainda há controversas na literatura. Assim é necessário estudar biomarcadores, tais como EGFR e KRAS, com a finalidade de detecção, diagnóstico, prognóstico e resposta ao tratamento[8, 12, 30, 42].

O Cetuximab (Erbitux® Merck) é um anticorpo monoclonal IgG1 que inibe a ligação de proteínas como o EGF com o EGFR[45] e estimula a citotoxicidade mediada por células dependentes de anticorpos[46]. Além disso, aumenta a efetividade de outros quimioterapêuticos[47, 48]. Porém, *EGFR* é superexpresso na maioria dos pacientes com câncer que recebem como tratamento quimioterápico o Cetuximab apresentando resistência intrínseca[49]. Fato este que pode ser explicado devido a presença de células tronco tumorais nestes tumores[20, 21, 32, 38, 42].

O Paclitaxel é considerado um quimioterápico com múltiplos alvos, dentre eles, um agente inibidor de antimicrotúbulo, o qual é importante na divisão celular e em outras funções da célula. Este principal mecanismo de ação inibe a dinâmica dos microtúbulos, por meio da montagem da microtubulina que estabiliza os polímeros contra a despolimerização. Assim, os microtúbulos passam a ser uma estrutura sem

função biomolecular, o que altera profundamente a mitose e causa a morte celular[50, 51]. Portanto, a angiogênese, um exemplo de via de sinalização que pode se basear nos processos afetados pelo Paclitaxel é o EGFR, no qual os sinais são transmitidos a partir da superfície da célula para o núcleo por meio de uma variedade de proteínas efetoras, tais como a KRAS.

A cisplatina, cis-Diaminodicloroplatina (II), reage com o DNA para produzir ligações cruzadas, é um dos medicamentos anticancerígenos mais eficazes e é amplamente utilizado no tratamento do CCP[52]. O 5-fluorouracil é um antimetabólito antineoplásico como objetivo de terapia direcionada em criar um estado sem timina disponível para ser incorporada ao DNA, isso seria tóxico para dividir rapidamente as células cancerígenas[53]. Assim, ambos os medicamentos prejudicam a replicação e a transcrição do DNA, no entanto, a eficácia é frequentemente limitada devido ao desenvolvimento de resistência e dos efeitos tóxicos ao organismo[52, 53]. A cisplatina pode induzir a ativação de Ras e suas efetoras Raf/MEK/ERK e PI3K/Akt sugerindo que este medicamento anticâncer ativa a via do sinal de sobrevivência que podem desempenhar um papel considerável na resistência à cisplatina no CEC[52].

Assim, o entendimento do mecanismo de ação da cisplatina, 5-fluorouracil, Cetuximabe, Paclitaxel e de drogas combinadas, adicionada com a compreensão das alterações genéticas e moleculares do CCP em CTTs poderão permitir a identificação de pacientes mais adequados para tratamentos específicos e mais eficazes. Esta abordagem contribuirá para o progresso no tratamento do câncer dos padrões atuais, para terapias individualizadas, fornecendo melhores benefícios ao paciente e novas informações sobre estimativa de prognóstico e decisão de tratamentos.

1.1 Objetivos

1. Identificar e separar as células tronco tumorais por meio de biomarcadores moleculares CD44, CD133, CD117 em combinação ou ALDH isolado;
2. Avaliar o potencial tumorigênico das células tronco tumorais, bem como a eficácia do Cetuximabe, Paclitaxel, Cisplatina e 5-Florouracil utilizados em diferentes tempo de exposição;
3. Analisar a expressão dos genes *CD44, TrkB, EGFR e KRAS* relacionados à proliferação celular em células tronco tumorais de câncer de cabeça e pescoço.

2. RESULTADOS

2. ARTIGOS CIENTÍFICOS

Os resultados estão apresentados em forma de artigos. No total estão apresentados três artigos, um publicado e dois a serem submetidos.

Artigo 1

Título: Relationship between CD44high/CD133high/CD117high cancer stem cells phenotype and Cetuximab and Paclitaxel treatment response in head and neck cancer cell lines

Autores: Ana Livia Silva Galbiatti-Dias, **Glaucia Maria Mendonça Fernandes**, Marcia Maria Urbanin Castanhole-Nunes, Luiza Fernandes Hidalgo, Carlos Henrique Viesi Nascimento Filho, Rosa Sayoko Kawasaki-Oyama, Leticia Antunes Muniz Ferreira, Patricia Matos Biselli-Chicote, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo

Periódico: American Journal of Cancer Research, ISSN: 2156-6976, Impact Factor 2017: 3.998. **Publicado em 15 de Agosto de 2018.**

Artigo 2

Título: Anti-EGFR Treatment Effects on Laryngeal Cancer Stem Cells

Autores: Glaucia Maria de Mendonça Fernandes, Ana Lívia Silva Galbiatti-Dias, Vilson Serafim Junior, Leticia Antunes Muniz Ferreira, Rosa Sayoko Kwasaki Oyama, José Victor Maniglia, Erika Cristina Pavarino, Eny Maria Goloni Bertollo

Periódico: American Journal of Cancer Research, a ser submetido

Artigo 3

Título: Characterization of EGFR pathway in Head and Neck Cancer Stem Cells.

Autores: Glaucia Maria de Mendonça Fernandes, Ana Lívia Silva Galbiatti-Dia, Marcia Maria Urbanin Castanhole-Nunes, Vilson Serafim Junior, Leticia Antunes Muniz Ferreira, Caroline Izak Cuzziol, Maria Antonia dos Santos Bezerra, Rosa Sayoko Kwasaki Oyama, José Victor Maniglia, Erika Cristina Pavarino, Eny Maria Goloni Bertollo

Periódico: Oncogene, a ser submetido

ARTIGO CIENTÍFICO 1

Artigo 1

Título: Relationship between CD44high/CD133high/CD117high cancer stem cells phenotype and Cetuximab and Paclitaxel treatment response in head and neck cancer cell lines

Autores: Ana Livia Silva Galbiatti-Dias, **Glaucia Maria Mendonça Fernandes**, Marcia Maria Urbanin Castanhole-Nunes, Luiza Fernandes Hidalgo, Carlos Henrique Viesi Nascimento Filho, Rosa Sayoko Kawasaki-Oyama, Leticia Antunes Muniz Ferreira, Patricia Matos Biselli-Chicote, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo

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Artigo Original

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Original Article
Relationship between CD44^{high}/CD133^{high}/CD117^{high} cancer stem cells phenotype and Cetuximab and Paclitaxel treatment response in head and neck cancer cell lines

Ana Livia Silva Galbiatti-Dias, Glaucia Maria Mendonça Fernandes, Marcia Maria Urbanin Castanhole-Nunes, Luiza Fernandes Hidalgo, Carlos Henrique Viesi Nascimento Filho, Rosa Sayoko Kawasaki-Oyama, Leticia Antunes Muniz Ferreira, Patricia Matos Biselli-Chicote, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo

Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), SP, Brazil

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Abstract: Recent evidence suggests that cancer stem cells (CSCs), a small population of cancer cells that are highly tumourigenic, capable of self-renewal and have the ability to differentiate into cells that constitute the tumor, are the "drivers" of local recurrence and metastatic spread and may be associated with resistant to conventional therapy. The objectives of the study are to identify and characterize two head and neck cancer cell lines with regard CD44^{high}/CD133^{high}/CD117^{high} profile (CSCs) and CD44^{low}/CD133^{low}/CD117^{low} profile (Non-CSCs); to investigate the influence of chemotherapy treatment in CSCs and compare with Non-CSCs; to evaluate CD44 and EGFR gene expression in CSCs. Fluorescent-activated cell sorting (FACS) using specific cell surface marker combination (CD44, CD117 and CD133) was performed to isolate CSCs of Non-CSCs from cell lines. The Wound Healing assay was performed to confirm the presence of CSCs. After, the CSCs subpopulation and Non-CSCs were cultured and exposed for 24 h to Cetuximab and Paclitaxel treatment, separately. Cell proliferation was determined by MTS assay. CD44 and EGFR gene expression was quantified by quantitative real time PCR (qPCR) using TaqMan® Assay in both subpopulations. CSCs subpopulation untreated were considered as relative expression control. We firstly characterized CSCs in HN13 and HEP-2 cell lines with CD44, CD133 and CD117 biomarkers. We treated CSCs and Non-CSCs subpopulations with Cetuximab and Paclitaxel treatment and found that CSCs subpopulations demonstrated more resistance to Paclitaxel chemoterapy, when compared with Non-CSCs subpopulations of oral cancer cell line. These CSCs subpopulations presented up-regulation of *CD44* gene and down-regulation of *EGFR* gene in oral cancer cell line, and down-regulation of *CD44* gene and up-regulation of *EGFR* gene in laryngeal cancer cell line when compared with Non-CSCs subpopulations. We conclude that the combination of CD44, CD133 and CD117 biomarkers have stem cell properties in both cell lines. CSCs has ability to resist to Paclitaxel treatment in oral cancer cell line. CSCs present high expression of CD44 gene and down expression of EGFR gene in oral cancer cell line. CSCs in laryngeal cell line present down expression of *CD44* gene and high expression of *EGFR* gene when compared with cells without characteristics of cancer stem cells.

Keywords: Cancer stem cells, chemotherapy, head and neck neoplasias, gene, expression, CD44, EGFR, cell line

Introduction

Head and neck cancer (HNC) is an aggressive disease that accounts for more than 500,000 cases each year worldwide [1]. The high prevalence of the disease is due to high rates of recurrence and metastasis. Furthermore the rate of success in treatment still remains low [2-4]. The treatment options for HNC depend of tumoral stage and can be surgery, radiotherapy and/or chemoterapy [5]. Treatment for HNC in early stage (stage I and II) generally involves single-modality therapy: Surgery or radiotherapy. However, patients with HNC locally advanced (stage III and IV A/B) are treated with chemoradiotherapy with or without chemotherapy [3, 7].

Chemotherapy treatment has improved in the last years but the supportive care for patients in treatment has increased because still there are many collateral effects as mucositis, skin desquamation, depression, fatigue, nausea, vomiting and others. Furthermore some patients have no answer for chemotherapy treatment compared to other patients with the same tumoral stage and the overall survival rate remains low [2, 8-10]. The fact can be associated with the presence of cancer stem cell (CSC) in tumor [11, 12].

CSC are defined as a small subpopulation of cells located within the tumor mass with high capacity of tumorigenic potential, self-renewal properties and slow growth cycle which is responsible to resistance to therapies that firstly target cancer cells that present faster growth [13-15]. The identification of CSC can provide interesting data regarding new therapeutic approaches in HNC and they may be identified through molecular biomarkers as CD44, CD117 and CD133 [16-18].

In the current study, the aim was to identify and separate cancer stem cells through CD44, CD133 and CD117 biomarkers in two subpopulations of head and neck cancer cell lines (HN13 and HEP-2 cell lines): $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ (CSCs) and $CD44^{low}$/$CD133^{low}$/$CD117^{low}$ (Non-CSCs), to verify if these biomarkers have stem cell properties; to compare effectiveness of Cetuximab and Paclitaxel treatment in CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines, and to evaluate *CD44* and *EGFR* gene expression in the CSCs subpopulations.

Material and methods

Cell line and culture conditions

HN13 (squamous cell carcinoma of oral cancer cell line) and HEP-2 (laryngeal cancer cell line) cells were cultured in D-MEN (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1 mM L-glutamine, 100 units/mL penicillin, and 100 lg/mL streptomycin (all reagents were from Invitrogen, Grand Island, NY).

Flow cytometry (Indetification and isolation of CSCs)

The trypsinized cells were resuspended, incubated with monoclonal antibodies for 30 min at 4°C, washed twice with phosphate buffered saline (PBS). The antibodies utilized were CD44-phycoerythrin (PE), CD117-fluorescein isothiocyanate (FITC) and CD133-allophycocyanin (APC). Fluorescent-activated cell sorting (FACS) of live cells was used to separate subpopulations of HN13 and HEP-2 subpopulation of cells using specific cell surface biomarkers combinations (CD44/PE, CD117/FITC and CD133/APC) with BD FACSAria Fusion equipament (BD Biosciences).

The subpopulation of sorted cell lines were classified based on the expressions of CD44/CD117/CD133 in combination as: $CD44^{high}$/$CD133^{high}$/$CD117^{high}$: presence of CSCs and $CD44^{low}$/$CD133^{low}$/$CD117^{low}$ profile (Non-CSCs). CSCs and Non-CSCs were resuspended in D-MEN for further experiments.

Wound healing assay

For confirmation of presence of CSCs, the CSCs and Non-CSCs subpopulations cells were plated at a density of 2×10^6 cells/wells and cultured until they reached confluence. A diamet- ric scratch was created using a pipette tip and washed with PBS 3 times. The cells were photo- graphed in microscope (OLYMPUS - CKX61/40
× objective lens) in three pre-marked spots as 0 h. Images were then acquired at 24 h in the same spots for comparison.

Drug sensitivity and MTS assay

CSCs and Non-CSCs subpopulations were plated at a density of 2×10^6 cells/well in six well plates. Cetuximab (CT), Paclitxel (P) chemotherapeutic agents at 0.06 mg/ml and 0.05 mg/ml concentrations, respectively, were added in the CSCs and Non-CSCs subpopulations [19, 20]. The cultures were incubated at 37°C for 24 h. The proliferation of cell lines were measured at OD 490 nm using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI, USA). The experiments were repeated two times. The results were expressed as percentage relative to the control cells. The chemotherapeutics evaluated are widely utilized in patients with oral cancer, so they were included in the study.

Real-time quantitative RT-PCR

RNA isolation was performed using Trizol (Invitrogen) according to manufacturers' manuals. The concentration of RNA utilized was 2 *ug*

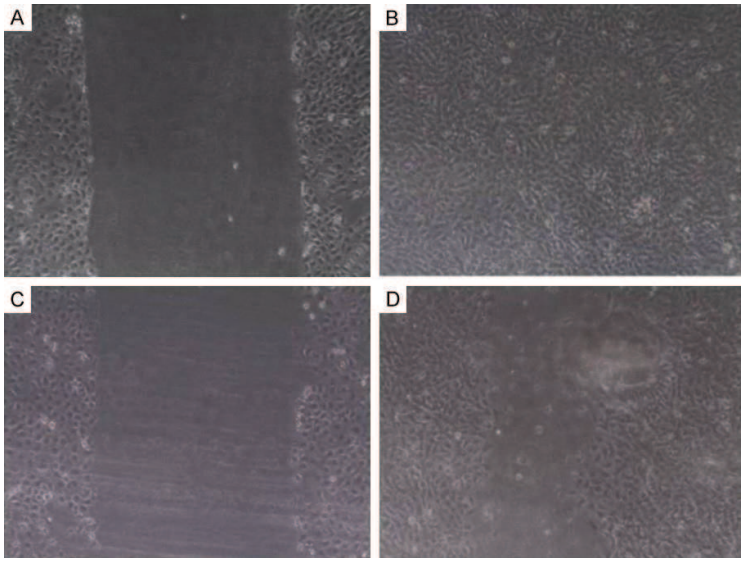

Figure 1. Cell Migration of CSCs and Non-CSCs subpopulations of HN13 cell line seeded in 6-well plates and cultured for 24 h. A. CSCs subpopulations of HN13: A diametric scratch using a pipette tip was made at 0 h; B. CSCs subpopulations of HN13 after 24 h; C. Non-CSCs subpopulations of HN13: A diametric scratch using a pipette tip was made at 0 h; D. Non-CSCs subpopulations of HN13 after 24 h.

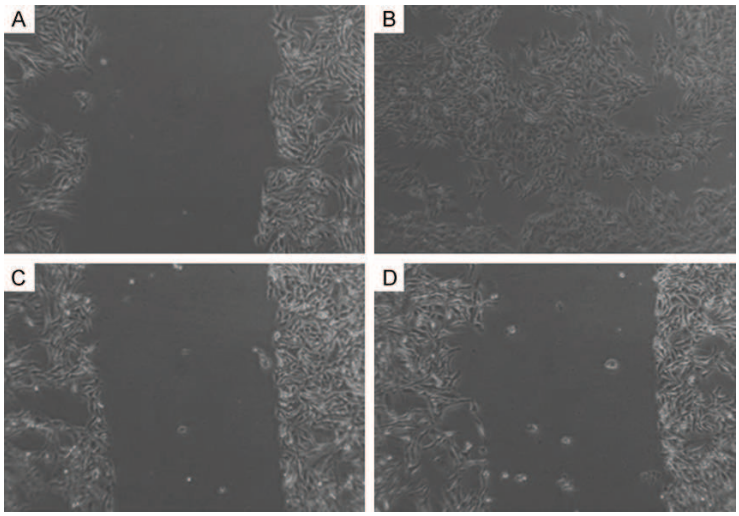

Figure 2. Cell Migration of CSCs and Non-CSCs subpopulations of HEP-2 cell line seeded in 6-well plates and cultured for 24 h. A. CSCs subpopulations of HEP-2: A diametric scratch using a pipette tip was made at 0 h; B. CSCs subpopulations of HEP-2 after 24 h; C. Non-CSCs subpopulations of HEP-2: A diametric scratch using a pipette tip was made at 0 h; D. Non-CSCs subpopulations of HEP-2 after 24 h.

(Picodrop Equipament). For cDNA synthesis, 1 ug RNA was used with primers by High capacity cDNA kit (Applied Biosystem®) according manufacturer's protocol. Genetic expression in all samples was evaluated by quantitative RT-PCR (qRT-PCR) with StepOnePlus™ Equipament (Applied Biosystems).

A polymerase chain reaction (PCR) was realized with 10 µL of Taqman Universal PCR Master Mix (Applied Biosystems), 80 nmol/L of primer, 2 nmol/L probe and 2 µL of cDNA. The cycling conditions were: 95°C for initial denaturation by 20 s, 40 cycles of 95°C for denaturation by 0,3 seconds, 60°C for annealing by 1 min and 72°C for extension by 30 seconds. TaqMan® Gene Expression Assay was pre-optimized PCR primer and probe sets for qRT-PCR formulated at 20 × concentration. Specific primers were utilized for quantification of genes evaluated through TaqMan® Custom Array Plate. Two reference genes (b-actin and Glyceraldehyde-3-phosp- hate dehydrogenase-GAPDH) and 2 target genes (CD44 and EGFR) were utilized. All reac- tions were realized in duplicate to better PCR specificity. Gene expression was normalized with ß-actin and GAPDH genes. Gene expression of CD44 and EGFR genes were compared in CSCs and Non-CSCs and it was calculated by delta thresh- old cycle (Ct) method accord- ing to mathematical following formula: Expression level of target gene = 2-(Delta Ct) × 1,000 Delta Ct = Ct of target gene - (Mean Ct of ß-actin and GAPDH genes).

Results

Identification and isolation of CSCs and Non-CSCs subpopulations in cell lines

The subpopulation of sorted HN13 cell line with $CD44^{high}/CD133^{high}/CD117^{high}$ (CSCs) was detected in 0.7% and isolated of Non-CSCs. The expression of CD44, CD117 and CD133 were 0.1%, 0.4% and 0.2%, respectively.

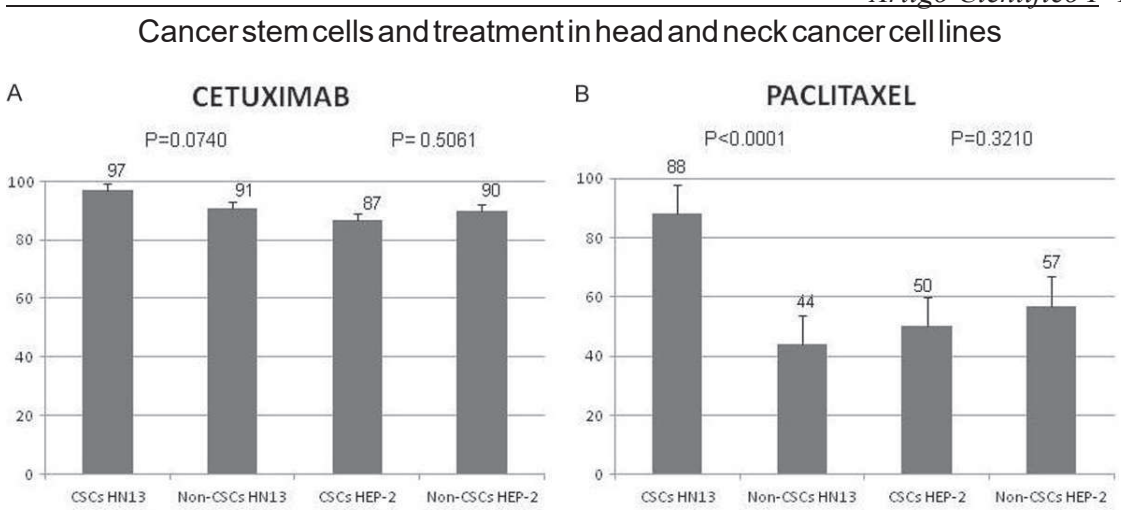

Figure 3. Cell proliferation of CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines treated with chemotherapies after 24 hours. A. CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines treated with Cetuximab. B. CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines treated with Paclitaxel chemotherapeutic.

The subpopulation of sorted HEP2 cell line with $CD44^{high}/CD133^{high}/CD117^{high}$ (CSCs) was detected in 0.8% and isolated of Non-CSCs. The expression of CD44, CD117 and CD133 were 0.3%, 0.4% and 0.1%, respectively.

Confirmation of presence of CSCs

After sorting, CSCs and Non-CSCs subpopulations were then collected and cultured separately under the same conditions, as described above. As shown in Figures 1 and 2, CSCs demonstrated increased invasive capacity as compared with Non-CSCs subpopulations after 24 hours in both cell lines. In CSC HN13 the migration was 92% and in Non-CSC HN13 was 53%. In CSC HEP-2 the migration was 94% and in Non-CSC HEP-2 was 13%.

Drug sensitivity of CSCs after treatment

Both CSCs and Non-CSCs subpopulations of cell lines were treated with Cetuximab and Paclitaxel agents, and then cell proliferation was assessed using MTS assay. As shown in Figure 3, CSCs subpopulation cells demonstrated more cell proliferation when compared with Non-CSCs subpopulation in HN13 and HEP-2 cell lines.

Expression of genes related to stem cell and cancer drug resistance in Non-CSCs and CSCs subpopulations

To exanimate the difference in the expression of genes related to stem cell and cancer drug resistance between Non-CSCs and CSCs subpopulations cells, we used delta threshold cycle (Ct) method according to mathematical following formula: Expression level of target gene = 2-(Delta Ct) × 1,000 Delta Ct = Ct of target gene - (Mean Ct of ß-actin and GAPDH genes). Regarding to HN13 cell line, we found that CD44 gene presented up-regulation (rate < 2.0) in CSCs when compared with Non-CSCs and, EGFR gene presented down-regulation (rate > 2.0) in CSCs when compared with Non-CSCs. For HEP-2 cell line the results showed that CD44 gene presented down-regulation (rate < 2.0) in CSCs when compared with Non-CSCs and, EGFR gene presented up-regulation (rate > 2.0) in CSCs when compared with Non-CSCs (Table 1).

Discussion

We firstly characterized CSCs in two head and neck cell lines with CD44, CD133 and CD117 biomarkers. So we treated CSCs and Non-CSCs subpopulations with Cetuximab and Paclitaxel chemoterapies and found that CSCs subpopulations demonstrated more resistance to Paclitaxel, as compared with Non-CSCs subpopulations in HN13 cell line. These HN13 CSCs subpopulations presented up-regulation of *CD44* gene and down-regulation of *EGFR* gene when compared with Non-CSCs subpopulations while HEP-2 CSCs presented down-regulation of *CD44* gene and up-regulation of *EGFR* gene when compared with Non-CSCs subpopulations.

Table 1. *CD44* and *EGFR* gene expression in CSCs HN13 and CSCs HEP-2 cell lines

Gene symbol	Gene description	GenBank	Fold change			
			CSCs HN13	Non-CSCs HN13	CSCs HEP-2	Non-CSCs HEP-2
CD44	The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration.	NM_000610.3	102.775859	1 (REF)	0.65892	1 (REF)
EGFR	EGFR and its ligands are cell signaling molecules involved in diverse cellular functions, including cell proliferation, differentiation, motility, and survival, and in tissue development	NM_001346897.1	0.741344907	1 (REF)	7.55986	1 (REF)

Regarding to characterization, the culture condition was capable of expanding $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cells from HN13 and HEP-2 cell lines. CD44 biomarker is a cell sur- face hyaluronan receptor protein involved in cell adhesion, cell-cell interactions and cell proliferation besides being receptor for hyaluronic acid [21, 22]. CD44 was firstly identified in head and neck cancer in 2007 by Prince and collaborators and found that positive CD44 cells initiated tumor growth with high tumorigenic potential and differentiation capacity when compared with negative CD44 cells confirming that positive CD44 population of human head and neck cancer has properties of cancer stem cells and head and neck cancer contain a subpopulation of CSC, which was confirmed in our study in HNC cell lines [16].

CD133 biomarker is a cell-surface glycoprotein comprising five trans-membrane domains associated with cell membrane topology organization. It is often expressed on adult stem cells with function of maintaining stem cell properties by suppressing differentiation [23, 24]. CD133 also has been identified human tongue, laryngeal and bucal cancer cell lines with ability of tumorigenic, power of cell proliferation and differentiation when compared to CD133-subpopulations, now we confirmed the identification of CD133+ cells in oral cancer cell line [25-28].

CD117 biomarker is a transmembrane receptor for MGF (mast cell growth factor, also known as stem cell factor) with cellular function not entirely known, however studies show that CD117 promotes the proliferation, survival, and metastasis of tumor cells and has been regarded as a cancer stem cell biomarker, but is not yet evaluated in oral cancer. We found CD117 high in oral cancer cell line, however more studies is needed to evaluate the importance of this biomarker is cancer stem cells development [30-32].

Regarding to treatment of CSCs and Non-CSCs subpopulations with Cetuximab and Paclitaxel we found more resistance to Paclitaxel chemoterapy. As compared with Non-CSC subpopulations in both cell lines suggesting that $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cells should be considered as targets in future therapies with Paclitaxel.

This is the first study that isolates cancer stem cells of head and neck cancer cell lines through of CD44/CD133/CD117 biomarkers in combination and evaluated the cancer treatment with Cetuximabe and Paclitaxel chemotherapies to single-modality treatment. Literature studies already evaluated these biomarkers alone and found that $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cells besides presenting stem cell properties also has ability to resist chemotherapeutic agents in cancer treatment, including head and neck cancer. Furthermore CSCs often have enhanced telomerase and DNA repair activities, as well as, membrane bound ATP-binding cassette transporters (ABC "drug" transporters) whose normal functions are to exclude xenobiotics, as chemotherapies [33-36].

Cetuximab is a monoclonal antibody binding the epidermal growth factor receptor (EGFR) on both normal and tumor cells. It is a functional antagonist of the EGF and TGF ligands and is thus inhibitors of the EGFR-dependent signaling pathways leading to inhibition of cancer cell division in the G1 phase and metastatic spread because of the lack of transcription factors [37]. In our study we found the Cetuximab is not effective in CSCs subpopulation of head and

neck cancer cell lines. There is a suggestion of pathways activated in head and neck cells by EGFR increase the migratory potential of cells and interfere with their sensitivity to single-modality treatment with cetuximab, as our study [38, 39].

Paclitaxel chemotherapy is a mitotic inhibitor used in cancer chemotherapy that interferes with the normal function of microtubule growth. It binds to the ß subunit of tubulin, that is the "building block" of mictotubules, and the bind- ing of paclitaxel locks these building blocks. The resulting microtubule/paclitaxel complex affects cell function leading to mitotic arrest, prevention of cell division, and eventually apop- tosis [40]. In our study the Paclitaxel is not effective in CSCs subpopulation of oral cancer cell line. Studies show that mesenchymal stem cells have been shown to be highly resistant to the cytotoxic effects of Paclitaxel and other chemotherapeutic agents due to regulation of the cell cycle [41, 42].

Besides that we found high expression of CD44 gene in HN13 CSCs and down expression of CD44 gene in HEP-2 CSCs suggesting that the exact influence of CD44 gene expression in resistant to chemotherapy is not entirely clear. The mechanistic origins can be associated with DNA repair, resistance to apoptosis, low mitotic rate, and increased tolerance of DNA damage [48, 49] According literature data the high expression of CD44 has been identified in treatment resistant in cancer with CSCs properties, including head and neck cancer, as our study [34, 43-47]. The high expression of CD44 gene in CSCs and resistance treatment can be explained due to association of this gene with cell-cell interactions, cell adhesion and migration that is increased in CSCs.

We also found down expression of CD44 in laryngeal cancer cell line, reports confirmed that levels of CD44 expression are linked to stem cell properties [50, 51]. The HEP-2 cell line presented decreased rate of population expansion with cancer stem cell characteristics which may justify this finding. However several signalling pathways can be associated with CSCs survival and therapies that target such pathways might be therapeutically effective [52].

Regarding to EGFR gene expression, our study found that the HN13 CSCs showed down expression of EGFR and HEP-2 CSCs showed high expression of EGFR. The EGFR is found in surface of cells to which epidermal growth factor (EGF) binds. When EGF attaches to EGFR, it activates tyrosine kinase activity, triggering reactions that cause the cells to grow and multiply this way activates a wide variety of intracellular cascades and induces the regulation of target genes, leading to a specific cellular response [53, 54].

The blocking EGFR signaling has provided less therapeutic benefit and this may be related to the presence of sub-populations of CSCs and heterogeneity of tumours [55, 56]. Literature data confirm that head and neck patient tumors express EGFR (~98%), however only approxi- mately 15-20% of patients respond positively and benefit from treatment [57, 58]. Our results suggest that 80-85% of patients may present tumor with CSCs and, consequently, alterations in EGFR expression, what can contribute to treatment resistance but the mechanisms are still unclear and need to be further studied in another cell lines and primary tumor.

In conclusion, our results show that the combination of CD44, CD133 and CD117 biomarkers have stem cell properties and ability to resist Paclitaxel chemoterapy. CSCs present high expression of CD44 gene and down expression of EGFR gene in oral cancer cell line. CSCs in laryngeal cell line presents down expression of CD44 gene and high expression of EGFR gene when compared with cells without characteristics of cancer stem cells.

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Disclosure of conflict of interest

None.

Address correspondence to: Eny Maria Goloni-Bertollo, Genetics and Molecular Biology Research

Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), SP, Brazil. E-mail: analiviagalbiattidias@gmail.com; eny.bertollo@famerp.br

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ARTIGO CIENTÍFICO 2

Artigo 2

Título: Anti-EGFR Treatment Effects on Laryngeal Cancer Stem Cells

Autores: Glaucia Maria de Mendonça Fernandes, Ana Lívia Silva Galbiatti-Dias, Vilson Serafim Junior, Leticia Antunes Muniz Ferreira, Rosa Sayoko Kwasaki Oyama, José Victor Maniglia, Erika Cristina Pavarino, Eny Maria Goloni Bertollo

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Original Article

Title: Anti-EGFR Treatment Effects on Laryngeal Cancer Stem Cells

Glaucia Maria de Mendonça Fernandes, Ana Lívia Silva Galbiatti-Dias, Vilson Serafim Junior, Leticia Antunes Muniz Ferreira, Patricia Matos Biselli-Chicote, Rosa Sayoko Kawasaki-Oyama, Erika Cristina Pavarino, Eny Maria Goloni-Bertollo.

Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), SP, Brazil.

Address correspondence to: Eny Maria Goloni-Bertollo, Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), SP, Brazil. E-mail: eny.goloni@famerp.br; fernandes_glaucia@hotmail.com.

Running title: Treatment Effects on Laryngeal Cancer Stem Cells

Abstract

Laryngeal cancer (LC) is one of the common head and neck neoplasms and is characterized by resistance to conventional therapy and poor prognosis. This may result from the presence of cancer stem cells (CSCs), which form a small population in tumors and possess metastatic potential, high invasive capacity, self-renewal, and differentiation. This study aimed to evaluate the effectiveness of 5-fluorouracil and cisplatin individually as well as the combination of cetuximab and paclitaxel in a CSC subpopulation separated with CD44, CD117, and CD133 biomarkers. In addition, *TrkB* and *KRAS* expression was evaluated in this subpopulation. The *CD44*, *CD133*, and *CD117* biomarkers were used to analyze the identification and separation of both subpopulations using FACSAria Fusion. Subpopulations that possessed or lacked these biomarkers were classified as laryngeal cancer stem cells (LCSCs) or laryngeal cancer non-stem cells (non-LCSCs), respectively. Matrigel invasion and colony forming assays were performed to confirm LCSCs presence. Then, the LCSCs and non-LCSCs subpopulations were cultured and exposed to 5-fluorouracil, cisplatin, or both cetuximab and paclitaxel for 24 h. Cell proliferation was determined by MTS assay. *KRAS* and *TrkB* gene expression levels were quantified by quantitative real time PCR using TaqMan® Assay in both subpopulations. The non-LCSC subpopulation was considered as the control for relative expression. We found that the CSC subpopulation demonstrated greater resistance to cetuximab and paclitaxel combination chemotherapy when compared with the non-CSC subpopulation of the LC cell line. These CSC subpopulations presented up-regulation of the *KRAS* gene and no *TrkB* gene expression in the LC cell line when compared with the non-CSC subpopulation. In conclusion, the combination of CD44, CD133, and CD117 biomarkers exhibited stem cell properties in an LC cell line, LCSCs were capable of resisting treatment, and LCSCs presented high *KRAS* gene expression.

Keywords: Cancer stem cells, chemotherapy, head and neck neoplasms, gene expression, cell line.

Introduction

Laryngeal Cancer (LC) is one of the most common head and neck neoplasms, representing 2% of all malignant neoplasms (3). Estimates show that by 2020, 9,491 new cases and 5,202 deaths may occur due to this disease (54). Chemotherapy treatment with docetaxel, bleomycin, hydroxyurea, pembrolizaumab, nivolumab, methotrexate, cetuximab (55), and paclitaxel (56) can be used. Despite advances in drug therapy, individuals with LC show low survival due to the locoregional recurrence and metastasis onset (2).

A small group of cells known as cancer stem cells (CSCs) may be responsible for tumor maintenance and dissemination. These cells possess self-renewal and differentiation potential and also play an important role in tumor initiation and progression (12). These features can be associated with poor prognosis (57) and provide resistance to tumors, leading to ineffective treatments (13, 58, 59). CSCs can be identified by cell surface biomarkers such as *CD44*, *CD117*, and *CD133* (12, 60, 61), and studies show that CSCs present high expression of these biomarkers (9, 12, 62).

Many genes related to the cell proliferation pathway are also involved in tumor progression and poor prognosis. Studies have shown that overexpression of genes such

as *tropomyosin-related kinase B* (*TrkB*) (63), *rat sarcoma* (*RAS*), and *epidermal growth factor receptor* (*EGFR*) are overexpressed in different tumor types (27, 63-67). Both EGFR and TrkB are cell surface receptors that are activated by binding to epidermal growth factors (EGF) and brain-derived neurotrophic factor (BDNF), respectively. These tyrosine kinase receptors are responsible for activating some downstream intracellular signals, such as the *Ras-Raf-MEK-ERK* pathway (28, 64).

The *RAS* oncogene family has three isoforms: Harvey (*HRAS*), neuroblastoma (*NRAS*), and Kirsten (*KRAS*) (65). They encode small GTPase proteins, which have essential roles in cell proliferation, growth, survival, migration, and epithelial-mesenchymal transition (EMT), as well as important roles in tumor relapse and chemotherapeutic resistance (27, 67). About 30% of tumors display mutations in *RAS* genes. *KRAS* mutations are associated with benefits from anti-EGFR antibody therapy, consequently improving progression-free survival and overall survival (65). Nevertheless, mutated *KRAS* can regulate the GDP–GTP process and activate Ras-Raf-MEK-ERK downstream effectors independent of EGFR and TrkB receptor activation, leading to chemotherapy resistance (29, 65).

This study aimed to evaluate the effectiveness of 5-fluorouracil and Cisplatin individually as well as the combination of cetuximab and paclitaxel in CSC subpopulations separated using CD44, CD117, and CD133 biomarkers. In addition, *TrkB* and *KRAS* expression was evaluated in these subpopulations.

Materials and Methods

Sample

A HEP2 cell line originally established and described as coming from laryngeal squamous cell carcinoma with HeLa cell contamination (American Type Culture Collection / ATCC, Rockville, Maryland, USA). HEP2 Authentication was performed using the AmpFLSTR Identifiler PCR Amplification kit (life Technoligies) and our cell line show 100% identify compare to ATCC database. The cell was cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco™, Carlsbad, CA, USA), 1% L-glutamine (Gibco™), and 1% penicillin/streptomycin/amphotericin B (Gibco™) in a humidified 5% CO_2 atmosphere at 37 °C..

Cell Sorting

Two HEP2 cell subpopulations were identified using the combination of three antibodies: CD44, phycoerythrin (PE; BD Biosciences, San Jose, CA, USA); CD117, fluorescein isothiocyanate (FITC; BD Biosciences); and CD133, allophyllocyanine (APC; Miltenyi Biotec, Bergisch Gladbach, Germany), and sorted by fluorescence-activated cell sorting (FACS) using FACSAria Fusion equipment (BD Biosciences) and FACSDiva Software Version 6.1.3 for analysis. Positively labeled cells ($CD44^+/CD117^+/CD133^+$) were classified as laryngeal cancer stem cells (LCSCs), and negatively labeled cells ($CD44^-/CD117^-/CD133^-$) were considered laryngeal cancer non-stem cells (non-LCSCs). Both cell subpopulations were cultured in DMEM to obtain sufficient cells for subsequent analysis.

Invasion Assay

Quantitative analysis of invasive potential was performed using Matrigel invasion chambers with 8 μm PET membranes in 24-well plates (Corning® BioCoat™,

Corning Inc., Corning, NY, USA). Cells were seeded in the upper compartment of the transwell chamber at a density of 2×10^4 cells per insert in 100 µL serum-free DMEM. Well bottoms were filled with 750 µL DMEM supplemented with 10% FBS, which acts as a chemoattractant. Cells were incubated for 24 h at 37 °C. Cells that invaded the lower membrane surface were fixed with 4% paraformaldehyde for 20 min and stained with 5% Giemsa for 10 min. Four fields were photographed from each insert at 100× magnification using an Olympus BX53 Microscope (Olympus Life Science, Waltham, MA, USA), and the cells were counted.

Spehre-forming Assay

Clonogenic characteristics were evaluated by observing the cells' capacity to generate tumor spheres. LCSC and non-LCSC cells were cultured in low-adherence 6-well plates (Ultra-low Attachment Plates, Corning) in triplicate. Then, 1×10^4 cells/well were cultured in DMEM without FBS and supplemented with 10 ng/mL EGF, 10 ng/mL fibroblast growth factor, and 1% antibiotic/antimycotic solution. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 5 days (120 h). The formed colonies were counted and photographed.

Treatments and MTS Assay

Cell viability was determined by colorimetrically by MTS assay using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), as described by the manufacturer. A total of 5×10^3 cells were seeded into 96-well plates and treated with 0.37 mg/mL 5-fluorouracil, 2.0 mg/mL cisplatin, and 0.06 mg/mL cetuximab combined with 0.05 mg/mL paclitaxel. After 24 h of treatment, cell viability was determined by absorbance analysis on an ELISA plate reader (Multiskan FC; Thermo Scientific – Uniscience, São Paulo, Brazil) at 490 nm.

Gene Expression

RNA was extracted from 1×10^6 cells by cell lysis with 750 µL Trizol® (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration was estimated using the Qubit™ RNA HS Assay Kit with the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Total RNA (1 µg) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA). For real-time PCR, TaqMan™ (Applied Biosystems™) probes for the *TrkB* (HS00178811_m1) and *KRAS* (HS00364284_g1)genes were used in custom microplates using the TaqMan™ Universal Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and the CFX96 Touch™ Deep Well Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The comparative expression level of each condition was calculated as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta$Ct1 method), The Ct values of the samples and controls were normalized by the amount of β-actin and GAPDH.

Statistical Analysis

Results were expressed independently as the mean ± standard deviation and compared by one-way variance analysis with the Bonferroni correlation. Analyses were performed using the GraphPad PRISM 6 software. Significance was set at $p<0.05$.

Results

$CD44^+/CD133^+/CD117^+$ subpopulation has cancer stem cell properties

Cells from a HEP2 cell line were sorted using the set of *CD44*, *CD133*, and *CD117* biomarkers. LCSCs were representative in 0.8% cells, whereas non-LCSCs were representative in 4.8% cells (Figure1). The invasive potential of the LCSC and non-LCSC subpopulations were evaluated *in vitro*. Figure 2 shows increased invasive capacity of the LCSCs when compared with the non-LCSC subpopulation after 24 h. The results were found to be statistically significant and showed that LCSCs have a higher invasive potential than non-LCSCs (p=0.0022).

The colony-forming assay was conducted for the LCSC and non-LCSC subpopulations of the HEP2 cell line. Clone formation was quantified, and LCSCs presented more colonies than Non-LCSCs (p=0.0117), as shown in Figure 3.

LCSCs are treatment-resistant

The results showed no statistical differences between LCSCs and Non-LCSCs when treated with 5-fluorouracil, but statistically significant differences were found with cisplatin (p=0.0024) as well as cetuximab combined with paclitaxel (p=0.0069) (Figure 4). LCSCs had higher viability than non-LCSCs. Furthermore, cetuximab and paclitaxel combination treatment was observed to have a greater influence on subpopulation elimination than did 5-fluorouracil and cisplatin treatments (Figure 5).

LCSC subpopulation presents high KRAS gene expression

The *KRAS* gene presented up-regulation (RQ=1,48205) in LCSCs compared with non-LCSCs (RQ=1). The *TrkB* gene showed no expression in both subpopulations of the Hep2 cell line.

Discussion

In our previous study, we showed that $CD44^+/CD133^+/CD117^+$ cells, classified as LCSC and obtained from a HEP2 cell line, presented 81% more migration capacity than $CD44^-/CD133^-/CD117^-$ cells, designated as non-LCSCs (42). In this current study, invasion and colony-forming assays were also performed to confirm CSC presence. The results of these assays demonstrated increased tumorigenic potential in the LCSC subpopulation of the HEP2 cell line.

To evaluate the effect of anticancer drug therapy in LCSCs and non-LCSCs, these cells were treated with 5-fluorouracil and cisplatin individually or with the combination of cetuximab and paclitaxel. These drugs were chosen because they are the most commonly used to treat head and neck cancer patients. Cisplatin reacts with DNA to produce crosslinks, and 5-fluorouracil is an antineoplastic antimetabolite; both drugs impair DNA replication and transcription (52, 53). Cetuximab is a monoclonal antibody that functions by blocking EGF from binding to EGFR (66), therefore interrupting the cascade that activates *KRAS* (63). Paclitaxel is a chemotherapeutic that inhibits mitotic spindle fiber dynamics (15).

We found that 5-fluorouracil was ineffective at eliminating either subpopulation. The LCSC subpopulation demonstrated greater resistance to cisplatin and the combination of cetuximab and paclitaxel compared with the non-LCSC subpopulation of the HEP2 cell line. Moreover, the cetuximab and paclitaxel combination treatment was most effective in both subpopulations compared to other treatments, especially in the non-LCSC subpopulation. In previous study, our research group demonstrated that individual cetuximab and paclitaxel treatments showed no statistically significant differences between LCSCs and non-LCSCs from the HEP2 cell line (42).

Our results align with those of other studies performed in CSCs from head and neck cancers, which showed resistance to 5-fluorouracil, cisplatin, and cetuximab when used individually (14, 21, 32, 68). Grau and collaborators (32) observed cisplatin and cetuximab resistance in CSCs from head and neck carcinoma squamous cell (HNCSC) lines that had high expression of the CD44 biomarker. It has also been shown that CSCs from HNCSC cell lines, which used Aldehyde dehydrogenases (ALDH) as a biomarker, were resistant to 5-fluorouracil, cisplatin, and etoposide (68). Others studies in HNCSC cell lines that were conducted with FACS to isolate CSCs using both CD44 and ALDH biomarkers also showed resistance to docetaxel, cetuximab, and PI3K inhibitor (ZSTK474 and PX-866) in these subpopulations in addition to radiation, photon irradiation (2 Gy/min), and carbon ion irradiation (75MeV/n) resistance (14, 21). On the other hand, CSCs from HNCSC cell lines sorted with $CD44^{high}/EGFR^{low}$ presented sensitivity to cisplatin, cetuximab, gefitinib, and radiation compared to $CD44^{high}/EGFR^{high}$ (22).

To our knowledge, there are no studies on combination therapy in LCSC. Herein, we hypothesized that the combined action of cetuximab and paclitaxel drugs may contribute to eliminating LCSCs, consequently reducing tumor aggressiveness and recurrence. However, further studies with combination therapy are required to better understand chemotherapy response in LCSCs.

This is the first study that has evaluated *TrkB* and *KRAS* gene expression in CSC and non-CSC subpopulations of head and neck cancer. Considering the role of these two genes in cell proliferation, we expected that both would be overexpressed in the HEP2 cell line, especially in the CSC subpopulation; however, the *TrkB* gene was not expressed. A recent review demonstrated that *TrkB* and its ligand *brain-derived neurotrophic factor* (*BNDF*) are expressed in 30–50% of human HNCSC (69-71). One limitation in our study is that only one cell line was assessed; hence, results may not be representative. Therefore, studies with a larger sample size are needed, as *TrkB* activation has been associated with cell migration, invasion, EMT, cisplatin resistance, and poor prognosis *in vivo* (69, 70, 72-74). Indeed, some studies in HNC have shown that *TrkB* inhibition can suppress tumor growth, cell proliferation, and migration as well as sensitize cells to cisplatin (23, 69, 75-77).

In this current study, *KRAS* presented high expression, which may be explained by *EGFR*-mediated signaling that is responsible for phosphorylating and activating *KRAS*, as shown in Figure 6. In our previous study, we observed EGFR overexpression in LCSCs from the HEP2 cell line (42); therefore, we suggested that this CSC subpopulation may contribute via *EGFR*-signaling to promote tumor cell growth, chemotherapy resistance, invasion, and migration, resulting in head and neck cancer progression.

Our results, although limited, suggest for the first time that the combined action of cetuximab and paclitaxel drugs may be more efficient at eliminating LCSCs than isolated therapies and provide evidence that higher *KRAS* expression in LCSCs could then contribute to aggressive tumor behavior and poor prognosis in LC. Thus, understanding of the molecular mechanisms that control CSC proliferation may contribute to better strategies for treating head and neck cancer.

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Disclosure of conflict of interest

None.

Abbreviations

BNDF	Brain-derived neurotrophic factor
CSCs	Cancer stem cells
DMEM	Dulbecco's modified Eagle medium
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
HRAS	Harvey rat sarcoma
KRAS	Kirsten rat sarcoma
LC	Laryngeal cancer
LCSCs	Laryngeal cancer stem cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
non-CSCs	Cancer non-stem cells
non-LCSCs	Laryngeal cancer non-stem cells
NRAS	Neuroblastoma rat sarcoma
RAS	Rat sarcoma
SCF	Stem cell factors
TrkB	Tropomyosin-related kinase B

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Figure 1: Cell sorting grafics with CD44, CD 117 and CD 133 in FACSAria Fusion using FACSDiva Software. Cells in quadrants above 10^3 (P2, P3 and P4) were considered positive for the marker, and cells in quadrants below 10^3 (P5, P6 and P7) were considered negative for the markers. Then the positive cells for FITC-CD117 (P2) were selected from these cells, those that were positive for the marker PE-CD44 (P3) were selected and then those positive for the APC-CD133 (P4) were selected. Therefore forming the triple cell group positive for the three tumor stem cell biomarkers. The negative cells for FITC-CD117 (P5) were selected from these cells, those that were negative for the marker PE-CD44 (P6) were selected and then those negative for the APC-CD133 (P7) were selected. Therefore forming the triple cell group negative for the three tumor stem cell biomarkers.

Figure 2: Cell invasion assay of LCSC and non-LCSC subpopulations of the Hep2 cell line. Cells were seeded in matrigel inserts and cultured for 24 h. A. LCSC subpopulation; B. non-LCSC subpopulation. Grayscale pictures are shown at 400× magnification.

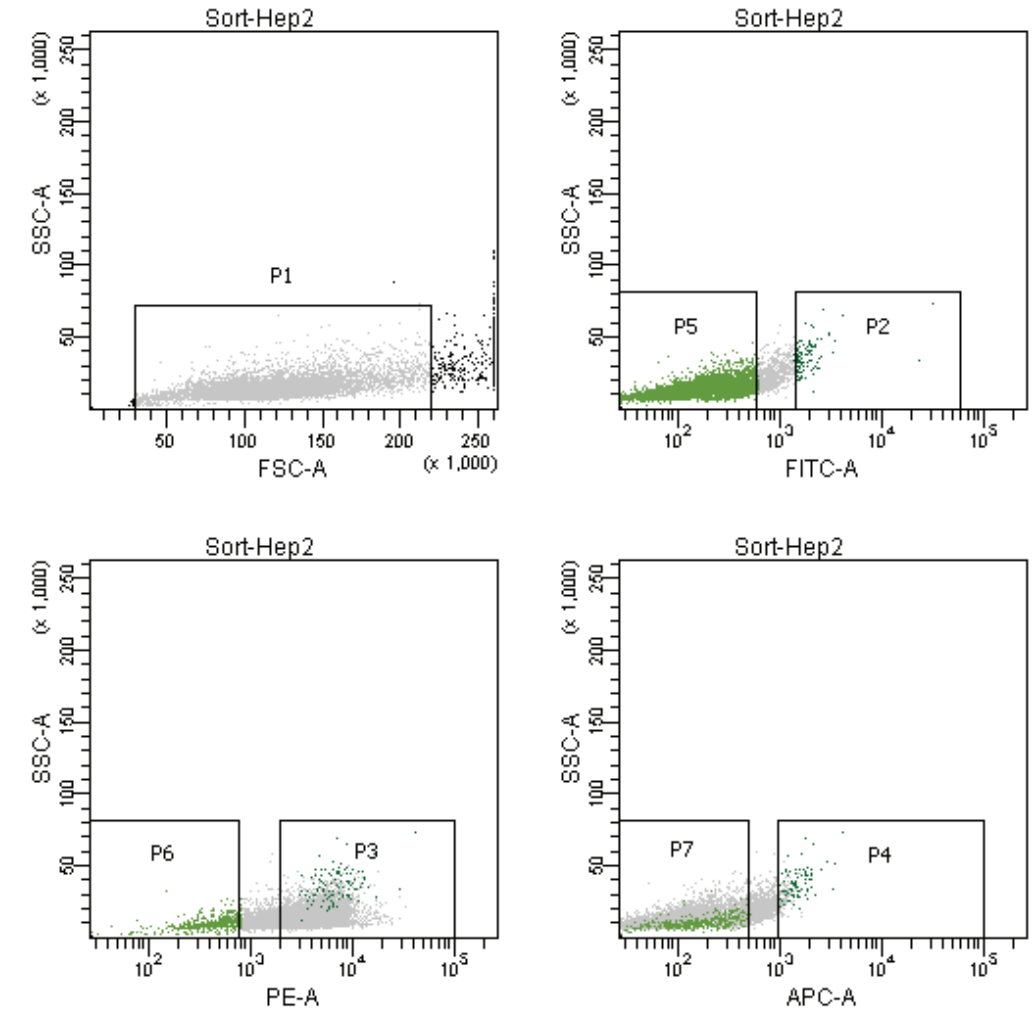
Figure 3: Sphere forming LCSC and non-LCSC subpopulations of the Hep2 cell line. The cells were seeded in ultra-low attachment surface 6-well plates and cultured for five days (120 h). A. Non-LCSC and C. LCSC subpopulations at 0 h; B. non-LCSC subpopulation after five days; and D. spheres formed in the Hep2 LCSC subpopulation after five days. Grayscale pictures are shown at 40× magnification.

Figure4: Cell viability after 24 h in HEP2. A. LCSC and non-LCSC subpopulations treated with 5-fluorouracil, cisplatin, and the combination of cetuximab and paclitaxel. Comparison of responses to 5-fluorouracil, cisplatin, and the combination of cetuximab and paclitaxel in Hep2Hep2 LCSC and non-LCSC subpopulations. Data and p-values are shown for B. LCSCs and C. non-LCSCs. *$p \leq 0{,}05$; ** $p \leq 0{,}001$; ***$p \leq 0{,}0001$.

Figure 5: Graph showing the relative values of the differential expression of the *KRAS*.

Figure 6: Summarized molecular mechanisms of the signaling pathway involving the EGFR, TrkB and KRAS genes; adapted from Fernandes et al. 2019 (29). 1) Phosphorylation resulting from BDNF/TrkB binding can also activate KRAS; however, TrkB gene expression was late. This suggests that only EGFR is activating the KRAS gene. 2) Phosphorylation resulting from EGFR/EGF binding activates KRAS, which leads to cell proliferation. The results of our present and previous studies showed high KRAS and EGFR expression (42). 3) Only cetuximab (42) binds with EGFR, which blocks EGFR/EGF binding; we suggest that this isolated treatment does not inhibit KRAS inactivation.

Figure 1.



Experiment Name: Experimento 01

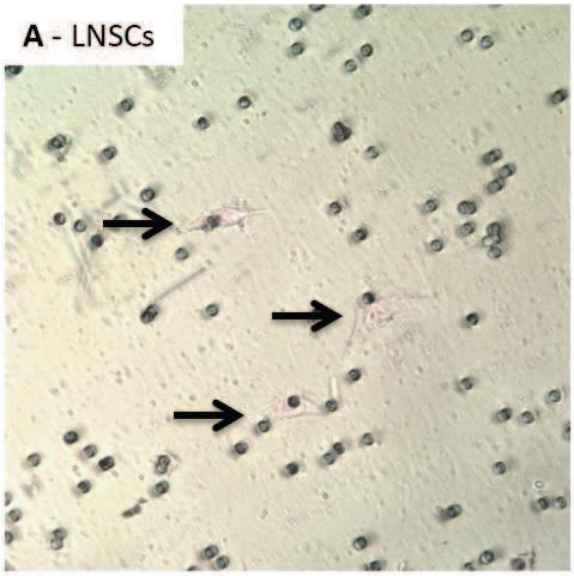
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	9,620	96.2	96.2
P2	107	1.1	1.1
P3	107	100.0	1.1
P4	82	76.6	0.8
P5	8,670	90.1	86.7
P6	485	5.6	4.9
P7	483	99.6	4.8

Tube: Hep2

Population

All Events
P1
P2
P3
P4
P5
P6
P7

Figure 2.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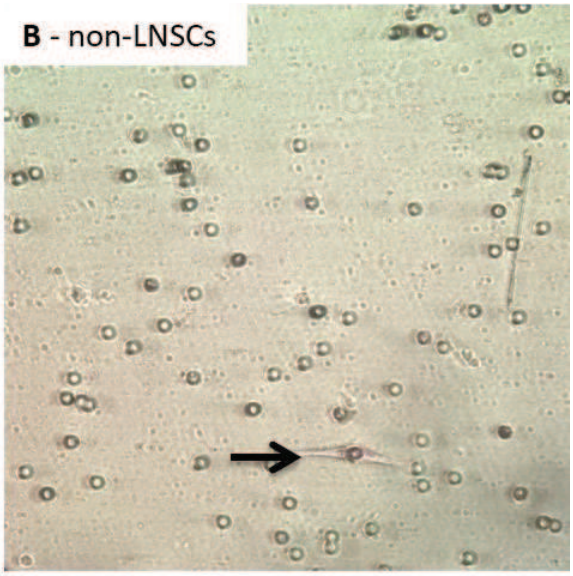
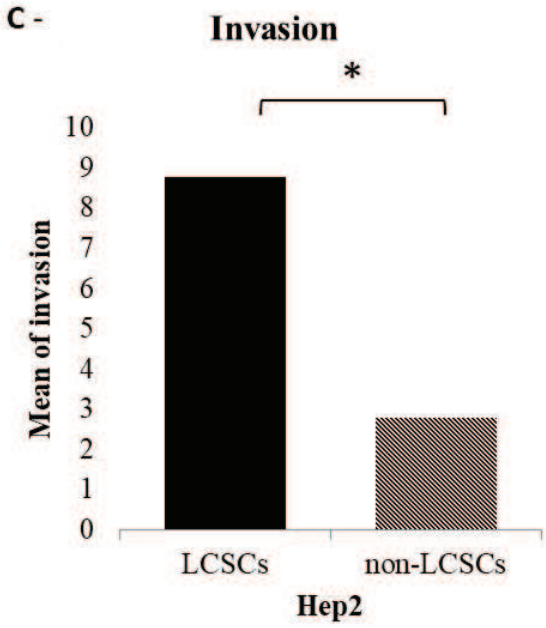

B - non-LNSCs


C -
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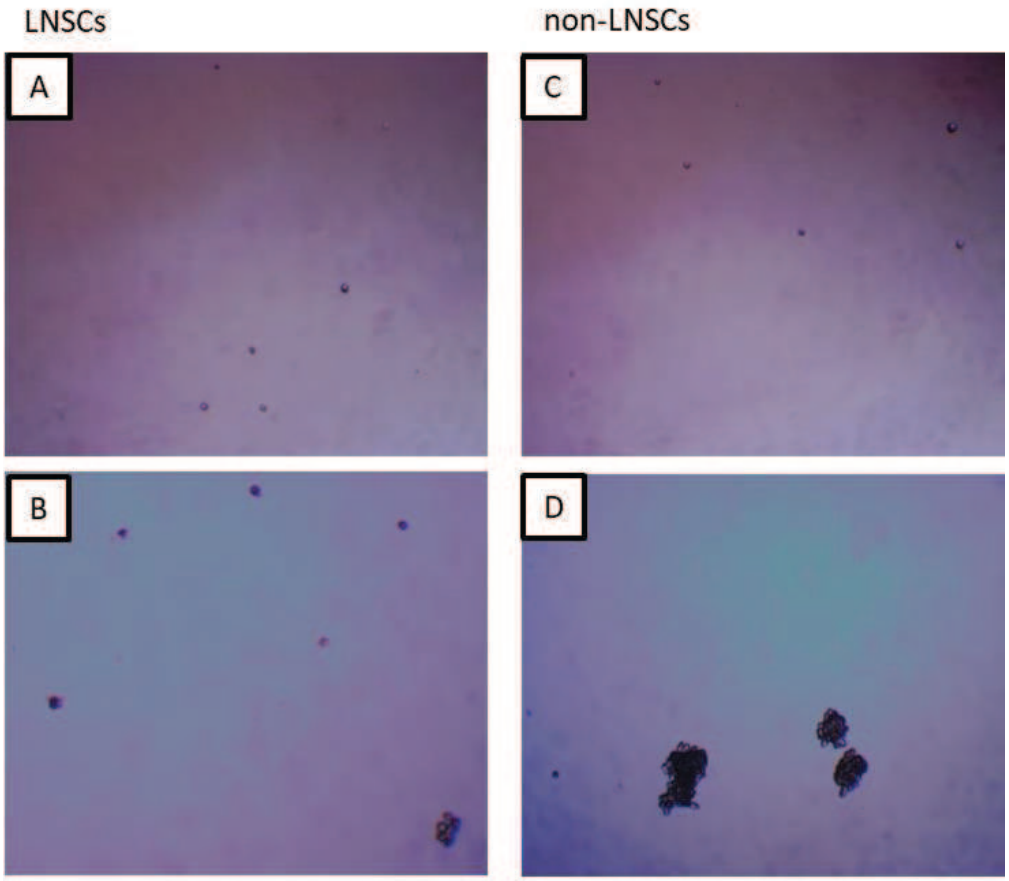
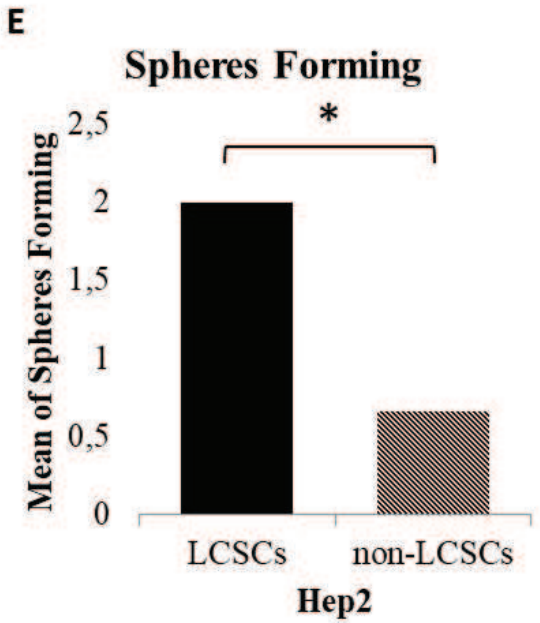
LCSCs
non-LCSCs
Hep2

Figure 3.


LNSCs
non-LNSCs
A
C
B
D


E
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2,5
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LCSCs
non-LCSCs
Hep2

Figure 4.

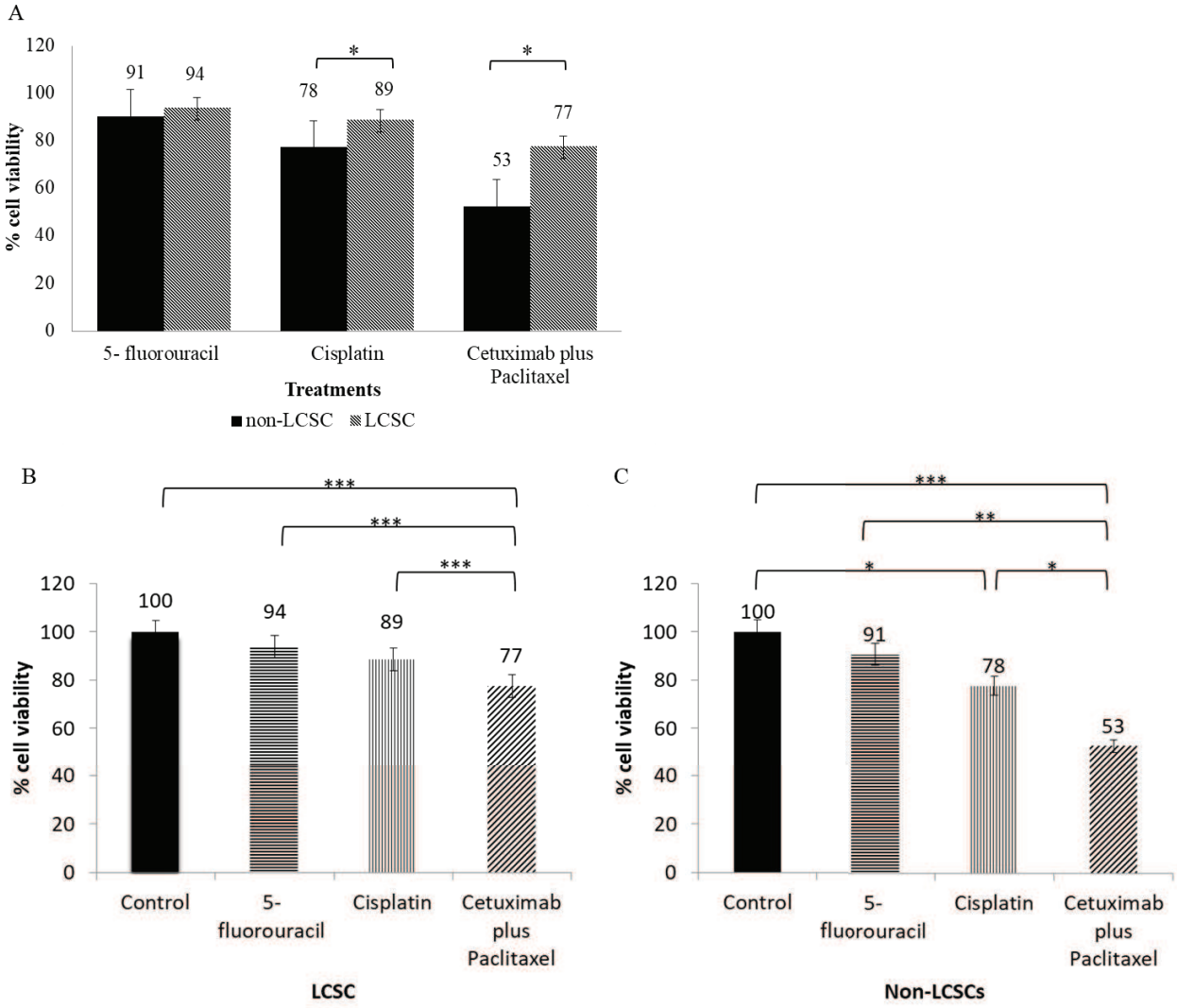


Figure 5.

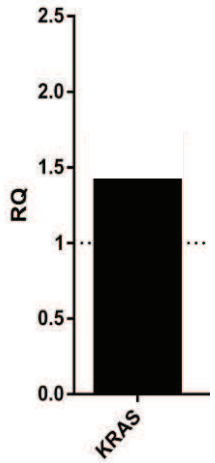
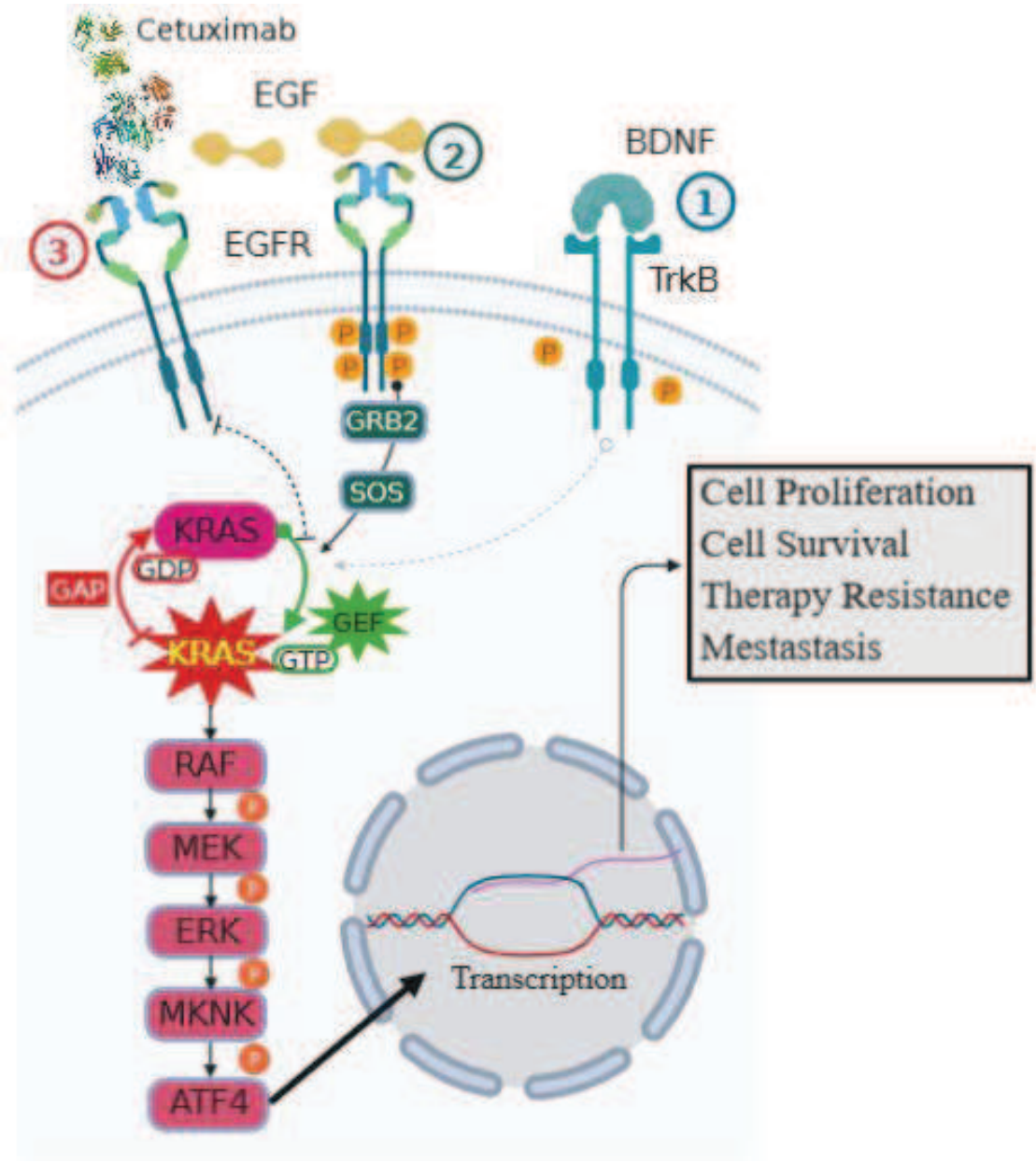


Figure 6.


Cetuximab
EGF
BDNF
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EGFR
TrkB
P
GRB2
SOS
KRAS
GDP
GAP
GEF
KRAS
GTP
Cell Proliferation
Cell Survival
Therapy Resistance
Mestastasis
RAF
MEK
ERK
MKNK
ATF4
Transcription

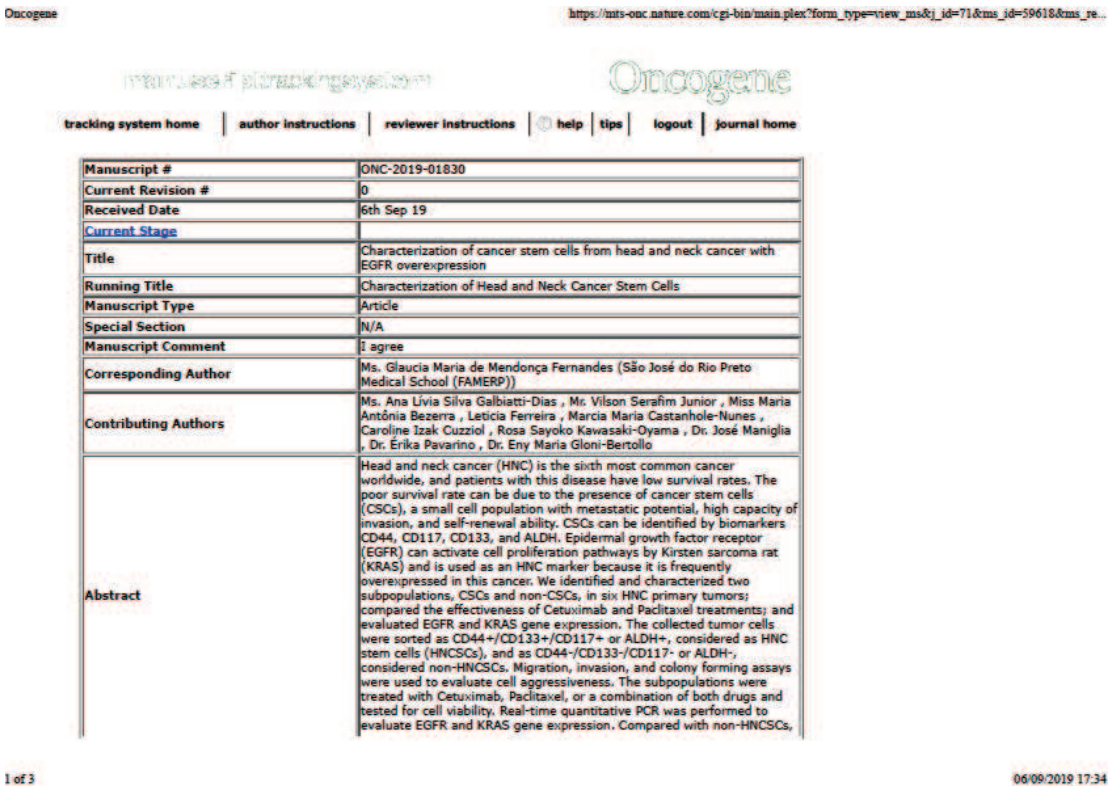
ARTIGO CIENTÍFICO 3

Artigo 3

Título: Characterization of EGFR pathway in Head and Neck Cancer Stem Cells.

Autores: Glaucia Maria de Mendonça Fernandes, Ana Lívia Silva Galbiatti-Dia, Marcia Maria Urbanin Castanhole-Nunes, Vilson Serafim Junior, Leticia Antunes Muniz Ferreira, Caroline Izak Cuzziol, Maria Antonia dos Santos Bezerra, Rosa Sayoko Kwasaki Oyama, José Victor Maniglia, Erika Cristina Pavarino, Eny Maria Goloni Bertollo

Periódico: Oncogene, a ser submetido



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Running Title	Characterization of Head and Neck Cancer Stem Cells
Manuscript Type	Article
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Corresponding Author	Ms. Glaucia Maria de Mendonça Fernandes (São José do Rio Preto Medical School (FAMERP))
Contributing Authors	Ms. Ana Lívia Silva Galbiatti-Dias , Mr. Vilson Serafim Junior , Miss Maria Antônia Bezerra , Leticia Ferreira , Marcia Maria Castanhole-Nunes , Caroline Izak Cuzziol , Rosa Sayoko Kawasaki-Oyama , Dr. José Maniglia , Dr. Érika Pavarino , Dr. Eny Maria Gloni-Bertollo
Abstract	Head and neck cancer (HNC) is the sixth most common cancer worldwide, and patients with this disease have low survival rates. The poor survival rate can be due to the presence of cancer stem cells (CSCs), a small cell population with metastatic potential, high capacity of invasion, and self-renewal ability. CSCs can be identified by biomarkers CD44, CD117, CD133, and ALDH. Epidermal growth factor receptor (EGFR) can activate cell proliferation pathways by Kirsten sarcoma rat (KRAS) and is used as an HNC marker because it is frequently overexpressed in this cancer. We identified and characterized two subpopulations, CSCs and non-CSCs, in six HNC primary tumors; compared the effectiveness of Cetuximab and Paclitaxel treatments; and evaluated EGFR and KRAS gene expression. The collected tumor cells were sorted as CD44+/CD133+/CD117+ or ALDH+, considered as HNC stem cells (HNCSCs), and as CD44-/CD133-/CD117- or ALDH-, considered non-HNCSCs. Migration, invasion, and colony forming assays were used to evaluate cell aggressiveness. The subpopulations were treated with Cetuximab, Paclitaxel, or a combination of both drugs and tested for cell viability. Real-time quantitative PCR was performed to evaluate EGFR and KRAS gene expression. Compared with non-HNCSCs,

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Original Article

Characterization of EGFR pathway in Head and Neck Cancer Stem Cells

Running title: Characterization of Head and Neck Cancer Stem Cells

Glaucia Maria de Mendonça Fernandes, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Ana Lívia Silva Galbiatti-Dias, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Marcia Maria Urbanin Castanhole-Nunes, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Vilson Serafim Junior, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Leticia Antunes Muniz Ferreira, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Caroline Izak Cuzziol, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Maria Antonia dos Santos Bezerra, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Rosa Sayoko Kawasaki-Oyama, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

José Victor Maniglia, *Department of Otolaryngology and Head and Neck Surgery, São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Erika Cristina Pavarino, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Eny Maria Goloni-Bertollo, *Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, São Paulo, Brazil.*

Address correspondence to: Eny Maria Goloni-Bertollo, Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), SP, Brazil. E-mail: eny.goloni@famerp.br; and Glaucia Maria de Mendonça Fernandes, Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), SP, Brazil. E-mail: fernandes_glaucia@hotmail.com.

Competing interest statement: The authors declare that they have no conflict of interest.

Abstract

Head and neck cancer (HNC) is the sixth most common cancer worldwide, and patients with this disease have low survival rates. The poor survival rate can be due to the presence of cancer stem cells (CSCs), a small cell population with metastatic potential, high capacity of invasion, and self-renewal ability. CSCs can be identified by biomarkers *CD44*, *CD117*, *CD133*, and *ALDH*. Epidermal growth factor receptor (EGFR) can activate cell proliferation pathways by Kirsten sarcoma rat (*KRAS*) and is used as an HNC marker because it is frequently overexpressed in this cancer. We identified and characterized two subpopulations, CSCs and non-CSCs, in six HNC primary tumors; compared the effectiveness of Cetuximab and Paclitaxel treatments; and evaluated EGFR and KRAS gene expression. The collected tumor cells were sorted as *CD44+/CD133+/CD117+* or *ALDH+*, considered as HNC stem cells (HNCSCs), and as *CD44-/CD133-/CD117-* or *ALDH-*, considered non-HNCSCs. Migration, invasion, and colony forming assays were used to evaluate cell aggressiveness. The subpopulations were treated with Cetuximab, Paclitaxel, or a combination of both drugs and tested for cell viability. Real-time quantitative PCR was performed to evaluate EGFR and *KRAS* gene expression. Compared with non-HNCSCs, HNCSCs presented more colonies and appeared to be more sensitive to the drug combination. The EGFR and *KRAS* genes were upregulated in CSCs compared with non-HNCSCs, thus explaining the drug resistance. Overall, the drug combination seems to be more beneficial for the elimination of HNCSCs, which show EGFR and the *KRAS* gene upregulation, than for non-HNCSCs.

71 **Introduction**

72 Head and Neck Cancer (HNC) comprises a wide range of tumors in the lips, oral
73 cavity (2.0%), hypopharynx (0.4%), oropharynx (0.5%), nasopharynx (0.7%), and
74 larynx (1.0%), and is the sixth most common cancer worldwide [1]. The risk factors
75 associated with HNC include smoking, alcohol consumption, human papillomavirus and
76 Epstein-Barr virus infections [2, 3]. HNC patients at all stages of the disease have a low
77 five-year survival rate, and the prognosis for patients with recurrent or metastatic
78 disease is poor [1, 2].

79 The standard treatment for HNC depends on the site of the primary tumor and
80 the stage of the disease. HNC in an early stage (I/II) is usually treated with surgery or
81 radiotherapy, while locally advanced disease (stage III/IV) requires the combination of
82 radiotherapy, surgery, and chemotherapy with the anti-mitotic agent Paclitaxel [4]. One
83 strategy aimed at improving the efficacy of the treatment is to add molecular target
84 agents, such as Cetuximab, to standard chemotherapy. Cetuximab is a chimeric
85 monoclonal antibody against the epidermal growth factor receptor (EGFR) that can be
86 safely combined with Paclitaxel in HNC treatment [5-9]. Despite the advances in drug
87 therapy, HNC patients still present a low survival rate and high metastatic rates [10].
88 One hypothesis that could explain the low survival is the presence of a small group of
89 cells named cancer stem cells (CSCs) that are present in many solid tumors, including
90 HNC. CSCs have metastatic potential, high capacity of invasion [11, 12], and the
91 abilities of self-renewal and differentiation, as well as having a substantial function in
92 the initiation and progression of the tumor. These features, which may provide tumor
93 resistance leading to treatment ineffectiveness [13], are all associated with poor
94 prognosis [14]. CSCs have been identified to express the biomarkers *CD44*, *CD117*,

CD133, and *ALDH*, which have also been found to be overexpressed in tumors with CSCs [15-18].

CSCs are thought to arise from progenitor cells or normal stem cells showing aberrant behavior of key regulatory genes, specifically, proto-oncogenes and tumor suppressors [19]. Important proto-oncogenes that play a key role in HNC tumorigenesis are *EGFR* and *Akt* [20]. EGFR is a member of the ErbB family of receptor tyrosine kinases and has several known growth factor ligands that activate many downstream effectors involved in the Rat Sarcoma/raf1/mitogen-activated protein kinase pathway (RAS/raf1/MAPK pathway) [12, 14, 20]. This activation leads to the expression of other proteins responsible for coordination of cell growth, promotion of tumor initiation, and disease progression [20]. EGFR is highly expressed in many cancers of epithelial origin, including head and neck squamous cell carcinoma (HNSCC), and is correlated with an increased risk of local relapse, adverse overall survival, and poor clinical outcome [2, 3, 20].

RAS is a family of proto-oncogenes encoding proteins that are members of the small GTPases superfamily, which has essential roles in several signaling pathways controlling cell growth. Kirsten rat sarcoma virus (*KRAS*) is the most important gene of the family [21] because mutations in this proto-oncogene are related to independent activation of pathways associated with growth and cell survival, and contribute to tumor maintenance [21, 23].

Thus, the aims of this study were: to identify and characterize two HNC cell subpopulations, namely, Head and Neck Cancer Stem Cells (HNCSCs) and Head and Neck Cancer non Stem Cells (non-HNCSCs) in six primary tumors of HNC patients; to

compare the effectiveness of Cetuximab and Paclitaxel treatment; and to evaluate *EGFR* and *KRAS* expression in both subpopulations.

Results

HNCSC subpopulation has cancer stem cell properties

The primary tumors cells were identified and sorted with *CD44*, *CD133*, and *CD117*, or *ALDH* biomarkers (Figure 1). Our results showed that the $CD44^{+}/CD133^{+}/CD117^{+}$, or $ALDH^{+}$ HNC cell population, designated as HNCSCs, had a higher potential for migration, invasion, and colony formation compared with the $CD44^{-}$ / $CD133^{-}$ / $CD117^{-}$, or $ALDH^{-}$ population, the so-called non-HNCSCs. Thus, by migration, invasion, and colony formation assays we have confirmed that the subpopulation of HNCSCs had higher tumorigenic potential and formed spheres, a unique characteristic of non-HNCSCs. The cell migration and invasion capacity of HNCSC and non-HNCSC subpopulations of the primary tumor were evaluated *in vitro*. After 24 h, HNCSCs demonstrated an increased migration and invasive potential compared with non-HNCSC subpopulations as shown in Figure 2 ($p<0.0001$ and $p=0.0324$, respectively).

The colony formation assay of the primary tumors showed more tumorspheres in HNCSCs than in non-HNCSC subpopulations ($p=0.0013$), as depicted in Figure 2.

HNCSCs are treatment resistant

Both HNCSCs and non-HNCSC subpopulations of primary tumors were treated with Cetuximab, Paclitaxel, and a combination of both drugs (CP). The viability of the two populations did not show statistical differences ($p>0.05$) (Figure 3). However, HNCSCs seemed to be more sensitive to the treatment with CP. Moreover, when

comparing the treatments in each subpopulation, only Cetuximab was not effective in both subpopulations; although the drug could potentiate the effects of Paclitaxel chemotherapy (Figure 4).

HNCSC subpopulations presented overexpression of *EGFR* and *KRAS* genes

EGFR and *KRAS* genes presented were up-regulated (mean RQ=7.081 and 1.568, respectively) in HNCSCs compared with non-HNCSCs, with no significant differences between the two subpopulations (p=0.5625 and p=0.5296, respectively). The differential quantitative gene expression and statistical analysis are shown in Figure 5.

Discussion

We found that HNC cells with biomarking of *CD44*, *CD133*, and *CD117*, or *ALDH* showed more migration potential and invasion, and formed more and larger colonies than non-HNCSCs demonstrating the growth tumorigenic potential of the HNCSC subpopulation. The results showed that these biomarkers were effective in sorting the CSC subpopulation from the non-CSCs one. Furthermore, the migration potential, invasion, and formation of larger colonies are related to the higher aggressiveness of the HNCSCs compared with the non-HNCSCs. In a previous study from our research group using HN13 and HEP2 cell lines, the HNCSC subpopulation demonstrated a migration capacity 81% higher than that of the non-HNCSC subpopulation lines[24].

The literature reports the use of *CD44*, *CD133*, *CD117*, and *ALDH* genes to separate and characterize tumor stem cells. The *CD44* biomarker is an integral membrane glycoprotein as well as a receptor for hyaluronic acid [25]. Proto-oncogene

CD117 (c-kit) is a member of the tyrosine kinase receptor family that interacts with stem cell factors [26, 27]. *CD133* (prominin-1) is a transmembrane glycoprotein [28] while *ALDH* is an intracellular cytosolic isoenzyme that converts acetaldehyde into acetate. High activity of the biomarkers has been considered as a reliable marker for CSCs. These biomarkers are involved in embryogenesis, hematopoietic stem, and progenitor cells as well as carcinogenesis, and their expression is correlated with tumor progression, differentiation suppression, resistance to radio and chemotherapy, self-renewal, relapse, and metastasis. [29-31].

We have found no statistical difference between HNCSCs and non-HNCSC subpopulations in primary tumors treated with Cetuximab, Paclitaxel, or CP. Moreover, CP resulted in the most effective treatment for the HNCSC subpopulation. Cetuximab seems to potentiate the effects of Paclitaxel in the HNCSC subpopulation. Paclitaxel chemotherapy inhibits the fibers of the mitotic spindle and consequently interrupts cell proliferation [32]. Cetuximab binds to *EGFR* and inhibits the cascade of cell proliferation slowing down the disease progression and increasing the survival rate of cancer patients. *KRAS* is a gene encoding an intracellular signaling protein indirectly activated by *EGFR*, resulting in an exacerbated cellular proliferation. However, if there are changes in this cascade, such as the high expression of the *KRAS* gene, the signaling may not depend on the EGFR receptor activation and therefore, there is no benefit in administering Cetuximab [21, 33].

Literature reports are inconclusive regarding HNCSCs treatment with Cetuximab. Studies showed that cells with CSC features are more sensitive to Cetuximab in hypoxic conditions [12] or when they depict the $CD44^{high}/EGFR^{low}$ phenotype in flow cytometry [34]. However, other contributions demonstrated that cells

189 with CSCs features and *CD44* overexpression were resistant to the Cetuximab treatment
190 [35]. Furthermore, CSC sorted using *ALDH* and *CD44* as biomarkers were resistant to
191 Cetuximab and Docetaxel (similar to Paclitaxel chemotherapy) [36]. The CSC
192 subpopulation sorted using Side Population through Hoechst exclusion, $CD44^{High}$, and
193 $ALDH^{High}$ did not show reduced proliferation when treated with Cetuximab [37].
194 Studies on the combination of Cetuximab with other chemotherapeutic agents
195 such as Paclitaxel, Docetaxel, Cisplatin, and/or 5-Fluorouracil are still being performed
196 to select the best treatment approaches [8, 9, 37-40]. Head and neck squamous cell
197 carcinoma patients with recurrence or metastasis after platinum-based
198 chemoradiotherapy were treated with CP and presented tolerance and a positive
199 response to the treatment [8, 9]. Another randomized study in HNC patients found that
200 the combination of Cetuximab with Paclitaxel and Cisplatin; or Cetuximab with
201 Docetaxel, Cisplatin and 5-Fluorouracil increased the progression-free survival by 20%
202 in two years compared with that of the control [39].
203 In the present study, we demonstrated the high expression of *EGFR* and *KRAS*
204 genes, confirmed by tumor proliferation, progression migration, and invasion assays, in
205 HNCSCs compared with that in non-HNCSCs. This is, to our knowledge, the first
206 research evaluating the influence the *KRAS* gene in subpopulations of stem and non-
207 stem tumor cells in head and neck primary tumor.
208 Literature showed that the *EGFR* gene can activate *KRAS*, one of the genes
209 responsible for cell growth and tumor recurrence. The *EGFR* gene expression is
210 controversial. Some studies showed *EGFR* [12, 41] and p-*EGFR* downregulation [35,
211 41]; however, others demonstrated *EGFR* [42, 43] and p-*EGFR* overexpression. In a
212 previous work we reported the down-regulation of the *CD44* gene and the up-

expression of the *EGFR* gene in laryngeal CSCs cell line; and the up-regulation of the *CD44* gene and the down-expression of the *EGFR* gene in an oral CSCs cell line [24]. The *KRAS* gene expression was not evaluated in HNCSCs. Thus, we decided to evaluate the expression of *EGFR* and *KRAS* genes in primary tumors and its real representativeness.

Our results reinforce the relation between the *KRAS* pathway activated by *EGFR* phosphorylation and a significant role in cell proliferation, tumor progression, and resistance to chemotherapy in HNC. It is known that gene expression is extremely variable among the tumor subsets of HNC. Therefore, it does not mean that this genes is unrelated to CSCs in HNC and more studies are needed on other tumor sites. Limitations of our study were the small sample size and the difficulties maintaining the tumor stem cells in primary tumors due to their extreme fragility.

We concluded that cells with the triple biomarking *CD44*, *CD133*, and *CD117*, or *ALDH* only biomarkers form more colonies, an exclusive characteristic of CSCs, and show more aggressive cellular features. The CP seems to be more beneficial in the elimination of both cellular subpopulations of HNC cells. *EGFR* and *KRAS* genes overexpress in HNCSCs. The relation between the combination of the Cetuximab with Paclitaxel and the high expression of the genes may contribute to elucidate tumor resistance and progression processes. However, more studies are necessary to understand the role of these genes in the chemoresistance of CSCs.

Materials and Methods

Sample

HNC tissues were collected from patients who underwent surgical resection at the Service of Otorhinolaryngology and Head and Neck Surgery of the Medical School of São José do Rio Preto-FAMERP. All patients signed consent letters and the study was approved by the Institutional Research Ethics Committee of the Medical School São José do Rio Preto-FAMERP, SP, Brazil (903.775). Exclusion criteria was patients that have been initiate chemo or radiotherapy treatment. Table 1 presents the clinical features and surgical staging from the patients' six primary tumors included in the study. Data were retrospectively obtained from medical records.

All samples were cultured in Dulbecco's Modified Eagle Medium, (DMEM, Sigma-Aldrich Co.) supplemented with 20% Ham's Nutrient Mixture F12, (HAMF12, Sigma-Aldrich Co.), 10% fetal bovine serum (FBS, Gibco™), 1% L-glutamine (Gibco™), 1% of penicillin, streptomycin, and anfotericin B (Gibco™) in 5% CO2 at 37 °C.

Cell sorting

Identification and separation of CSCs were performed using the Cell Sorting BD FACSAria Fusion flow cytometer (BD Biosciences) and specific antibodies for labeling, following manufacturers' recommendations. Cells that were positively marked with the three antibodies together *CD44*-phycoerythrin (PE) (BD Biosciences), CD117-fluorescein isothiocyanate (FITC) (BD Biosciences), and CD133-allophycocyanin (APC) (Miltenyi Biotec) or only for *ALDH*-aldehyde dehydrogenase-bright (ALDEFLUOR™ - STEMCELL Technologies) were classified as HNCSCs (*CD44+/CD133+/CD117+*, or only *ALDH+*). Cells that were negative for labeling with the three antibodies together CD44/PE, CD133/APC, and CD117/FITC, or ALDH/FITC alone were considered non-HNCSCs (*CD44-/CD133-/CD117-*, or only *ALDH-*).

Migration and invasion assay

For the migration analysis, confluent cells grown in 2 ml of culture medium supplemented with 10% FBS within the well of a 6-well plate were "wounded" by scraping off an area using a plastic pipette tip. After the procedure, plates were incubated at 37 °C for 24 h in a CO_2 incubator. Images were obtained with an inverted microscope at the beginning of the experiment and after 24 h. Six fields per well were photographed in triplicate at 40x magnification. Subsequently, the quantitative analysis was performed by measuring the invaded area at the beginning of the experiment and after 24 h using the ImageJ application. The percentage of the invaded area was calculated for each well and results subjected to statistical analysis.

The transwell invasion assay was carried out performed in duplicates in a Corning® BioCoat™ Matrigel® Invasion Chamber (Discovery Labware, Inc ©Corning Inc.). A total of $2x10^4$ cells were placed in a serum-free medium in the upper chamber, while medium containing 10% FBS was added as a chemoattractant to the lower chamber. Invading cells were fixed with 4% paraformaldehyde for 2 min followed by methanol for 20 min, and stained with 0.5% crystal violet. Four fields were randomly selected and photographed under a light microscope (Olympus Microscope BX53, Olympus Life Science) at 100x magnification. The cells that invaded the inserts were counted and results statistically analyzed.

Colony forming assay

Cells were placed seeded into 6-well ultra-low plates (Ultra-Low Attachment Multiple Well Plate, Corning® Costar®) at a density of $2x10^4$ cells/well in 2 ml culture medium supplemented with 10% of FBS and incubated for 5 days. The colonies formed

were counted and photo documented in an inverted microscope at 40x magnification. The procedure was performed in triplicates.

Cell viability assay

Cell viability was determined using the MTS cell proliferation kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay) after treating the cells with 0.06 mg/ml of Cetuximab, 0.05 mg/ml of Paclitaxel, or Cetuximab combined with Paclitaxel (CP), and untreated cells as a control. The therapeutic agent concentrations were chosen based in clinical treatments. Thus, $5x10^3$ cells were resuspended in 100 µl of DMEM with no supplementation and were placed into 96-well plates. Twenty microliters of MTS were added after 24h and absorbance measured with an ELISA plate reader (Multiskan FC, Thermo Scientific – Uniscience) at 490 nm filter.

Gene expression

Total RNA was extracted from $1x10^6$ cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA quantification was performed with the Qubit™ RNA HS Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies'). Complementary DNA (cDNA) for all samples was synthesized in a 20 µl reaction tube containing 2-5 µg of total RNA, using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The RNA concentration was 1 µg/µL. Quantification of the *EGFR* and *KRAS* gene expression was carried out in duplicates using the TaqMan Universal Master Mix and probes (Life Technologies). Two endogenous controls, Beta Actin (MUC1) and GAPDH (FAM dye and MGB probe) were included. The relative expression of *EGFR* and *KRAS* was calculated through the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The Kolmogorov–Smirnov test was used to assess normality. Independent t-test and ANOVA Bonferroni post hoc tests were used to calculate the significance between both groups. All data were evaluated with the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). A significance level of 5% was used.

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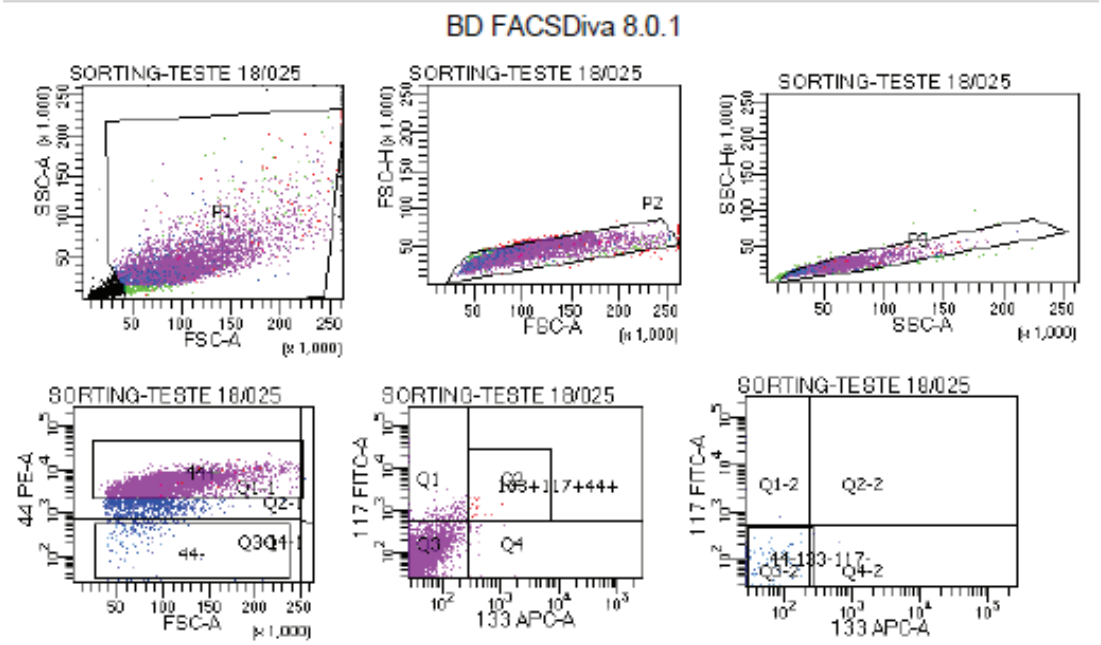
Table 1: Clinical features and surgical staging characterization of the group of patients with head and neck cancer.

Sample	Age	Gender	Smoking Habit	Exposure/Time	Alcoholic Habit	Exposure/Time	Primary Site	Staging (TNM*)	Labeling
HNC-1	72	Male	Yes	≥1 pack/≥ 15 years	Yes	≥400ml/≥35anos	Oral Cavity	T2N0M0	CD44/CD117/CD1
HNC-2	57	Male	Yes	≥1 pack/≥ 15 years	Yes	≥400ml/≥35anos	Pharynx	T1N0M0	CD44/CD117/CD1
HNC-3	44	Male	Yes	≥1 pack/≥ 15 years	Yes	≥400ml/≥35anos	Oral Cavity	T4N0M0	CD44/CD117/CD1
HNC-4	68	Male	Yes	≥1 pack/≥ 15 years	No	Never	Larynx	T1N0M0	CD44/CD117/CD1
HNC-5	48	Female	No	Never	No	Never	Oral Cavity	T2N0M0	CD44/CD117/CD1
HNC-6	71	Male	Yes	≥1 pack/≥ 15 years	No	Never	Larynx	T3N0M0	ALDH

TNM* = T: size tumor; N: lymph nodes affected; M: presence of metatasis



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Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	5,713	57.1	57.1
P2	5,575	97.6	55.8
P3	5,235	93.9	52.3
Q1-1	5,035	96.2	50.3
Q2-1	5	0.1	0.0
Q3-1	195	3.7	2.0
Q4-1	0	0.0	0.0
44+	4,291	82.0	42.9
133+117+44+	16	0.4	0.2
Q1	129	3.0	1.3
Q2	17	0.4	0.2
Q3	4.126	96.2	41.3
Q4	19	0.4	0.2
44-	118	2.3	1.2
Q1-2	4	3.4	0.0
Q2-2	0	0.0	0.0
Q3-2	111	94.1	1.1
Q4-2	3	2.5	0.0
44-133-117-	60	50.8	0.6

Figure 1: Graph example of cell sorting with triple positively marked with the three antibodies together were classified as HNCSCs (CD44+/CD133+/CD117+,). Cells that were negative for labeling with the three antibodies were considered non-HNCSCs (CD44-/CD133-/CD117-).

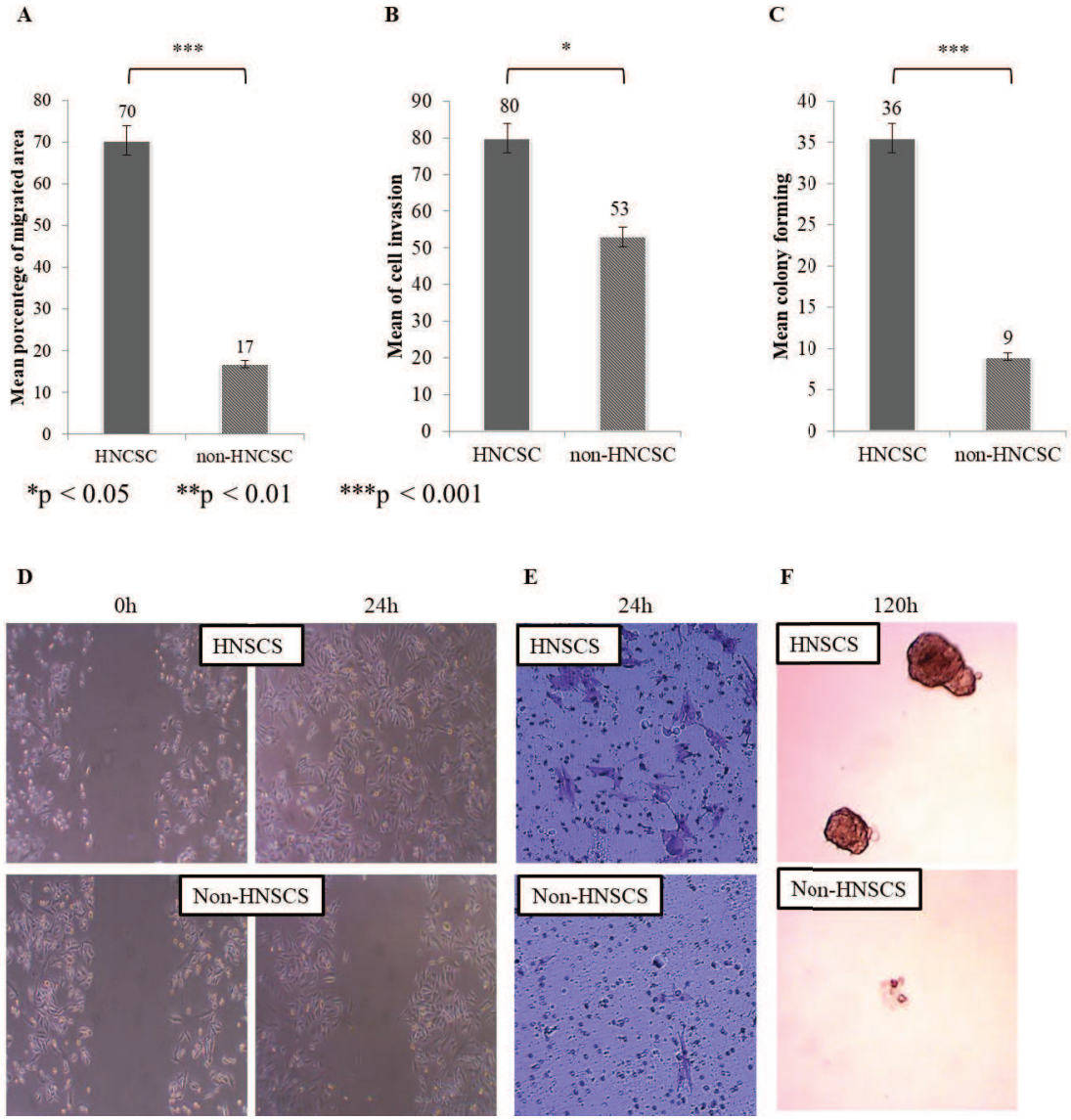
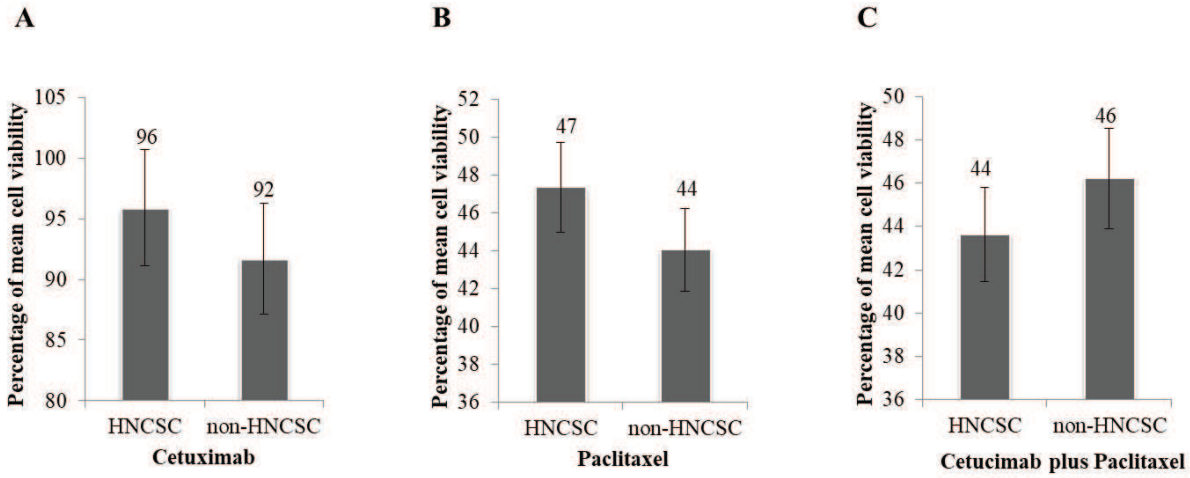
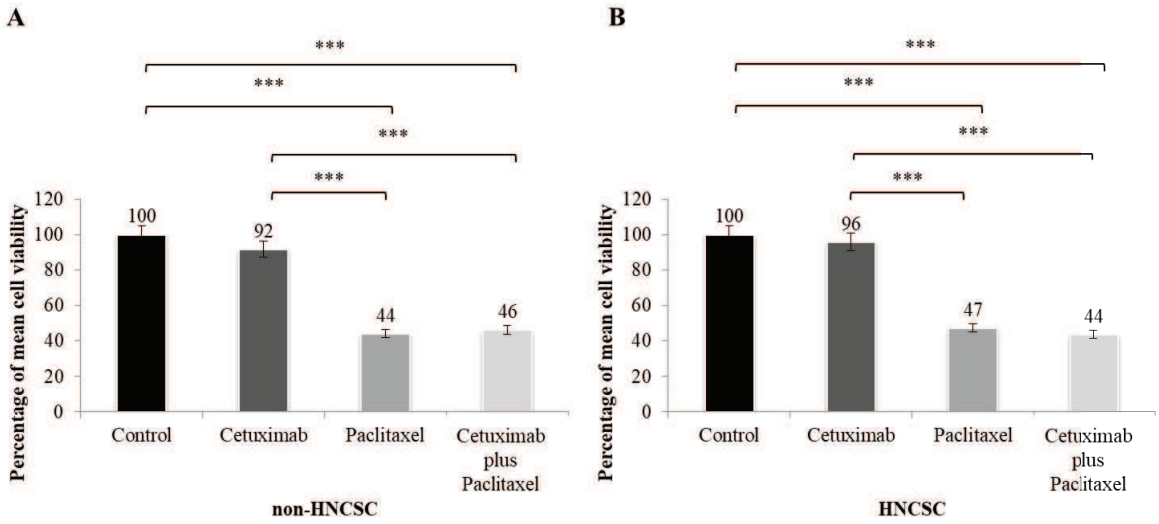


Figure 2: Graph comparing the HNCSC and non-HNCSC subpopulations. It shows statistical analysis data (ANOVA) for: A) migration performed; B) invasion and C) colony formation tests performed in triplicate. Image samples of primary tumors comparing the HNCSC and non-HNCSC subpopulations. For the tests: D) cell migration 0h and after 24h in 40x magnification; E) cell invasion after 24h in 200x magnification and F) colony forming after 120h in 40x magnification.



*p < 0.05

Figure 3: Cell viability of HNCSCs and Non-HNCSCs subpopulations of primary tumors treated with A) Cetuximab (p>0.05), B) Paclitaxel (p>0.05) and C) Cetuximab plus Paclitaxel combination (p>0.05), after 24 hours.



*p < 0.05 **p < 0.01 ***p < 0.001

Figure 4: Comparison of responses to Cetuximab, Paclitacel, Cetuximab plus Paclitaxel combination and controls regarding to untreated cell in HNCSCs and Non-HNCSCs subpopulations of primary tumors. A) Data and p values of the comparisons between treatments for the LCSC are presented. B) The data and p values of the comparisons between treatments for the non-LCSC are presented.

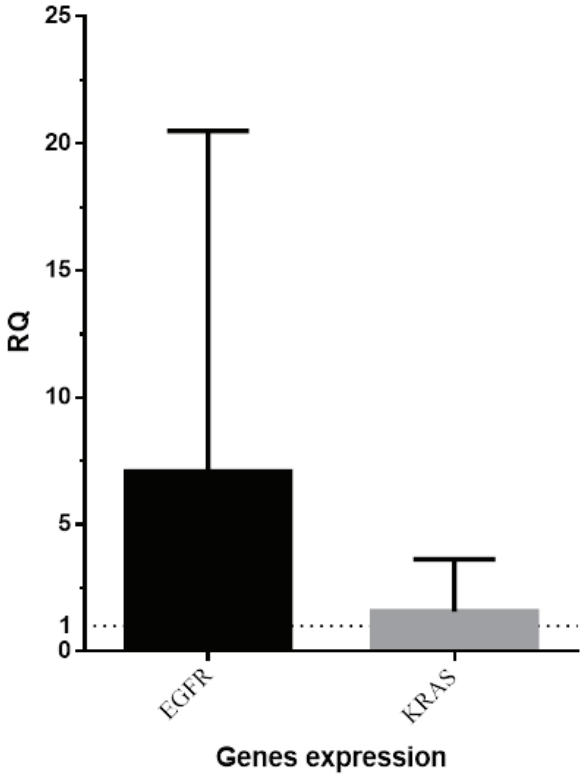


Figure 5: Graph showing the relative values of the differential expression and the values of the Wilcoxon Signed Rank Test and One sample t test, respectively, of the *EGFR* and *KRAS* genes, respectively.

Supplementary Material

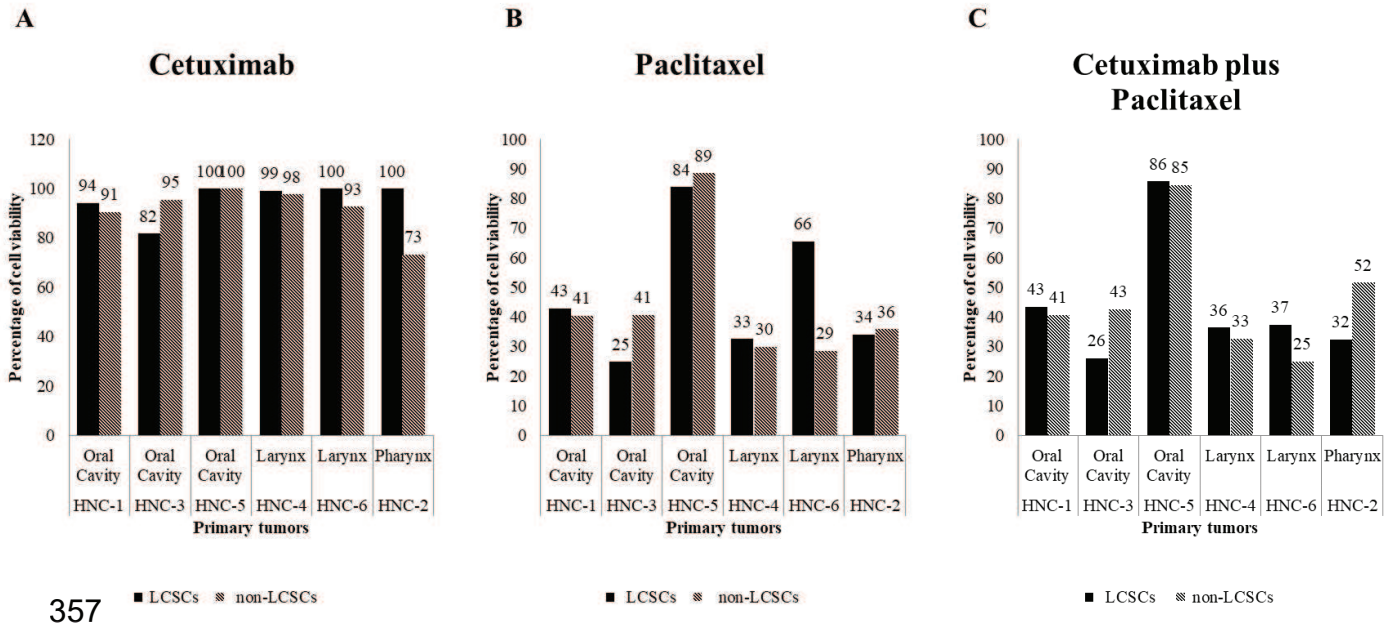


Figure Suplementary 1: Cell viability of HNCSCs and Non-HNCSCs subpopulations of primary tumors separated by tumor site, treated with A)

Cetuximab, B) Paclitaxel and C) Cetuximab plus Paclitaxel combination, after 24 hours.

Author contributions GMMF and ALSG-D designed the experiments; GMMF analyzed data, prepared igures, and wrote the manuscript. GMMF, ALSG-D, LAMF, VSJ, CIC, and MASB performed the experiments. GMMF, ALSG-D, MMUC-N, RSK-O, JVM, ECP, and EMG-B critically readthe manuscript, provided intellectual insights, and critically discussed the project.

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4. CONCLUSÕES

4. CONCLUSÕES

1. Os biomarcadores moleculares CD44, CD133 e CD117 em combinação e o ALDH isolado são eficientes em identificar e separar células tronco tumorais em câncer de cabeça e pescoço;

2. As células tronco tumorais identificadas e separadas por meio dos biomarcadores CD44, CD133 e CD117 em combinação e o ALDH isolado mostram maior potencial tumorigênico evidenciado pelo aumento da migração e invasão celular e pela formação de colônias. As células tronco tumorais da linhagem celular HEP2 é resistente ao tratamento com 5-fluorouracil, entretanto é sensível ao tratamento com a cisplatina. Nos tumores primários bem como nas linhagens celulares HEP2 e HN13 de câncer de cabeça e pescoço, as células tronco tumorais mostram habilidade de resistir ao Paclitaxel e Cetuximab. Enquanto que a combinação do Cetuximab com o Paclitaxel é mais eficaz em eliminar a subpopulação células tronco tumorais que os outros tratamentos na linhagem celular HEP2 e nos tumores primários.

3. As células tronco tumorais apresentam alta expressão do gene CD44 e subexpressão do EGFR na linhagem celular HN13. A HEP2 apresenta subexpressão *CD44*, superexpressão do *EGFR* e *KRAS* e não expressa o gene *TrkB*. Nos tumores primários de câncer de cabeça e pescoço, os genes *EGFR* e *KRAS* estão superexpressos.

Sabe-se que no Brasil, ainda há poucos estudos relacionados com células-tronco tumorais em câncer de cabeça e pescoço bem como relacionados à resposta de tratamento com os quimioterápicos, o que reforça a importância deste estudo. Os ensaios moleculares *in vitro* realizados nesta pesquisa mostram a importância da identificação das CTTs na escolha de tratamento da doença, tal como na identificação farmacogenética de quimioterápicos por meio da detecção de biomarcadores

relacionados às CTTs. Assim, os pacientes estarão melhor caracterizados e tratados de maneira uniforme e sistematicamente avaliados para resposta à quimioterapia. Dessa forma, contribui-se para identificação de subgrupos de pacientes que são predispostos a melhores respostas clínicas a drogas específicas e combinadas, com menor toxicidade, proporcionando novos protocolos de tratamento.

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6.ANEXOS

FACULDADE DE MEDICINA DE SÃO JOSE DO RIO PRETO- FAMERP - SP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Identificação de células tronco tumorais em câncer de cabeça e pescoço: Expressão gênica, quantificação de proteínas e resposta a quimioterapia

Pesquisador: Ana Lívia Silva Galbiatti

Área Temática:

Versão: 1

CAAE: 37632114.9.0000.5415

Instituição Proponente: Faculdade de Medicina de São Jose do Rio Preto- FAMERP - SP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 903.775

Data da Relatoria: 08/12/2014

Apresentação do Projeto:

O entendimento da biologia das CTT poderá auxiliar em tratamentos mais eficazes para a doença, bem como a não formação de metástase.

Estudos associados à resposta clinica são necessários para obter-se uma combinação de fatores prognósticos com parâmetros moleculares,

podendo assim trazer benefícios aos pacientes e novas informações sobre estimativa de prognóstico e decisão de tratamentos. Espera-se com os

resultados a serem obtidos com este projeto, trazer novas informações a respeito da identificação de CTT e a resposta de tratamento com os

quimioterápicos utilizados na terapia do CCP. Espera-se concluir, através dos ensaios moleculares in vitro propostos neste estudo, a importância da

identificação das CTT na escolha de tratamento da doença, bem como a identificação fármacogenética de quimioterápicos através da detecção da

expressão de genes relacionados às CTT para fornecer novos protocolos de tratamento onde os pacientes estão bem caracterizados e tratados de

maneira uniforme e sistematicamente avaliados para resposta a quimioterapia e, assim, podem ser uteis para identificação de subgrupos de

Endereço: BRIGADEIRO FARIA LIMA, 5416
Bairro: VILA SAO JOAO **CEP:** 15.090-000
UF: SP **Município:** SAO JOSE DO RIO PRETO
Telefone: (17)3201-5813 **Fax:** (17)3201-5813 **E-mail:** cepfamerp@famerp.br

pacientes que são predispostos a ter melhores respostas clínicas a drogas específicas com menor toxicidade.

Objetivo da Pesquisa:

Objetivo Primário:

Identificar a presença de CTT em tumores de cabeça e pescoço e em linhagens celulares, verificar a eficácia dos quimioterápicos utilizados no tratamento do câncer de cabeça e pescoço na eliminação das CTT em diferentes condições de tempo de exposição, analisar quantitativamente a expressão de RNAm de genes relacionados a presença das CTT: CD44, CD133, CD117 e ALDH1 e verificar a associação da expressão dos RNAm com o nível de agressividade do tumor.

Objetivo Secundário:

- identificar CTT em tumores de cabeça e pescoço de pacientes submetidos à cirurgia - Identificar CTT em duas linhagens celulares de cabeça e pescoço (HEP-2 – carcinoma de laringe e HN13- carcinoma de cavidade oral); - Aplicar os quimioterápicos

utilizados no tratamento do câncer de cabeça e pescoço nas CTT e verificar a viabilidade celular em relação ao tempo de exposição ao

quimioterápico - Comparar o grau de resistência das CTT sem a aplicação dos quimioterápicos com as CTT expostas aos quimioterápicos utilizados em tratamento de câncer de cabeça e pescoço - Avaliar a expressão dos genes CD44, CD133, CD117 e ALDH1 relacionados com CTT em tumores de cabeça e pescoço e nas linhagens celulares de acordo com o tempo de exposição - Quantificar a expressão de RNAm de proteínas dos genes

CD44, CD133, CD117 e ALDH1 em células tratadas com os quimioterápicos e correlacionar com a expressão gênica. - Verificar a associação da expressão dos genes CD44, CD133, CD117 e ALDH1com o nível de agressividade do tumor.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos são mínimos, uma vez que as amostras a serem obtidas serão retiradas no momento da cirugia do paciente.

Benefícios:

Descoberta de células tronco tumorais e avaliação da resposta de quimioterápicos a essas células. Além de avaliar a expressão genética de

marcadores relacionados a presença de células tronco tumorais e verificar associação com a resposta a quimioterápicos utilizados no tratamento do câncer de cabeça e pescoço. Os resultados poderão contribuir para novas estratégias terapêuticas que possam diminuir a morbidade e

FACULDADE DE MEDICINA DE SÃO JOSE DO RIO PRETO- FAMERP - SP

Continuação do Parecer: 903.775

mortalidade desta doença através de terapias que eliminem as CTT.

Comentários e Considerações sobre a Pesquisa:

Da apreciação geral, a proposta de pesquisa é muito boa. O projeto é pertinente aos seus objetivos.Os métodos são atuais, e bem fundamentado.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos obrigatório (folha de rosto e TCLE) foram preenchidos adequadamente.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

projeto aprovado Deve ser solicitado:

1. Substituição da palavra banco de amostra por biorepositório no termo de consentimento.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Projeto Aprovado.

SAO JOSE DO RIO PRETO, 09 de Dezembro de 2014

Assinado por:
LUCIANO GARCIA LOURENCAO
(Coordenador)

Endereço: BRIGADEIRO FARIA LIMA, 5416
Bairro: VILA SAO JOAO **CEP:** 15.090-000
UF: SP **Município:** SAO JOSE DO RIO PRETO
Telefone: (17)3201-5813 **Fax:** (17)3201-5813 **E-mail:** cepfamerp@famerp.br

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(Conselho Nacional de Saúde, resolução 466/12)

Título da Pesquisa: **Identificação de células tronco tumorais em câncer de cabeça e pescoço: Expressão gênica, quantificação de proteínas e resposta a quimioterapia**

Pesquisadora Responsável: Profa. Dra. Ana Lívia Silva Galbiatti

Eu ..... RG ..... nascido(a) em ...../...../..... e domiciliado(a) à ....., município de .... usuário (a) (ou responsável legal pelo usuário) ...., declaro que consinto em participar como voluntário (a) do projeto de pesquisa "**Identificação de células tronco tumorais em câncer de cabeça e pescoço: Expressão gênica, quantificação de proteínas e resposta a quimioterapia.**" e que também fui satisfatoriamente esclarecido(a) que:

A) Os objetivos do estudo são: investigar a presença de células-tronco tumorais em amostras de câncer de cabeça e pescoço, após cultivo celular; a eficácia dos quimioterápicos utilizados no tratamento do câncer de cabeça e pescoço na eliminação das células tronco tumorais em diferentes condições de tempo de exposição; analisar quantitativamente a expressão de RNAm de genes relacionados a presença das células tronco tumorais e verificar a associação da expressão dos RNAm com o nível de agressividade do tumor.

B) Durante a cirurgia, o médico irá remover o tumor e um pedaço dele não será usado para diagnóstico e poderá ser congelado e armazenado no laboratório para posteriormente ser utilizado para estudo genético/molecular compondo um banco de amostras biológicas, podendo ser utilizado em futuros estudos após aprovação de um novo projeto pelo Comitê de Ética em Pesquisa - CEP. A obtenção deste fragmento não implicará em riscos adicionais na sua cirurgia e não resultará em aumento no tempo de operação ou na extensão da cirurgia;

C) O material será identificado no laboratório por código formado por números e letras e, portanto, minha privacidade e identidade serão preservadas;

D) Todas as informações obtidas por meio da história clínica e os resultados serão mantidos em sigilo e que, estes só serão utilizados para divulgação em reuniões e revistas científicas;

E) Se eu concordar em participar desta pesquisa e se eu concordar com a retirada e uso do material, do modo descrito acima, não terei quaisquer benefícios ou direitos financeiros sobre os eventuais resultados decorrentes da pesquisa. Se eu não concordar, ou decidir retirar meu consentimento em qualquer momento, minha decisão não influenciará, de modo algum, o meu tratamento;

F) Esse estudo é importante porque pode colaborar para o conhecimento científico dos mecanismos envolvidos no desenvolvimento do tumor e auxiliar na identificação de marcadores para as células-tronco tumorais;

G) Os resultados serão divulgados após a conclusão do estudo em forma de artigos científicos ou trabalhos apresentados em congressos.

Declaro que, após ter convenientemente esclarecido pelo pesquisador, consinto em participar livre e espontaneamente deste estudo sem que tenha sido submetido a qualquer tipo de pressão. Assim, consinto em participar do projeto de pesquisa em questão.

RG do prontuário médico:
Data: ...../...../..... Assinatura:.....

—

Declaração de responsabilidade: Expliquei a natureza, objetivos, riscos e benefícios deste estudo. Coloquei-me a disposição para perguntas e respondi a todas. Obtive o consentimento de maneira livre e me coloquei à disposição para esclarecimento de qualquer dúvida sobre o estudo pelo endereço abaixo indicado.

Pesquisador responsável:
Data: ...../...../..... Assinatura:.....

Profa. Dra. Ana Lívia Silva Galbiatti – Unidade de Pesquisa em Genética e Biologia Molecular- UPGEM
Av. Brigadeiro Faria Lima, no. 5416
São José do Rio Preto, SP - CEP 15090-000 Fone: (17) 3201-5906 e-mail: analivia_sg@yahoo.com.br

Em caso de dúvidas contatar a secretaria do Comitê de Ética em Pesquisa da Faculdade de Medicina de São José do Rio Preto, telefone: (0xx17)3201-5700, ramal 5813