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AVALIAÇÃO DE MARCADORES MOLECULARES RELACIONADOS AO PROGNÓSTICO DE PACIENTES COM CARCINOMA EPIDERMÓIDE DE CAVIDADE ORAL E OROFARINGE

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Avaliação de marcadores moleculares relacionados ao prognóstico de pacientes com carcinoma epidermóide de cavidade oral e orofaringe

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO) do Ponto focal da Universidade Federal do Espírito Santo, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

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"Independentemente das circunstâncias,

devemos ser sempre humildes,

recatados e despidos de orgulho."

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RESUMO

O câncer de cabeça e pescoço é o quarto em incidência e o quinto em mortalidade na lista das neoplasias mais frequentes no mundo. Para o ano de 2014, são estimados pouco mais de 15 mil novos casos de câncer oral e de orofaringe no Brasil.

Assim como outros tumores, o câncer oral e de orofaringe é uma doença multifatorial decorrente de fatores ambientais e genéticos, envolvendo diversas alterações em mecanismos moleculares importantes para a homeostase celular.

A investigação de marcadores moleculares envolvidos nesses mecanismos tem sido o objeto de estudo de muitos grupos de pesquisa, uma vez que, apesar do crescente avanço em técnicas terapêuticas, a sobrevida desses pacientes pouco tem aumentado nas ultimas décadas.

É sabido que a progressão do tumor depende de que suas células adquirem algumas competências, como por exemplo, evasão da apoptose mediada pelo sistema imunológico, disfunção no controle da proliferação celular, proliferação celular facilitada pela ativação angiogênica, adaptações celulares como resposta à hipóxia tumoral, ativação do mecanismo de sobrevivência celular e modificações epigenéticas dependentes de hipóxia.

Considerando sua atuação nesses mecanismos, o presente trabalho teve o objetivo de avaliar o potencial das proteínas FAS, FASL, FGFR4, LEPR, HIF1-a, NDRG1 e JMJD1a e os polimorfismos Gly388Arg no gene FGFR4 e Gln223Arg no gene LEPR, como possíveis marcadores moleculares para as características clinicopatológicas e o prognóstico de pacientes com o carcinoma epidermóide oral e de orofaringe.

Nossos resultados mostraram a expressão HIF1-alpha relacionada com a recidiva local da doença e sobrevida livre de doença local nos pacientes submetidos à radioterapia pós-operatória, sendo também relacionada com a microdensidade vascular tumoral.

Adicionalmente, a expressão NDRG1 foi diferente quando comparadas as amostras de tecido tumoral e margem cirurgica não tumoral, também mostrando relação com a sobrevida da doença.

Com relação ao FGFR4, a expressão e o polimorfismo Gly388Arg mostraram relação com a ocorrência do óbito e com a sobrevida da doença. Contudo, apenas a expressão FGFR4

mostrou relação com com a metástase linfonodal e a ocorrência de recidiva. O perfil FGFR4 proposto mostrou relação com a sobrevida da doença.

Sobre o sistema FAS/FASL, ambas expressões mostraram relação com a ocorrência do óbito e o perfil FAS/FASL proposto foi significantemente relacionado com a sobrevida da doença.

O polimorfismo Gln223Arg no gene LEPR mostrou relação com a sobrevida livre de doença e da doença específica, enquanto que a expressão LEPR mostrou relação com a metástase linfonodal.

Á respeito da proteína JMJD1A, tanto a expressão nuclear quanto a citoplasmática mostraram relação com a metastase linfonodal. Contudo, apenas a expressão nuclear JMJD1A mostrou relação com a ocorrência de recidiva e com a sobrevida da doença.

Em conclusão, nossos resultados sugerem que as proteínas e polimorfismos avaliados podem ser utilizados como marcadores moleculares para auxiliar na predição prognóstica de pacientes com carcinoma epidermóide oral e de orofaringe.

Palavras chaves: Câncer cabeça e pescoço; prognóstico; apoptose; proliferação celular; hipóxia; angiogênese.

ABSTRACT

Head and neck cancer is the fourth in incidence and the fifth in mortality among the most frequent malignancies worldwide. For 2014, there has been estimated over 15 thousand new oral and oropharynx cancers in Brasil.

Similar to other tumors, oral and oropharynx cancer is a multifactorial disease, caused by multiple environmental and genetic factors, involving alterations in molecular pathways and cellular homeostasis.

The investigation of molecular markers involved in this process has been the object of my studies, especially because in spite of great advances in the molecular aspects of cancer, little progress has been made in the clinical outcome during the last decades.

It is known that tumor progression depends on cellular aquisition of competences, such as apoptosis evasion, cell proliferation disregulation, angiogenic activation, cellular adaptation to hipoxia, cell survival mechanism activation and hypoxia dependent epigenetic changes.

Therefore, the present work had the purpose to evaluate the potential of proteins FAS, FASL, FGFR4, LEPR, HIF1-a, NDRG1 and JMJD1a, as well as polymorphisms FGFR4 Gly388Arg and LEPR Gln223Arg, as putative molecular markers for clinicopathological tumor features or oral and oropharynx squamous cell carcinoma.

Our results show that HIF1-1 expression was associated with local disease relapse and local disease-free survival in patients who undertook pos-operative radiotherapy and were also related to tumor vascular microdensity.

In addition, NDRG1 expression was different in tumor tissue and non tumoral margins, but also showing an association with disease survival.

FGFR4 polymorphism and expression showed a relation with death and disease survival. However, FGFR4 expression alone showed an association with lymph node metastasis and relapse. FGFR4 profile showed a relation with disease survival.

FAS/FASL expression showed a correlation with death and its proposed profise was related with disease survival.

LEPR Gln223Arg polymorphism was realted with disease free survival and disease specific survival, whereas LEPR expression was realted with lymph node metastasis.

JMJD1A nuclear and cytoplasmic expression were related with lymph node metastasis. However, only nuclear expression was related with relapse and disease survival.

In conclusion, our results suggest that proteins ans polymorphisms can be used as molecular markers to help predict prognosis in oral and oropharynx squamous cell carcinoma.

Keywords: Head and neck cancer; prognosis; apoptosis; cell proliferation; hypoxia; angiogenesis.

LISTA DE SIGLAS E ABREVIATURAS

A - Adenina

ARD1 - *Acetyltransferase Arrest-defective-1*

Arg - Arginina

ARNT - *Aryl Hydrocarbon Receptor Nuclear Translocator*

bHLH-PAS - *basic Helix-Loop-Helix-Per-ARNT-Sim*

CD178 - Nomenclatura alternativa para FASL

CD95 - Nomenclatura alternativa para FAS

C-TAD - C-terminal

DNA - *Deoxyribonucleic Acid* (em português, Ácido desoxirribonucleico)

FADD - *FAS-associated Death Domain*

FAS - *Fas Cell Surface Death Receptor*

FASL - *Fas Cell Surface Death Receptor Ligand*

Fe - Ferro

FGF - *Fibroblast growth factor*

FGFR - *Fibroblast Growth Factor Receptor*

G - Guanina

Gln - Glutamina

Gln223Arg - Substituição de uma glutamina por uma arginina no aminoácido 223

Gly - Glicina

Gly388Arg - Substituição de uma glicina por uma arginina no aminoácido 388

H3K9me1 - Lisinas 9 das Histonas H3 monometilada

H3K9me2 - Lisinas 9 das Histonas H3 dimetilada

HIF - *Hypoxia-inducible factor*

HIF1-a - *Hypoxia-inducible factor 1-alpha*

HPV - *Human Papillomavirus*

HRE - *Hypoxia Response Element*

JAK - *Janus Kinase*

JMJD1A - *JMJ C-domain-containing histone demethylase 1A*

LEPR - *Leptin Receptor*

Lys - Lisina

MMP-9 - *Matriz Metalloproteinase-9*

NDRG1 - *N-myc downstream regulated gene 1*

N-TAD - N-terminal

ODD - *Oxygen-regulated Degradation*

p53 - *Protein 53* (em português, proteína 53)

PHD - *Prolyl Hydroxylation*

pVHL - *protein von Hippel-Lindau*

SIRT1 - *Sirtuin 1*

SNP - *Single Nucleotide* Polymorphism

STAT - *Signal Transducer and Activator of Transcription*

TNM - Tumor, Linfonodo e Metástase

SUMÁRIO

1. INTRODUÇÃO

O carcinoma epidermóide de cabeça e pescoço é uma causa significativa de morbidade e mortalidade em todo o mundo, com aproximadamente 780.000 casos e 391.000 mortes por ano, sua taxa de mortalidade atinge 50% dos casos em 5 anos [1,2].

A doença diagnosticada em seu estágio inicial tem expectativa de sobrevida livre da doença variando entre 60 a 90%. Nos casos restantes, quando a doença é detectada em estágio avançado, cerca de 50% dos casos têm tumores potencialmente ressecáveis com chance de sobrevida variando em 40-50%. Nos casos avançados e não ressecáveis, o prognóstico é pior, com uma taxa de sobrevida variando de 10 a 40% em 5 anos [3-5].

O câncer de cabeça pescoço é uma doença multifatorial, decorrente de fatores ambientais e genéticos. A maioria das alterações genéticas e epigenéticas importantes para o desenvolvimento do câncer ocorrem em genes atuantes em vias de sinalização [6-9].

O comportamento tumoral, assim como seu prognóstico, pode ser definido molecularmente pelo quantitativo e qualitativo destas alterações, incluindo um potencial ilimitado de replicação, sinalização da proliferação autossuficiente, habilidade de evadir à apoptose, angiogênese, ineficiente reparo do DNA, invasão e metástase [10].

A agressividade da doença está diretamente relacionada com a capacidade de adaptação da célula tumoral às condições desfavoráveis, ao crescimento da massa tumoral, tais como escape da vigília imunológica e sobrevivência em condições de hipóxia, além de migração para outras regiões do corpo no desenvolvimento de metástases [11,12].

A maior precisão prognóstica do carcinoma epidermóide oral e de orofaringe pode ser realizada através de testes moleculares com expectativa de aumento da sobrevida dos pacientes em 80%. Estes dados confirmam a importância da investigação do potencial de marcadores moleculares para a doença [13,14].

Pesquisa envolvendo expressão e polimorfismos de genes que fazem parte de vias metabólicas complexas abre perspectiva de melhor compreensão da doença e de novos alvos terapêuticos. Dessa forma, o trabalho teve o objetivo de avaliar o potencial das proteínas *Fas*

Cell Surface Death Receptor (FAS), *Fas Cell Surface Death Receptor Ligand* (FASL), *Fibroblast growth factor receptor 4* (FGFR4), *Leptin Receptor* (LEPR), *Hypoxia-inducible factor 1-alpha* (HIF1-a), *N-myc downstream regulated gene 1* (NDRG1) e *JMJ C-domain-containing histone demethylase 1A* (JMJD1A) e os polimorfismos Gly388Arg no gene FGFR4 e Gln223Arg no gene LEPR, como possíveis marcadores moleculares para as características clinicopatológicas e o prognóstico de pacientes com o carcinoma epidermóide oral e de orofaringe.

A presente tese será apresentada na seguinte forma: Introdução, com a contextualização, importância e objetivos; Revisão da literatura e as respectivas referências bibliográficas. A metodologia, bem como os resultados e as discussões serão apresentados na forma de artigos que foram publicados ou submetidos para periódicos.

2. REVISÃO BIBLIOGRÁFICA

2.1. Carcinoma Epidermóide Oral e de Orofaringe

O câncer de cabeça e pescoço é o quarto em incidência e o quinto em mortalidade na lista das neoplasias mais frequentes no mundo [1], sendo a sobrevida de 5 anos alcançada por apenas 30% dos pacientes com o câncer oral ou de orofaringe [5].

No Brasil, estimam-se para que no ano de 2014 sejam detectados 11.280 novos casos de câncer oral em homens e 4.010 em mulheres. Tais valores correspondem a um risco estimado de 11,54 casos novos a cada 100 mil homens e 3,92 a cada 100 mil mulheres [15]. O carcinoma epidermóide é o tipo histológico predominante, ocorrendo em mais de 90% dos casos [16].

Do ponto de vista epidemiológico, sabe-se que existe uma associação entre o consumo de tabaco e o aparecimento de tumores na cavidade oral e orofaringe. O risco de desenvolvimento do câncer é de até nove vezes maior em indivíduos tabagistas, do que em não tabagistas, mantendo relação direta com a quantidade consumida [13,17]. O uso do álcool também é fator de risco para o desenvolvimento do câncer das vias aerodigestivas superiores, além de atuar como potencializador dos efeitos carcinogênicos do tabaco [18].

Além dos fatores de risco químicos, o carcinoma epidermóide oral e de orofaringe pode ser decorrente de infecção por *Human Papillomavirus* (HPV) ou de fatores de suscetibilidade genética [19]. Existe ainda a associação entre a condição socioeconômica do paciente e o aparecimento do tumor, essa associação pode ser explicada pela pobreza, alimentação deficiente e dificuldade de acesso ao serviço de saúde [20,21].

O tratamento do carcinoma epidermóide oral e de orofaringe é frequentemente baseado em uma estratégia de multimodalidade terapêutica, envolvendo cirurgia, quimioterapia e radioterapia. O planejamento terapêutico e prognóstico da doença é estabelecido com base nos parâmetros clínicos, radiológicos e histopatológicos, os quais

consistem no local do tumor primário e o sistema de classificação TNM (T - Tumor, N - Linfonodo, M - Metástase) [22,23].

Apesar dos avanços das técnicas cirúrgicas e do surgimento de novas abordagens terapêuticas, a sobrevida média dos pacientes em estágio avançado da doença não tem aumentado substancialmente nas últimas décadas. Pacientes portadores de carcinoma epidermóide oral e de orofaringe geralmente apresentam um padrão clínico heterogêneo, com um prognóstico ruim relacionado ao estágio avançado da doença. Enquanto o tratamento do tumor primário e da metástase regional permite um aumento da sobrevida, mais de 50% dos pacientes desenvolvem, em um período de cinco anos, recorrência da lesão primária ou metástases [24].

O tamanho do tumor primário, a presença de metástases linfonodais, margens positivas após excisão cirúrgica, têm sido indicadores de uma pior evolução clínica em pacientes com carcinoma epidermóide oral e de orofaringe. No entanto, pacientes com características clínicas semelhantes podem apresentar padrões diferentes de crescimento e evolução tumoral [25].

Muitos relatos mostram que as variações nos mecanismos moleculares da ativação apoptótica, estímulos da proliferação celular e na resposta às condições de hipóxia estão relacionadas com a agressividade e o prognóstico dos pacientes oncológicos [11,12]. Isto demonstra a necessidade da análise de outros fatores complementares capazes de nortear com maior precisão o tratamento e prognóstico da doença.

Desta forma estudos nos mecanismos de evasão da apoptose mediada pelo sistema imunológico (FAS/FASL), disfunção no controle da proliferação celular (FGFR4), proliferação celular facilitada pela ativação angiogênica (LEPR), adaptações celulares como resposta à hipóxia tumoral (HIF1-a), ativação do mecanismo de sobrevivência celular (NDRG1) e modificações epigenéticas dependentes de hipóxia (JMJD1A) podem oferecer caminhos para a descoberta de marcadores moleculares prognósticos.

2.2. Evasão da apoptose mediada pelo sistema imunológico: FAS/FASL

Múltiplos fatores são responsáveis pela modulação do crescimento tumoral e modulação do prognóstico de pacientes com tumores malignos. Acredita-se que o desequilíbrio entre os mecanismos de apoptose e proliferação seja o essencial para o desenvolvimento e prognóstico tumoral, podendo fornecer uma previsão mais realista do comportamento do tumor [26].

A apoptose é um programa de morte celular com função crucial no desenvolvimento e homeostase de organismo multicelulares. Este processo complexo envolve muitos genes, mutações e polimorfismos, podendo levar à sinalização de morte deficiente, potencializando a agressividade tumoral [27].

Algumas células tumorais podem adquirir com sucesso a habilidade de resistir ao estímulo apoptótico ou de induzir a apoptose de linfócitos tumor específico, favorecendo a progressão tumoral [28]. A capacidade de resistir aos estímulos apoptóticos é compartilhada por quase todos os tipos de doenças malignas. Modificações nos componentes da via apoptótica são associadas ao desenvolvimento do câncer, à agressividade tumoral e ao prognóstico dos pacientes [29,30].

A apoptose pode ser ativada por duas vias, a intrínseca, também conhecida como via mitocondrial, e a extrínseca, também conhecida por via citoplasmática. A via intrínseca ocorre através da presença de sinais de estresse intracelular, ativando o apoptossomo executor, com a liberação do Citocromo-c do espaço intramembranoso da mitocôndria para o citoplasma. A apoptose pela via extrínseca ocorre através de estímulos externos, por meio da ativação de receptores específicos presentes na membrana celular denominados receptores da morte. Ambas as vias culminam na ativação de proteases conhecidas como caspases executoras, que clivam o DNA nuclear levando a célula à morte [31-34].

Dentre os oito receptores pertencentes à família de proteínas transmembrânicas do tipo 1, o FAS, também conhecido como CD95, tem sido extensamente investigado pela sua atuação apoptótica, assim como seu ligante, o FASL, alternativamente denominado como CD178 [35,36].

Assim como os outros membros de sua família, o FAS é caracterizado por conter três domínios extracelulares ricos em cisteína, seguidos por uma região intracelular composta por uma sequência de 80 aminoácidos denominada domínio de morte. A molécula é funcional apenas depois de oligomerizada em trímero, ação dependente de uma sinalização extracelular desencadeada pelo seu ligante FASL [37].

O FASL é uma proteína de membrana tipo II e apresenta 281 resíduos de aminoácidos, sendo 150 presentes na sua região extracelular e 77 na região citoplasmática. O FASL também pode clivado por proteólise, tomando uma forma livre solúvel bioativa [38,39].

Logo após a ativação do receptor FAS pelo seu ligante FASL, o sinal apoptótico é transmitido por transdução de sinal através da molécula adaptadora intracelular *FAS-associated Death Domain* (FADD), que é recrutada por possuir complementação ao domínio de morte do receptor FAS. Esta fará a adaptação da forma zimogênica da cisteíno-aspartato protease (caspase-8) que sofrerá autoclivagem passando para sua forma ativa, dando início à cascata de ativação de diversas outras caspases. Por fim, esta cascata irá culminar na ativação da DNAse que irá fragmentar o DNA nuclear entre os nucleossomos, levando à modificações morfológicas e bioquímicas características da apoptose, com consequente morte celular [32,36,40].

Enquanto a expressão do FAS é uma característica comum da maioria dos tecidos não neoplásicos, a expressão do FASL é restrita às estruturas anatomicamente bem definidas. Além de células do sistema imune, como linfócitos T e células *Natural Killers*, a expressão FASL é constitutiva nas células de Sertoli nos testículos, células epiteliais da câmara anterior do olho e células de Kupffer dos sinusóides hepáticos. Estudos revelaram que a expressão do FASL nestes sítios confere um privilégio imunológico ao tecido, onde os linfócitos FAS positivos são rapidamente destruídos após ligação com FASL [38].

O sistema FAS/FASL já demonstrou desempenhar um papel importante na regulação da apoptose em células tumorais. Estudos em diversos tipos tumorais indicam que imunomodulações causadas pela alteração da expressão do FAS/FASL pode ter um impacto significante na sobrevida dos pacientes [40].

A perda ou redução da expressão FAS ocorre frequentemente e progressivamente no processo de progressão tumoral em seres humanos. A expressão FAS já foi relatada como diminuída em câncer colorretal, esôfago, mama, melanoma, hepático, gástrico e pulmão [39-43].

O baixo índice apoptótico é associado com baixa ou nula expressão FAS e consequente piora da sobrevida, sugerindo que a expressão tumoral FAS seja um fator biológico importante no controle da doença, mas que é perdido de acordo com a evolução tumoral [26].

Estudos tem demonstrado que o FASL pode ser expresso por vários tipos de tumores sólidos, tais como no câncer de mama [44,45], ovário [46], fígado [47,48], colorretal [49], melanoma [50] e em cabeça e pescoço [51,52], frequentemente sendo associado com o pior prognóstico.

Esses resultados sugerem que tumores FASL positivos podem induzir à supressão imunológica contra os linfócitos do infiltrado tumoral, representando o modelo de evasão imunológica tumoral denominada "contra-ataque" (do inglês, *counterattack*) [53]. Neste modelo, as células tumorais podem, através da expressão FASL, explorar a sinalização extrínseca da apoptose para se livrar das células linfocitárias FAS positivas, de modo a minimizar seu reconhecimento e combate pelo sistema imunológico [54].

Contudo, evidências controversas dificultam a compreensão clara do papel exercido pela expressão tumoral FASL, na qual está relacionada com a rejeição tumoral em modelos experimentais [55,56]. Colocando em dúvida a capacidade real de tumores malignos expressarem FASL [57].

Os conflitos de evidências sugerem que a função e expressão FASL podem ser diferentes entre os diversos tipos tumorais. No carcinoma epidermóide oral e de orofaringe, a relação entre as expressões FAS e FASL e seu prognóstico não é clara, tornando necessário este tipo de investigação.

2.3. Disfunção no controle da proliferação celular: FGFR4

O câncer é causado por uma série de modificações moleculares na maquinaria regulatória celular [58]. Assim como descrito anteriormente, o desequilíbrio entre apoptose e proliferação celular é o ponto chave para o desenvolvimento e progressão tumoral [26]. A perda do controle da proliferação celular pode ser decorrente da superexpressão ou variações genéticas em fatores críticos para o seu bom funcionamento [59].

Atuantes no controle da proliferação celular, os membros da família do Receptor do Fator de Crescimento de Fibroblasto (FGFR) estão divididos em duas principais classes. A primeira composta por FGFRs com baixa afinidade, que necessitam da ligação de moléculas de heparano sulfato proteoglicanos na superfície celular para acoplamento de seus ligantes. A segunda classe dos FGFRs são as de alta afinidade e englobam os FGFRs de 1 a 4. Cada subtipo pode ser diferentemente expresso gerando isoformas, ativadas por ligantes distintos, levando à dimerização e fosforilação de resíduo tirosino-quinase na porção citoplasmática da célula [11,60,61].

Ainda não completamente compreendidas, essas sinalizações são mediadoras de processos celulares importantes como proliferação, diferenciação, sobrevida e motilidade, ocorrendo principalmente durante o desenvolvimento embrionário. Em adição ao FGFR1 e FGFR3, o FGFR4 pode induzir a transformação celular, atuando como oncogenes. [62-64]. Estes receptores possuem um papel decisivo no desenvolvimento do câncer e estão envolvidos no crescimento da célula tumoral e sua migração [11].

Diversos estudos tem examinado o papel do FGFR4 na carcinogênese de diferentes tipos tumorais, provendo evidências da complexidade envolvida na via de sinalização FGF/FGFR, devido ao grande número de proteínas que interagem diretamente com o FGF e o FGFR4 [65-69].

No câncer de cabeça e pescoço, a investigação de 104 casos por imunohistoquímica apontou a expressão forte FGFR4 relacionada com o pior prognóstico, sugerindo que a maior quantidade de proteína FGFR4 pode levar à maior ativação para a proliferação das células tumorais [70].

Adicionalmente, trabalhos apontam para alterações genéticas nos genes FGFRs, associadas a diversos tumores. A maioria dos SNPs é silenciosa, porém, alguns estão diretamente relacionados com a progressão da doença. [61,70].

Assim como a expressão FGFR4 tem sido relacionada com diversos aspectos da progressão tumoral, a transição de uma Guanina por uma Adenina (G>A) que ocorre no gene FGFR4, tem sido associada com a progressão e prognóstico de várias neoplasias como sarcomas, mama, próstata, bexiga e de cabeça e pescoço [11,61,70-79].

Presente no éxon 9 do gene FGFR4, este polimorfismo resulta na substituição de uma glicina (Gly) por uma arginina (Arg) na posição 388 do domínio transmembrânico, localizada em uma região hidrofóbica e altamente conservada [59,80].

Contudo, o número de evidências que confirmam a importância da expressão FGFR4 e o do polimorfismo Gly399Arg na progressão tumoral e prognóstico é insuficiente para um consenso final. Adicionalmente, o mecanismo de sinalização FGF/FGFR pode ser variável entre os diversos tipos tumorais, tornando incerto o papel do FGFR4 no carcinoma epidermóide oral e de orofaringe.

2.4. Proliferação celular facilitada pela ativação angiogênica: LEPR

Para o crescimento de tumores sólidos é essencial a manutenção dos níveis de oxigênio e nutrientes para que a maquinaria celular esteja em constante funcionamento, sendo a falta destes um bloqueio em seu desenvolvimento. A proliferação celular tumoral pode ser facilitada por estímulos angiogênicos desencadeados pela ação do hormônio Leptina. Atuando no crescimento, proliferação e migração de células endoteliais, a Leptina tem sido associada com o surgimento de diversas neoplasias, tais como o câncer de mama, colorretal, fígado e próstata [81-83].

Esse hormônio atua como mitógeno em diversos tipos de células, como em células endoteliais, células musculares lisas, células normais e neoplásicas epiteliais. A Leptina atua também no processo de formação de vasos sanguíneos em tecidos normais e neoplásicos, atuando como promotora na expressão de genes que promove a angiogênese, resultando no aumento da vascularização endotelial [84-86].

A Leptina é um hormônio produzido predominantemente pelo tecido adiposo branco, responsável pela regulação da massa de tecido adiposo através dos efeitos hipotalâmicos da saciedade e gasto de energia [87,88]. Além da regulação da massa corpórea, a Leptina e seu receptor estão relacionados com os processos de hematopoiese, formação dos ossos, angiogênese, cicatrização, reprodução e resposta imunológica, dentre outras funções ainda em estudo [82,89,90].

A Leptina atua através de seu receptor, o LEPR. Um receptor transmembrânico da família de receptores de citocinas de classe 1, conhecido por estimular a transcrição gênica através da ativação citosólica de proteínas S*ignal Transducer and Activator of Transcription* (STAT) [88,91,92]. O LEPR é expresso principalmente no hipotálamo, sistema vascular humano e em culturas primárias de células endoteliais [93].

Quando a Leptina se liga com o seu receptor, ocorre uma mudança na conformação da molécula, levando a fosforilação cruzada de moléculas *Janus Kinase* (JAK) e consequente fosforilação das proteínas (STAT). Posteriormente, a STAT transloca-se para o núcleo, onde se liga na região promotora de genes alvos da ativação transcricional. Dentre os genes

ativados pela STAT, muitos são enzimas conhecidas por sua atuação no desenvolvimento e progressão de diversos tipos tumorais [90,94].

O LEPR tem sido frequentemente identificado em células malignas de diferentes origens, incluindo o câncer de mama, pulmão, gástrico e leucemias [95-98]. Algumas investigações têm relacionado a expressão LEPR com o desenvolvimento, diferenciação e prognóstico em câncer de mama e gástrico, sugerindo que sua atuação no controle da proliferação celular seja importante nestes aspectos [98-101].

A eficiente sinalização via Leptina/LEPR em tumores foi associada à expressão aumentada da *Matriz Metalloproteinase-9* (MMP-9). Por sua vez, a MMP-9 atua na degradação do colágeno tipo IV constituinte da membrana basal, processo relacionado com a invasão e metastatização tumoral, sugerindo seu impacto no prognóstico destas neoplasias [102].

Dentre os diversos polimorfismos descritos no gene LEPR, a transição de uma Adenina para uma Guanina leva à troca de uma Glutamina (Gln) para Arginina (Arg) no aminoácido 223. Localizado no domínio extracelular do receptor, esta troca de um aminoácido neutro por um aminoácido com carga positiva, altera a função e capacidade de sinalização do receptor [103,104].

O polimorfismo Gln223Arg no gene LEPR foi relacionado com o risco do câncer de mama, sendo o risco aumentado nos indivíduos homozigotos para o alelo Arg223 [98]. Resultados contrários foram apresentados em estudos com câncer de próstata, onde os indivíduos homozigotos para o alelo Gln223 possuem um maior risco de desenvolver esta neoplasia [105,106].

A primeira investigação deste polimorfismo no câncer de cabeça e pescoço confirma sua relação com o desenvolvimento da doença. O autor ainda sugere que seus achados reforçam o importante papel do LEPR na promoção do crescimento tumoral angiogênese, metástase e invasão tumoral [107].

Quando moléculas pró-angiogênicas tem sua expressão aumentada em relação às moléculas angiostáticas, o tumor adquire o fenótipo angiogênico que permite a formação de novos vasos sanguíneos [108].

A terapia tumoral antiangiogênica representa uma estratégia promissora para o tratamento do câncer e provavelmente exibirá potencial clínico conjuntamente com as terapias antitumorais clássicas [109]. No entanto, muitos dos mecanismos envolvidos neste processo devem ser mais bem esclarecidos de modo a ser utilizado no carcinoma epidermóide oral e de orofaringe.

2.5. Adaptações celulares como resposta à hipóxia tumoral: HIF1-a

O sistema de resposta celular às condições de hipóxia é essencial para a progressão de tumores sólidos [110,111], como por exemplo, os tumores de mama, pulmão, ovário e cabeça e pescoço [112-116].

A hipóxia é encontrada na maioria dos tumores sólidos devido às alterações estruturais e quantitativas nos vasos, levando a diminuição das concentrações de oxigênio [117]. Contudo, a hipóxia não é um mecanismo exclusivo da progressão tumoral [118], sendo também encontrada durante o desenvolvimento embrionário [119-121] e em doenças isquêmicas [122-126].

Para manter a homeostase do oxigênio, muitos organismos eucariotos adaptaram evolutivamente um mecanismo especializado para reforçar a ingestão e distribuição do oxigênio. Consequentemente, uma via responsiva dependente de oxigênio foi conservada e presente em quase todas as células de mamíferos [127,128].

O complexo transcricional *Hypoxia-inducible factor 1* (HIF1) é o principal mecanismo de regulação para a homeostase do oxigênio celular e sistêmico [119,127,129-137].

Quando ligado ao DNA genômico, o complexo HIF1 pode promover a transcrição de mais de 100 genes que atuam em mecanismos específicos para evitar a morte celular causada pela hipóxia. Por sua vez, as proteínas produzidas aumentam as concentrações de oxigênio através da angiogênese [138], além de enzimas do transporte de glicose e metabolismo [116,136].

O complexo HIF1 é um fator de transcrição composto por uma subunidade -alpha e uma -beta, a última também conhecida como *Aryl Hydrocarbon Receptor Nuclear Translocator* (ARNT), ambas pertencem a família de proteínas *basic Helix-Loop-Helix-Per-ARNT-Sim* (bHLH-PAS) [139,140].

A subunidade HIF1-beta é expressa constitutivamente, mantendo os seus níveis de mRNA e proteína constantes, independente das condições de oxigênio [141]. Igualmente, a transcrição e síntese protéica da HIF1-alpha são constitutivas e particularmente não são

afetadas pelo teor de oxigênio [139,141-144]. Contudo, sob normóxia, a HIF1-alpha é rapidamente degradada quando detectada [139].

Assim como as proteínas da família bHLH-PAS, a subunidade HIF1-alpha possui os dois domínios bHLH e PAS, necessários para a heterodimerização com a subunidade HIF1-beta [145]. Mais dois domínios presentes na HIF1-alpha, o N-terminal (N-TAD) e o C-terminal (C-TAD), localizados nas porções C-terminal e mediana da proteína [146].

Outro importante domínio presente na subunidade HIF1-alpha é o *Oxygen-regulated Degradation* (ODD – Degradação regulada por oxigênio), que media sua estabilidade de acordo com a disponibilidade de oxigênio [147]. Modificações pós-traducionais, como a hidroxilação, acetilação e ubiquitinação no domínio ODD da proteína HIF1-alpha é o primeiro passo de sua degradação [148-150].

Sob condições normais de oxigênio, a HIF1-alpha é hidroxilada pela proteína *Prolyl Hydroxylation* (PHD) em seu domínio ODD [134,135, 151-155]. Em seguida, a proteína *von Hippel-Lindau* (pVHL) reconhece a hidroxilação adicionando ubiquitina, marcando-a para a degradação proteossomal [133,156-162].

Alternativamente, a degradação da HIF1-alpha pode ser dada através da acetilação pela proteína *Acetyltransferase Arrest-defective-1* (ARD1), no resíduo de lisina 532 (Lys532) de seu domínio ODD, e consequente ubiquitinação e degradação proteossomal [135,151,163].

Sob condição de hipóxia, a subunidade HIF1-alpha é estabilizada e traslocada do citoplasma para o núcleo, onde dimeriza com a subunidade HIF1-beta, formando o complexo HIF1 trascricionalmente ativo [136,141,164].

Entre os genes regulados pela HIF1 importantes para o processo tumorigênico estão os fatores angiogênicos, de proliferação celular, transportadores de glicose, enzimas glicolíticas anaerobióticas [136,165,166]. O aumento da densidade vascular, a permeabilidade tecidual e, consequente diminuição da distância para a difusão do oxigênio são resultados da ativação angiogênica, favorecendo a progressão tumoral [161,167].

Em diversos tipos tumorais, a expressão da HIF1-alpha foi associada com o processo de carcinogênese e o prognóstico e sobrevida dos pacientes, como por exemplo, no câncer renal [168], bexiga [169], colorretal [170], mama [171,172], ovário [173] e útero [174,175].

No carcinoma epidermóide de cabeça e pescoço, os trabalhos apontam a expressão HIF1-alpha associada à carcinogênese e fatores prognósticos e de sobrevida [176-182], porém, sem um consenso entre os trabalhos. Dê um lado, a expressão HIF1-alpha fraca é relacionada com a pior sobrevida dos pacientes [177,183], enquanto que outros trabalhos sugerem que a pior sobrevida seja determinada pela expressão forte [176,184,185].

Os marcadores de hipóxia, como a proteína HIF1-alpha, podem auxiliar na estratificação dos pacientes em categorias, facilitando identificar a conduta terapêutica mais adequada para cada estrato, assim como predizer o prognóstico com maior precisão [186]. Deste modo, a expressão HIF1-alpha é um forte candidato à marcador molecular no carcinoma epidermóide oral e de orofaringe, principalmente pelo tratamento destas neoplasia visarem, em segundo plano, a preservação da estrutura e função de órgão essenciais para a qualidade de vida dos doentes [187].

2.6. Ativação do mecanismo de sobrevivência celular: NDRG1

A hipóxia e a indução da angiogênese são fatores cruciais para a progressão de tumores sólidos [110,111]. Nos mais de 100 genes conhecidos por serem alvos do complexo HIF, é comum a presença de Elementos Responsivos à Hipóxia (HRE - *Hypoxia Response Element*) em suas sequencias nucleotídicas [138].

Um gene que apresenta três motivos HRE em sua sequência e alvo do complexo HIF é o *N-myc downstream regulated gene 1* (NDRG1) [188,189]. A sequência do gene NDRG1 é altamente conservada [190] e está localizada na banda cromossômica 8q24 [191]. O gene codifica uma proteína com 394 aminoácidos e geralmente está presente no citoplasma e membrana de células epiteliais [192].

A proteína codificada pelo gene NDRG1 parece ter um papel no controle do ciclo celular, na diferenciação celular e no crescimento, na resposta hormonal e ao estresse, podendo atuar como uma proteína sinalizadora entre o citoplasma e o núcleo [193].

A expressão NDRG1 também pode ser regulada por outros fatores, como a presença de danos no DNA, níquel, androgênio e homocisteína, possivelmente atuando como supressor tumoral [194,195]. Estudos funcionais tem relatado que a expressão NDRG1, quando induzida por danos no DNA, está associada com a expressão da *Protein 53* (p53), sugerindo sua cumplicidade na indução da apoptose via p53 [196].

Outros trabalhos indicam ainda que a expressão do NDRG1 é regulada pela *Sirtuin 1* (SIRT1), uma proteína com função de promover a sobrevivência celular, com expressão aumentada em situações de hipóxia. Neste caso, aumentos nos níveis de NDRG1 reduzem a expressão de p53, levando à diferenciação de trofoblastos e diminuição da injúria celular [197].

O preciso papel do NDRG1 é desconhecido, mas sua proteína parece ser multifuncional, com participação em diversas vias moleculares [198]. Diversas evidências sugerem o envolvimento da proteína NDRG1 na proliferação e diferenciação celular, atuando no papel de supressor tumoral [199,200].

A expressão anormal NDRG1 tem sido observada em diferentes tipos de tumores, em algumas vezes associada com o estágio avançado da doença e o prognóstico [188,201-203].

Apesar das perspectivas dos resultados de pesquisas, o papel do gene NDRG1 no câncer ainda é incerto. Sua relação com a progressão tumoral e o prognóstico em diversos tipos tumorais sugere fortemente a atuação do NDRG1 como um mecanismo de sobrevivência celular [195], abrindo a possibilidade de considerar o NDRG1 como um potencial alvo de diagnóstico, prognóstico ou terapêutico no carcinoma epidermóide oral e de orofaringe.

2.7. Modificações epigenéticas dependentes de hipóxia: JMJD1A

O mecanismo de resposta da célula tumoral às condições de hipóxia envolve a ativação de mais de 100 genes [204]. Atualmente, pouco se sabe sobre a regulação epigenética durante a ativação transcricional gerada pelo sistema HIF [205]. Contudo, as adaptações trascricionais mediada pela hipóxia e pelas HIFs (HIF1a, HIF2a, HIF3a e HIF1b) sugerem a ocorrência de modificações epigenéticas das histonas [206].

Um número crescente de estudos tem identificado o papel da metilação de histonas na adaptação tumoral à hipóxia mediada pelo sistema HIF [207-209]. Sabe-se que o sistema HIF facilita a adaptação metabólica da célula tumoral por meio da transcrição direta de enzimas com atividade desmetilase, como a *Jumonji Domain-Containing Protein 1A* (JMJD1A) [209,210].

A JMJD1A regula a expressão de genes pró-angiogênicos induzidos pela hipóxia diminuindo a metilação das histonas em suas regiões promotoras. A diminuição do crescimento tumoral foi relata em estudo onde a JMJD1A foi silenciada [211].

A atuação da proteína JMJD1A consiste na desmetilação de histonas em resíduos de lisina e arginina em reação oxigênio dependente, sendo necessários íons de Fe (II) e α-cetoglutarato como cofatores. Seu alvo de ação são as Lisinas 9 das Histonas H3, dimetiladas ou monometiladas (H3K9me2 e H3K9me1) [212].

A metilação de histonas contribui para uma mudança na estrutura da cromatina, de modo a aumentar ou diminuir a facilidade do acesso de enzimas, podendo influenciar na expressão gênica, replicação e reparo do DNA. [213].

Um nível baixo da JMJD1A resulta em uma baixa atividade desmetiladora e consequentemente, à uma ativação gênica ineficiente. A indução da JMJD1A pela hipóxia pode aumentar a atividade das desmetilases de histonas nos promotores de genes alvos, podendo levar a célula tumoral à um fenótipo indiferenciado [209].

A perda global de metilação da H3K9 foi observada em muitos tipos de câncer [214]. Estas evidências sugerem que a regulação epigenética imposta pelas condições de hipóxia é um fator importante para caracterizar a agressividade tumoral [215,216].

A expressão da proteína JMJD1A tem sido relacionada com o desenvolvimento e prognóstico de diversos tipos de tumores, tais como o câncer colorretal [211], hepatocelular [216], nasofaríngeo [217].

Com relação ao de câncer de cabeça e pescoço, este tipo de investigação ainda não foi consolidada. Dessa forma, é necessário que a JMJD1A seja avaliada com relação ao seu potencial em ser um marcador molecular de prognóstico em pacientes com carcinoma epidermóide oral e de orofaringe.

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4. ARTIGOS DERIVADOS DA TESE

4.1. Artigo 1: HIF1-alpha expression predicts survival of patients with squamous cell carcinoma of the oral cavity

O artigo intitulado "*HIF1-alpha expression predicts survival of patients with squamous cell carcinoma of the oral cavity*" foi publicado em Setembro de 2012, pela revista *Plos One*, a qual possui fator de impacto de 3.73 (JCR2012).

HIF1-ALPHA EXPRESSION PREDICTS SURVIVAL OF PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE ORAL CAVITY

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Running title

HIF1α Expression and Prognosis in Oral Cancer

Keywords

Oral squamous cell carcinoma, HIF1-alpha expression, radiotherapy

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Authors declare that they have no competing interests.

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ABSTRACT

Background: Oral squamous cell carcinoma is an important cause of death and morbidity wordwide and effective prognostic markers are still to be discovered. HIF1α protein is associated with hypoxia response and neovascularization, essential conditions for solid tumors survival. The relationship between HIF1α expression, tumor progression and treatment response in head and neck cancer is still poorly understood.

Patients and methods: In this study, we investigated HIF1α expression by immunohistochemistry in tissue microarrays and its relationship with clinical findings, histopathological results and survival of 66 patients with squamous cell carcinoma of the lower mouth.

Results: Our results demonstrated that high HIF1α expression is associated with local disease-free survival, independently from the choice of treatment. Furthermore, high expression of HIF1α in patients treated with postoperative radiotherapy was associated with survival, therefore being a novel prognostic marker in squamous cell carcinoma of the mouth. Additionally, our results showed that MVD was associated with HIF1α expression and local disease relapse.

Conclusion: These findings suggest that HIF1α expression can be used as a prognostic marker and predictor of postoperative radiotherapy response, helping the oncologist choose the best treatment for each patient.

INTRODUCTION

Head and neck cancer is a significant cause of mortality and morbidity worldwide, presenting approximately 600,000 new cases yearly [1], whereas tumors of the oral cavity show 389,000 new cases per year, with a mortality rate of 50% [2].

Currently, the most important prognostic factor is the presence of regional lymph node metastases, which correlates with a 50% reduction in life expectancy [2-4]; however, micrometastases may not be detected by routine histology [5].

Oral squamous cell carcinoma is a solid tumor that relies on a hypoxia cellular response system for tumor progression [6-12]. Focal hypoxia is found in the majority of solid tumors due to quantitative and qualitative alterations in tumor vasculature, leading to local reduction of oxygen availability [13].

Tumoral response to radiotherapy has been studied through hypoxia measurements in cervical cancer, as well as other tumors, including head and neck tumors [14, 15]. Hypoxia-inducible factor-1 (HIF1) is a heterodimeric transcriptional complex that functions as the main regulator of systemic and cellular oxygen homeostasis [16-25]. When activated, HIF1 can induce the transcription of over 60 genes, as an attempt to avoid hypoxia-mediated dell death. Among HIF1-regulated genes, there are angiogenic and proliferating factors, glucose transporters, anaerobic glycolytic enzymes and others, that are important for tumorigenesis [25-28].

Expression of HIF1α has been studied in renal, bladder, colorectal, breast, ovary and cervical tumors and it was often associated with patient prognosis [29-36]. Positive HIF1α expression has been associated with improved prognosis in head and neck tumor patients that underwent surgery [37]. Additionally, Fillies and cols, described a better prognosis for patients with high HIF1α expression in squamous cell carcinomas of the tongue basis treated with radiotherapy [38]. Nonetheless, lower survival and higher disease relapse in irradiated patients has been associated with strong HIF1α protein expression, as reported by Aebersold et al. [39]. These contradicting results indicate a high complexity of the hypoxia signaling pathway and its participation in radiotherapy treatment response.

A tempting hypothesis to explain these observations would envision HIF1α activation as an inducer of higher tumor vascularization and oxygenation due to Vascular Endothelial Growth Factor (VEGF) expression [40], which would ultimately increase the concentration of intratumoral reactive oxygen species after radiotherapy and therefore render such tumors more responsive to this type of therapy.

In this study, we demonstrate that high HIF1α expression is associated with local disease-free survival. Moreover, in patients treated with postoperative radiotherapy, high HIF1α expression was associated with survival, therefore being a novel prognostic marker in squamous cell carcinoma of the oral cavity. We also show that microvessel density (MVD) is associated with HIF1α expression and local disease relapse. These findings suggest that HIF1α expression can be used as a prognostic marker and a tool for choosing the best treatment for each patient.

MATERIALS AND METHODS

Ethics

The present study was approved by the Ethics Committee of the Heliopolis Hospital on 06/10/2008 (CEP # 619).

Sample

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from 9 institutions in São Paulo State, Brazil, whose aim is to develop clinical, genetic and epidemiological analysis of head and neck squamous cell carcinomas. In this study, we analyzed 66 parafinized tumor samples of squamous cell carcinomas of the lower mouth, surgically treated in the Head and Neck Surgery Department of the Heliópolis Hospital, São Paulo, Brazil, during the period of January/2001 to December/2007. Exclusion criteria were: previous surgical or chemotherapy treatment, presence of distant metastasis, no removal of cervical lymph nodes and positive surgical margins.

Postoperative radiotherapy was indicated when the tumor invaded adjacent tissues (pT4) or cervical lymph nodes were compromised (pN+). Histopathologycal characteristics of all samples were revised by A.M.C.M. (pathologist,author) of the Heliópolis Hospital. According to TNM classification (3rd edition) [41], 27 tumors were T1 and T2, 13 tumors were T3 and 26 tumors were T4. Thirty six cases showed metastasis to cervical lymph nodes. Well differentiated tumors were found in 30 samples, moderately differentiated tumors in 31 and poorly differentiated in 5 (Table 1).

A gender and age characterization of the 66 patients showed a predominance of males (85%) and age varying from 34-81 years, with a mean age of 55 years. According to the anatomical localization of the tumor, 26 (39.4%) were on the tongue, 12 (18.2%) on inferior gums, 22 (33.3%) on the floor of the mouth and 6 (9.1%) on the retromolar area (Table 1).

Postoperative radiotherapy was indicated for 36 patients, but 3 deceased before the end of treatment and were excluded from the survival after radiotherapy analysis. After a follow up of at least 24 months, 36 patients (54.5%) were alive, 27 (41.0%) died due to the disease and 3 (4.5%) died of other causes. Local reccurrence was observed in 23 cases (34.8%).

Tissue microarray

Formalin-fixed, paraffin-embedded tissue sections from 66 primary oral squamous cell carcinomas treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, SP, were used for immunohistochemistry (IHC) analysis. Histological characterization of all samples was done by Hematoxylin and Eosin staining, followed by immunohistochemistry analysis of tissue microarrays (TMA). Two 1mm cylinders taken from tumor central regions were used to represent each sample in the TMA slide (Beecher Instruments®, Silver Spring, MD, USA).

Immunohistochemistry

Anti-HIF1α polyclonal antibody (Millipore Corporation®, USA) was used in the IHC reaction, at a 1:150 dilution [42-44]. Positive (breast cancer controls) and negative (absence of primary antibody) controls were used. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of HIF1α immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 10-50% of cells were positive and 3 when ≥50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2) and strong (3). Both scores were multiplied [45, 46] and the resulting score was used to categorize HIF1α expression as negative (≤1), weak (1-6) and strong (>6).

Angiogenic activity was assessed by MVD analysis using anti-CD34 antiboby (Santa Cruz Biotecnology®, USA) for the IHC reaction, at a 1:150 dilution. Endothelial cell cytoplasmic staining was considered the positivity criterion. MVD was scored in four areas of the tissue array and categorized as ≤20, 20-40 and >40%. These analyses were performed by A.M.C.M. (pathologist, author).

Statistical Analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $p < 0.05$). Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI ≥ 95%). Survival was calculated by the number of months between surgery and death for each patient or the last appointment in case patients were alive. In order to calculate disease-free survival, the time endpoint was the date of local disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon p-value and the Cox Proportional Hazards to adjust p-values and obtain hazard ratio (HR). Statistical calculations were performed using the Epi Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares.

RESULTS

HIF1α expression was detected in all 66 tumors. However, expression was considered weak in 34 samples (51.5%), strong in 32 (48.5%) and it did not show association with tumor characteristics, such as size (p=0.284), positive lymph nodes (p=0.787) and others (Table 1).

In spite of being more frequent in surviving cases, strong expression of HIF1α did not show a significant correlation with the status alive (p=0.094), but showed a significant association with cases with no local disease relapse (p=0.002, Table 2). Multivariate analysis, considering pTNM, showed that HIF1α weak expression is an independent marker for local disease relapse, representing an increased risk of over 7 times in relation to strong expression (OR=7.59, CI=1.94-29.75).

Although overall survival did not show a significant association with HIF1α expression intensity (p=0.185), a strong expression was associated with local disease-free survival (p=0.013, Figure 1). According to a 12 month after surgery follow up, approximately 10% of cases with high HIF1α expression showed local disease relapse, as compared to approximately 50% of relapse in patients with low HIF1α expression (Figure 1). Multivariate analysis, considering pTNM, revealed that a weak expression of HIF1α is an independent marker for a faster local disease relapse, with a 3-fold increased risk when compared to strong expression (HR=3.22, CI=1.16-8.93).

HIF1α low expression was associated with increased local disease relapse, independently from the choice of treatment (p=0.038 for operated and irradiated patients; p=0.039 for operated, but not irradiated patients), increasing the risk of relapse 11 times, both for operated and irradiated patients, as well as operated, but not irradiated cases (OR=11.47 for operated and irradiated patients; OR=11.48 for operated, but not irradiated patients. pTNM was considered in both analysis).

Most interestingly, when analyzing patients that undertook postoperative radiotherapy, low HIF1α expression correlated with a six-fold increased risk of death when compared to high expression (OR=6.13, IC=1.18-31.94, p=0.031, considering pTNM). In contrast, surgically treated patients that did not make use of postoperative radiotherapy did not show this association (p=0.366). Moreover, patients treated only with surgery showed no survival

or local disease relapse difference between cases with high or low expression of HIF1α protein (Figure 2b and Figure 2d).

Disease-free survival curves of patients treated with postoperative radiotherapy showed that half of the cases with low expression of HIF1α deceased in the first 12 months after surgery, as compared to less than 10% of death in the same period for patients with high HIF1α expression (Figure 2a). Multivariate analysis, considering pTNM, showed that weak expression of HIF1α is an independent prognostic marker, indicating a 3-fold increased risk of death for patients treated with postoperative radiotherapy (HR=3.41, 1.13-10.34, p=0.029).

Microvessel density was associated with HIF1α expression in operated and irradiated cases (p=0.036), as well as with lower local disease relapse (p=0.001, Table 3).

DISCUSSION AND CONCLUSIONS

HIF1α protein expression was observed in all squamous cell carcinomas of the lower mouth, being divided into weak and strong expression signals, according to semiquantitative immunohistochemistry staining suggested by Soini et al. [45] and modified by Campos et al. [46].

Our analysis showed a significant relationship between strong HIF1α protein expression and lower local disease relapse (p=0.002) and increased local disease-free survival (p=0.013), suggesting that weak HIF1α expression is an independent risk factor for local disease relapse. Similarly, we have shown a correlation between strong HIF1α protein expression and disease-free survival (Figure 2a, p=0.015) and local disease-free survival for patients that undertook postoperative radiotherapy (Figure 2c, p=0.005). Interestingly, surgery only cases did not show a correlation between HIF1α protein expression and disease-free survival (p=0.700) or local disease-free survival (p=0.391), suggesting an interaction between tumor vascularization and radiotherapy response. Because, no significant relationship between HIF1α expression and tumor size was found, we propose HIF1α expression as a TNM-independent prognostic marker.

Beasley et al. [37] and Fillies et al. [38] have described strong HIF1α protein expression as an independent marker for higher disease-free survival, as well as general survival in patients with head and neck squamous cell carcinomas.

In contrast, lower survival and higher disease relapse has been associated with strong HIF1α protein expression, as reported by Aebersold et al. [39]. However, his work analyzed radiotherapy treated squamous cell carcinomas of the oropharynx, a disease also associated with HPV and therefore with different characteristics [1].

Lin and cols. have described an association between strong HIF1α expression and lower survival in patients with oral squamous cell carcinomas [47]. In this case, immunohistochemistry was quantitative and the scores based on nuclear staining (strong signal attributed to over 60% of immunopositivity). Koukourakis et al. observed a relationship between HIF1α and HIF2α high protein expression and a more aggressive local disease or worse response to carboplatin chemotherapy in squamous cell carcinomas of the tongue, pharynx and larynx [48]. In 2008, a study by Koukourakis et al. showed a relation between HIF1α expression and local disease control in irradiated advanced head and neck tumors, but they did not find the same relation with HIF2α expression [49]. This observation might be explained by the fact HIF1α e HIF-2α can have different functions and tissue specificity, as HIF1α and HIF-2α knockout mice show different phenotypes [50, 51]. Above all, the authors attributed their findings to a higher tumor vascularization.

According to Astekar et al., MVD is directly related with VEGF expression and vascularization of HNSCCs [52]. We have observed that high HIF1α expression is related to higher MVD, probably as a result of VEGF pathway activation, according to previous reports [40]. Moreover, we have detected a correlation between MVD and local disease relapse in patients that underwent post operative radiotherapy. These results suggest that the best disease control is achieved when angiogenesis is stimulated by HIF1α and VEGF expression.

Hypoxia is commonly found in human solid tumors, serving as a selective environment for survival of aggressive cancer cells and as protection from anti-cancer therapies. Commonly, hypoxic tumors are resistant to radio and chemotherapies, since these treatments rely upon the generation of oxygen reactive species to induce lethal DNA damage [53, 54]; however, Zolzer and Streffer [55] showed an increased radiosensitivity of some

human tumor cell lines under chronic hypoxia conditions, including squamous cell carcinoma cell lines. This observation was probably due to breakdown of cellular energy metabolism and cessation of cell cycle progression [55].

In comparison, tumors with high expression of HIF1α activate transcription of genes associated with angiogenesis, such as VEGF, therefore it would be reasonable to predict a higher success rate for postoperative radiotherapy in conditions where tissue oxygen concentrations and its reactive species are high, causing a more efficient neoplastic cell death.

It has been shown that increased vascularization of solid tumors can result in higher oxygenation, which together with increased radionuclide uptake show great potential for optimizing treatment strategies, causing better tumor response to therapy [56].

This hypothesis is in complete accord with our results. We propose that head and neck tumors with high HIF1α expression are more sensitive to radiotherapy due to the facilitated generation of reactive oxygen species in a more vascularized microenvironment.

In conclusion, we suggest the utilization of HIF1α protein expression as a squamous cell carcinoma tumor marker to better evaluate the therapeutic options at hand, especially in the decision of postoperative radiotherapy and the establishment of local disease relapse prognosis. For instance, a low expression of HIF1α could indicate the need of more extensive surgical margins. We also suggest that a single immunohistochemistry scoring protocol is adopted, so that results are similarly interpreted worldwide.

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FIGURE LEGENDS

Figure 1. Local disease-free survival relation to HIF1α expression. High HIF1α expression is related to an increased local disease-free survival.

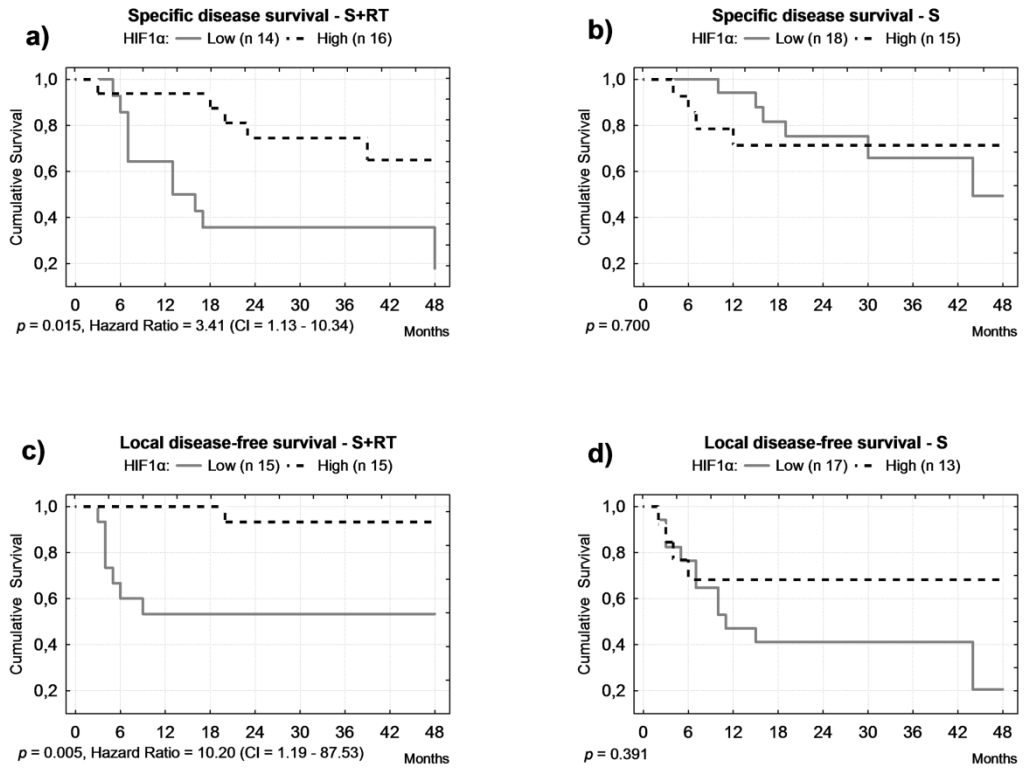
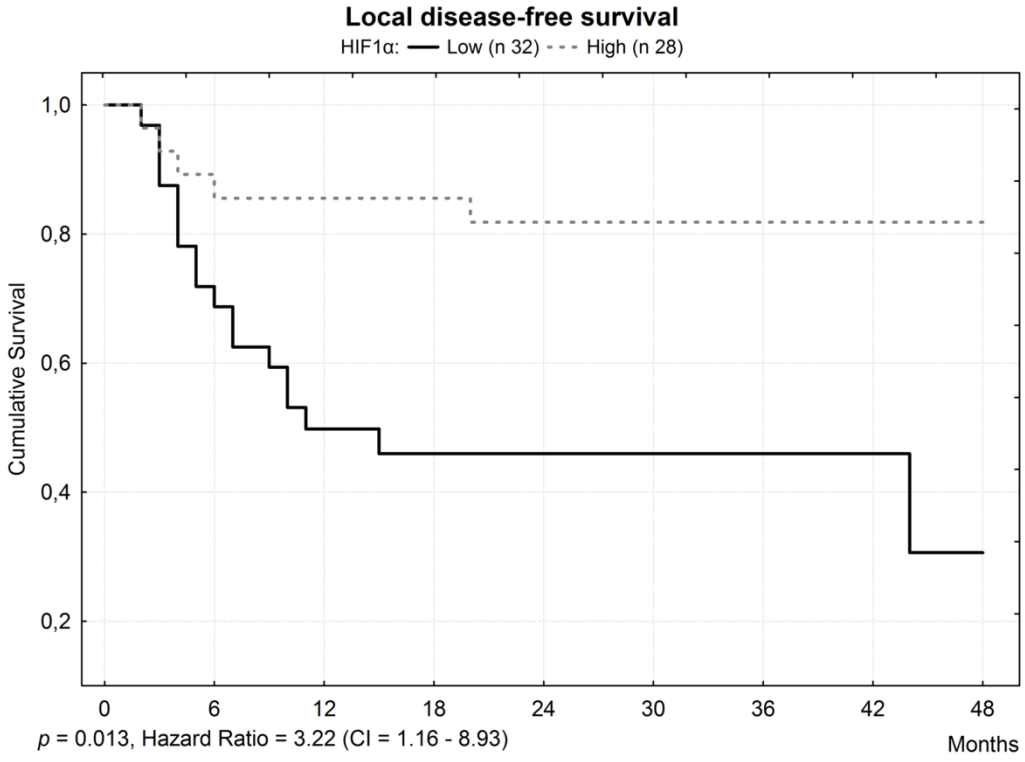


Figure 2. HIF1α expression and specific or local disease-free survival after treatment. Considering surgical (S) treatment only, high HIF1α expression predicts local disease-free survival. Considering surgery plus radiotherapy (S+RT), high HIF1α expression predicts both specific and local disease-free survival.



TABLES

Table 1. Correlation of tumor epidemiological and pathological features with HIF1α expression.

Features	Frequency		HIF1α expression				*p value*
			Weak		Strong		
	No.	(%)	No.	(%)	No.	(%)	
Gender							
Female	10	(15.2)	–	–	–	–	–
Male	56	(84.8)	–	–	–	–	–
Age, yr (median 55, df ±10,9)							
≤ 55	33	(50.0)	–	–	–	–	–
> 55	33	(50.0)	–	–	–	–	–
Treatment							
Only operated	33	(50.0)	–	–	–	–	–
Operated and irradiated	33	(50.0)	–	–	–	–	–
Site							
Tongue	26	(39.4)	–	–	–	–	–
Inferior gums	12	(18.2)	–	–	–	–	–
Floor of the mouth	22	(33.3)	–	–	–	–	–
Retromolar area	6	(9.1)	–	–	–	–	–
Stage							
I+II	16	(24.3)	8	(23.5)	8	(25.0)	
III	15	(22.7)	10	(29.4)	5	(15.6)	0.394
IV	35	(53.0)	16	(47.1)	19	59.4)	
Tumor size (pT)*							
pT1, pT2	27	(40.9)	14	(41.2)	13	(40.6)	
pT3	13	(19.7)	9	(26.4)	4	(12.5)	0.284
pT4	26	(39.4)	11	(32.4)	15	(46.9)	
Lymph node status (pN)*							
Absent	30	(45.5)	16	(47.1)	14	(43.8)	0.787
Present	36	(54.5)	18	(52.9)	18	(56.2)	
Differentiation grade							
Well	30	(45.4)	20	(58.8)	10	(31.2)	
Moderately	31	(47.0)	12	(35.3)	19	(59.4)	0.079
Poorly	5	(7.6)	2	(5.9)	3	(9.4)	
Lymphatic invasion							
Absent	21	(31.8)	11	(32.4)	10	(31.3)	0.923
Present	45	(68.2)	23	(67.6)	22	(68.7)	
Perineural invasion							
Absent	31	(47.0)	17	(50.0)	14	(43.3)	0.611
Present	35	(53.0)	17	(50.0)	18	(56.7)	
Total	**66**	**(100.0)**	**34**	**(51.5)**	**32**	**(48.5)**	

*TNM classification (3rd edition).

Table 2. HIF1α expression association with the status alive or local disease relapse.

Prognostic features	HIF1α Expression				*p value*
	Weak		Strong		
	No.	(%)	No.	(%)	
Survival status					
Alive	15	(46.9)	21	(67.7)	0.094
Deceased	17	(53.1)	10	(32.3)	
Local disease relapse					
No	14	(43.7)	23	(82.1)	0.002
Yes	18	(56.3)	5	(17.9)	

Table 3. Microvessel density relation to HIF1α expression and local disease relapse in operated and irradiated patients.

Microvessel density	HIF1α expression				*p value*	Local disease relapse				*p value*
	Weak		Strong			No		Yes		
	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
≤ 20	4	(26.7)	0	(0)		0	0	4	(50)	
20 - 40	10	(66.7)	10	(66.7)	0.036	16	(72.7)	4	(50)	0.001
> 40	1	(6.7)	5	(33.3)		6	(27.3)	0	(0)	

4.2. Artigo 2: Prognostic significance of NDRG1 expression in oral and oropharyngeal squamous cell carcinoma

O artigo intitulado "*Prognostic significance of NDRG1 expression in oral and oropharyngeal squamous cell carcinoma*" foi publicado em Setembro de 2012, pela revista *Molecular Biology Reports*, a qual possui fator de impacto de 2.506 (JCR2012).

PROGNOSTIC SIGNIFICANCE OF NDRG1 EXPRESSION IN ORAL AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

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Running title

NDRG1 Expression in Head and Neck Cancer

Key words

NDRG1; N-myc downstream-regulated gene 1 protein; Head and Neck carcinoma; Oral squamous cell carcinoma; Immunohistochemistry

Competing interests

Authors declare that they have no competing interests.

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ABSTRACT

Human N-myc downstream-regulated gene 1 (NDRG1) is a metastasis suppressor gene with several potential functions, including cell differentiation, cell cycle regulation and response to hormones, nickel and stress. The purpose of this study was to investigate the immunoexpression of NDRG1 in oral and oropharyngeal squamous cell carcinomas searching for its role in the clinical course of these tumors. We investigated immunohistochemical expression of NDRG1 protein in 412 tissue microarray cores of tumor samples from 103 patients with oral and oropharyngeal squamous cell carcinomas and in 110 paraffin-embedded surgical margin sections. The results showed NDRG1 up-regulation in 101/103 (98.1%) tumor samples, but no expression in any normal tissue sample. Western blot assays confirmed the immunohistochemical findings, suggesting that lowerleves of NDRG1 are associated with a high mortality rate. NDRG1 overexpression was related to long-term specific survival (HR=0.38; p=0.009), whereas the presence of lymph-node metastasis showed the opposite association with survival (HR=2.45; p=0.013). Our findings reinforce the idea that NDRG1 plays a metastasis suppressor role in oral and oropharyngeal squamous cell carcinomas and may be a useful marker for these tumors.

INTRODUCTION

The human N-myc downstream-regulated gene (NDRG) family includes four members (NDRG1-4) and belongs to the α/β hydrolase superfamily [1-4]. Because the catalytic triad residues in the NDRG proteins differ from the consensus on conventional hydrolases, they do not appear to have hydrolytic properties [5]. In spite of not presenting a common functional motif, these proteins share well-conserved residues [3,4] and have been detected in different species of metazoa, such as zebrafish, as well as in plants [6,7]. There are several reports suggesting that they are involved in cell proliferation and differentiation. NDRG1 and 2 have a potential role as tumor suppressors [8,9].

The human NDRG1 gene, also named DRG1, CAP43, GC4 or RTP, has been mapped to chromosome band 8q24.3 [10], the same region where MYC is located and frequently amplified in cancer [11,12], including head and neck squamous cell carcinomas [13]. Mutations in NDRG1 have been described as the cause of the Lom form of hereditary motor and sensory neuropathy, also called Charcot-Marie-Tooth disease type 4D (MIM 601455). The 5' region of the gene exhibits a CpG island, suggesting that NDRG1 may be regulated by DNA methylation. Experiments using histone deacetylase inhibitors as well as the analysis of the promoter region have shown that epigenetic mechanisms [8,14] and different transcription factors, such as MYC, also participate in the regulation of this gene [15,16].

Although ubiquitously expressed, NDRG1 is mostly observed in epithelial cells. Immunohistochemical and electron microscopy studies have shown that the protein has cytoplasmatic or nuclear localization depending on the tissue and is also associated with membranes, especially close to adherens junctions [17].

Human N-myc downstream-regulated gene 1 protein can be regulated by nickel, has a molecular weight of 43 kDa and contains 394 aminoacids [18], with a C-terminal region that is unique for the presence of three 10-amino acid tandem repeats [3]. The protein has phosphorylation sites for protein kinase A and decreased levels of phosphorylated forms have been related to low cell densities, linking NDRG1 to proliferation [19,20].

Several biological functions have been attributed to NDRG1, including differentiation [1,21], cell cycle regulation [22,23], maintenance of the myelin sheaths [24], vesicular

transport and recycling of the adhesion molecule E-cadherin [25], response to hormones [20] and stress, such as heavy metal [18], hypoxia [26,27] and DNA damage response [22].

Human N-myc downstream-regulated gene 1 transcript and protein have been reported downregulated in most tumors, especially in advanced stages, such as in breast, esophageal and colorectal cancers [1,28,29]. In prostate cancer, some studies have detected NDRG1 upregulation in neoplastic cells compared with normal cells [30,31], downregulation in advanced stages [22,32] or different expression patterns, probably reflecting differences in the response to hypoxia and androgens [33]. NDRG1 upregulation has also been observed in melanoma, as well as in lung, brain, liver, breast, renal, cervical and oral cancers [30,34-38]. The latter are very common malignancies which have been associated with high mortality and morbidity rates, mainly due to late diagnosis [39]. This disease has an unpredictable course and no sensitive biomarkers of aggressive behavior have been established. At the moment, the most important prognostic factor is the presence of regional lymph node metastases. However, micrometastases may not be detected by routine histology [40], making the identification of new efficient markers of diagnosis and prognosis an urgent necessity [41].

Motivated by our previous study findings showing NDRG1 upregulation in head and neck tumors [35], we investigated the immunoexpression of NDRG1 in primary oral and oropharyngeal squamous cell carcinomas and matched normal tissues, searching for the relationship between the expression of this protein and the clinical course of the disease.

MATERIAL AND METHODS

Case selection

Formalin-fixed, paraffin-embedded tissue sections from 137 primary carcinomas and 110 non-neoplastic mucosas were obtained from 103 patients with oral and oropharyngeal squamous cell carcinoma at the Head and Neck Surgery Department of Hospital Heliópolis and Hospital do Câncer Arnaldo Vieira de Carvalho, São Paulo, SP, between 2005 and 2007, and used for immunohistochemical analysis. A different subset of samples was analyzed by Western blot (17 oral squamous cell carcinomas and 7 non-neoplastic surgical margins) from 17 patients with surgically resected tumors at Hospital do Câncer Arnaldo Vieira de Carvalho,

São Paulo, SP. The samples were classified by the TNM system [42]. Both subsets of samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from nine institutions in São Paulo State, Brazil, whose aim is to develop clinical, genetic and epidemiological analysis of head and neck squamous cell carcinomas.

Cases with a positive histology report of oral and oropharyngeal squamous cell carcinoma from two different hospitals were included in the study. Histopathological slides were reviewed by senior pathologists to confirm the diagnosis and select appropriate areas for immunohistochemical and Western blot. Lip tumors were excluded because of their origin and characteristics.

The average age of the patients was 55.8 years (SD 24, range, 81 years), and the male/female sex ratio was 6:1. Most patients were smokers or former smokers (95.8%) and had a history of chronic alcohol abuse (85.0%; Table 1). The choice of treatment depended on tumor size and presence of metastases and included surgery, radiation therapy and/or systemic chemotherapy. The clinical follow-up was at least 48 months after surgery.

The study protocol was approved by the National Committee of Ethics in Research (CONEP 1763/05, 18/05/2005) and informed consent was obtained from all patients enrolled.

Tissue microarray

The tissue microarray (TMA) included 103 formalin-fixed, paraffin-embedded samples. For each case, one representative tumor area was selected from a hematoxylin- and eosin-stained section of a donor block. Four cylinders per tumor (diameter of 1 mm each) were punched out and arrayed in a recipient paraffin block using an arraying device (Beecher Instruments, Silver Spring, MD, USA). Therefore, the TMAs contained 412 cores of tumor samples.

Immunohistochemical analysis

Immunohistochemical analyses of TMA tumor specimens and of usual paraffin surgical margin blocks were performed using conventional protocols [43-47].

Sections of the tissue microarray block and the individual paraffin-embedded blocks were immunostained with antibody against NDRG1. Briefly, after deparaffinization in xylene and rehydration in graded ethanol, antigen epitope retrieval was performed using 10 mM citrate buffer, pH 6.0 in a vapor cooker. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min.

Primary goat anti-NDRG1 polyclonal antibody (SC-19464, Santa Cruz Biotechnology, Inc, Santa Cruz CA, USA), diluted 1:500, was incubated overnight at 8ºC followed by addition of the secondary antibody and streptavidin-horseradish peroxidase (LSAB+, code k0690, Dako, CA, USA). Reaction product color was developed by 3,3'-diaminobenzidine (DAB, Dako) and counterstaining was performed with Harris hematoxylin.

The primary antibody was absent in negative controls and a normal prostate sample was used as positive control. The immuno-expression of NDRG1 was graded subjectively as <50% of positive cells (low NDRG1 immunostaining) and ≥50% of positive cells (high NDRG1 immunostaining). Expression differences were evaluated between cases showing low and high immunostaining.

Protein extraction

Fresh samples of primary oral and oropharyngeal carcinomas and matched surgical margins were frozen in liquid nitrogen and stored at -80°C. Analysis of hematoxylin and eosin-stained sections indicated that each carcinoma sample contained at least 70% tumor cells and the corresponding surgical margins were free of tumor cells. After RNA extraction using TRIzol®LS Reagent (Invitrogen Corporation, CA, USA), total protein was extracted by 100% isopropyl alcohol, 0.3 M guanidine hydrochloride in 95% ethanol, 100% ethanol, and 1% SDS. Protein concentration was determined with a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA).

Western blot

Antibodies used were monoclonal antibody anti-NDRG1 (N-19:SC-19464, Santa Cruz Biotecnology), diluted 1:200, and monoclonal anti-*β*-Actin antibody diluted 1:1000 (Santa Cruz Biotecnology). In brief, protein samples (60 ug) were loaded onto 10% SDS-polyacrilamide gels. The molecular weight ladder was the BenchMark™ *Ladder* (Invitrogen,). The proteins were then transferred electrophoretically (Mini Protean, BioRad, CA, USA) to PVDF paper (Hybond, GE Healthcare Bio-Science, NJ, USA). After blocking, the PVDF membranes were incubated with anti-NDRG1 (Santa Cruz Biotechnology), followed by washing and incubation with the secondary antibody anti-goat horseradish peroxidase HRP-conjugated (Santa Cruz Biotechnology). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used for detection.

Statistical analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $p < 0.05$). Multivariate logistic regression was used to obtain odds ratio and confidence intervals (CI ≥ 95%). Survival analysis was performed through Kaplan-Meier curves and log-rank test, and predictors of cancer-specific survival were analyzed by Cox multiple regression. Statistical calculations were performed using the Epi Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares.

RESULTS

Immunohistochemical analysis was performed in 412 TMA cores of tumor samples from 103 patients with oral and oropharyngeal squamous cell carcinomas. One hundred and ten samples of surgical margins were also studied in sections of individual paraffin-embedded blocks. Figure 1 presents immunohistochemical staining for NDRG1 in non-tumoral and tumoral cells of these carcinomas.

The results showed drastic differences between immunopositive cell counts means for normal and tumor areas. A total of 101/103 tumor samples showed positive immunostaining (Fig.1a), mostly in cytoplasm (81/101 or 80.2%; Table 2). However, no expression was detected in any surgical margin (Fig. 1b).

Human N-myc downstream-regulated gene 1 expression was evaluated with respect to clinicopathological factors, to estimate the potential use of NDRG1 as a prognostic marker in oral cancer. However, no correlation between NDRG1 protein expression and TNM, degree of differentiation and other clinicopathological features was observed (Table 3).

When considering low (11.9%) and high (88.1%) NDRG1 expression level groups (total: 101 patients), the results suggested that low NDRG1 levels are associated with a high mortality rate (9 deaths/10 cases) and, conversely, high NDRG1 levels with low mortality (42 deaths/91 cases). Additionally, low NDRG1 levels are associated with a statistically significant shorter global (p=0.004) and specific survival (p=0.001).

The Kaplan-Meier survival curves showed better specific survival for those patients with high NDRG1 expression (log-rank=0.001; Fig. 2). Multivariate survival analysis using Cox's regression model indicated that NDRG1 expression and nodal metastasis were independent prognostic factors for survival. As expected, NDRG1 overexpression was related to long-term specific survival (HR=0.38; p=0,009), whereas the presence of lymph node metastasis showed the opposite association (HR=2.45; p=0.013; Table 4).

Human N-myc downstream-regulated gene 1 expression western blot results for 17 oral squamous cell carcinomas and seven non-neoplastic surgical margins are presented in Figure 3. As observed in the immunohistochemical analysis, surgical margins were negative for NDRG1 expression, whereas mostly tumors showed high levels of this protein.

DISCUSSION

The NDRG1 protein is expressed in most epithelial cells [17], where it may act on differentiation [1,21], cell cycle regulation [22,23], and response to stress [18,22]. This protein has phosphorylation sites for protein kinase A and phosphorylated forms have been

linked to proliferation [19,20]. These data as well as the fact that abnormal expression of NDRG1 has been observed in different tumors, sometimes associated with advanced carcinoma stages [30,34,35,37], opens the possibility of considering NDRG1 a potential target for cancer diagnosis, prognosis or therapy.

In this study, we have extended and elaborated on our previous observation that NDRG1 is overexpressed in oral cancer [35] and analyzed 412 TMA cores of tumor samples from 103 patients with oral and oropharyngeal squamous cell carcinomas and 110 paraffin surgical margin sections, as well as 24 fresh samples of these tumors. The results showed sharp differences between normal and tumor tissues. In fact, no expression of this protein was detected in surgical margins; however, almost all tumors revealed consistent expression of NDRG1. Although no correlation between expression and clinicopathological features was detected, higher levels of NDRG1 were related to long-term specific survival, whereas nodal metastasis showed the opposite association. These findings suggest that NDRG1 plays a metastasis suppressor role, a hypothesis previously proposed by Guan et al. [8]. These authors found that *NDRG1* inhibited in vitro invasion and in vivo colon cancer metastasis, probably by inducing cell differentiation. The data of Bandyopadhyay et al. [48] also support the idea that NDRG1 is a metastasis suppressor protein since it inhibits the invasive ability of tumor cells by downregulating ATF-3, a transcription factor with proinvasive and prometastatic effects.

In contrast, NDRG1 downregulation has been detected in breast, esophageal and colorectal cancers [1,28,29,32]. In prostate cancer, the results are controversial since under and overexpression have been described [22,30-33], which may be an adaptative response to different levels of hypoxia and androgens.

Supporting our findings, NDRG1 upregulation has been observed in melanoma, lung, brain, liver, breast, renal, cervical and pancreatic cancers [30,36-38]. In our previous study [35], increased NDRG1 mRNA levels in head and neck normal and neoplastic tissues were detected after a detailed informatics analysis of more than 134,000 ORESTES followed by experimental validation. In another study, Chang et al. [34], using a differential display technique, identified NDRG1 overexpression in oral cancer and correlated with poorer differentiation. Moreover, this gene has been observed to be upregulated during keratinocyte differentiation in vitro studies and in mouse skin carcinogenesis [1,21]. Unfortunately, we

could not confirm this correlation, although a higher frequency of tumors in the present analysis showed NDRG1 overexpression and moderate to poor differentiation.

Similarly to the findings of other authors [17], NDRG1 exhibited predominantly cytoplasmatic localization but was also found in the nuclei. Sugiki et al [49] demonstrated that NDRG1 interacts with the heat shock cognate protein 70 (Hsc70) and the complex moves from the cytosol to the nucleus after cell activation, supporting the idea that NDRG1 performs critical functions in the nucleus, acting as a transcription regulator, displaying antitumoral effects or causing a cell cycle arrest at the G0/G1 transition [8,22,38,48].

To our knowledge, this is the first study assessing NDRG1 expression in a large set of oral and oropharyngeal carcinomas, including intracellular distribution, except for Chang et al. [34] studying 20 cases of oral carcinomas. As suggested by these authors, NDRG1 overexpression may indicate a response of tumor cells to stress conditions, such as hypoxia or other stimuli, in an attempt to improve cell survival. Although many data have been published on NDRG1, its role in molecular pathways is not completely defined. Our study may facilitate the understanding of oral and oropharyngeal tumorigenesis, as well as clinical management of these carcinomas.

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APPENDIX

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FIGURE LEGENDS

Fig. 1 Immunohistochemical analysis of tumoral areas strongly express NDRG1 in nuclei and cytoplasm (400x) (**a**) and Negative immunostaining for NDRG1 in tumoral and non-tumoral areas (100x) (**b**).

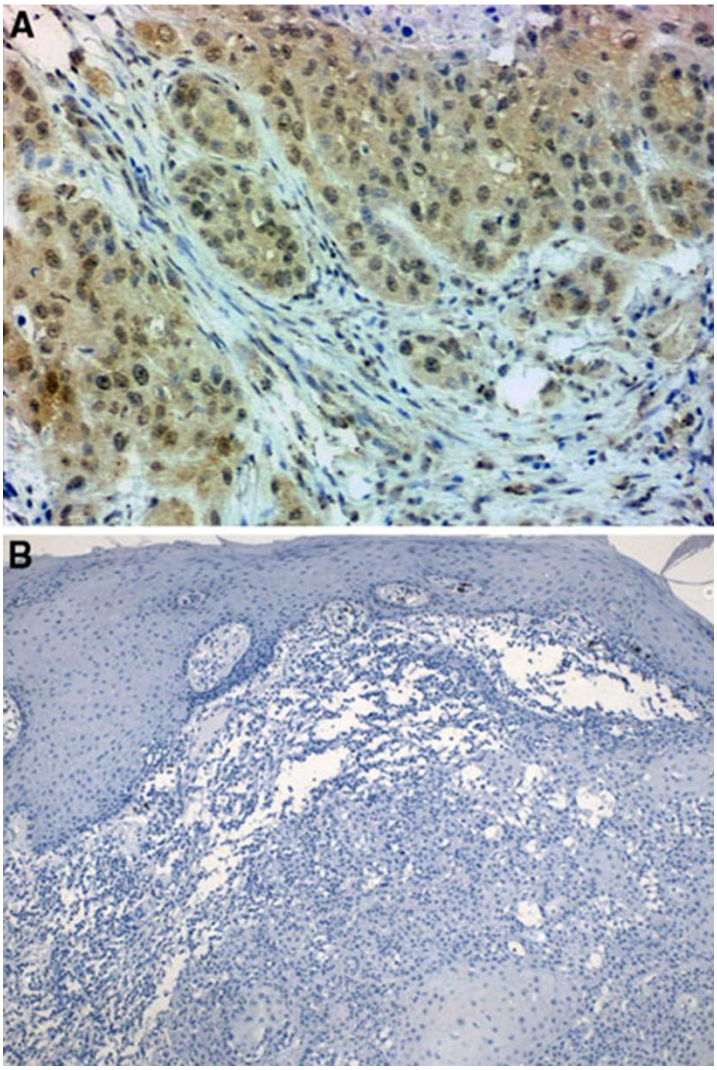


Fig. 2 Kaplan-Meier specific survival curves for patients with head and neck tumors showing low and high NDRG1 expression.

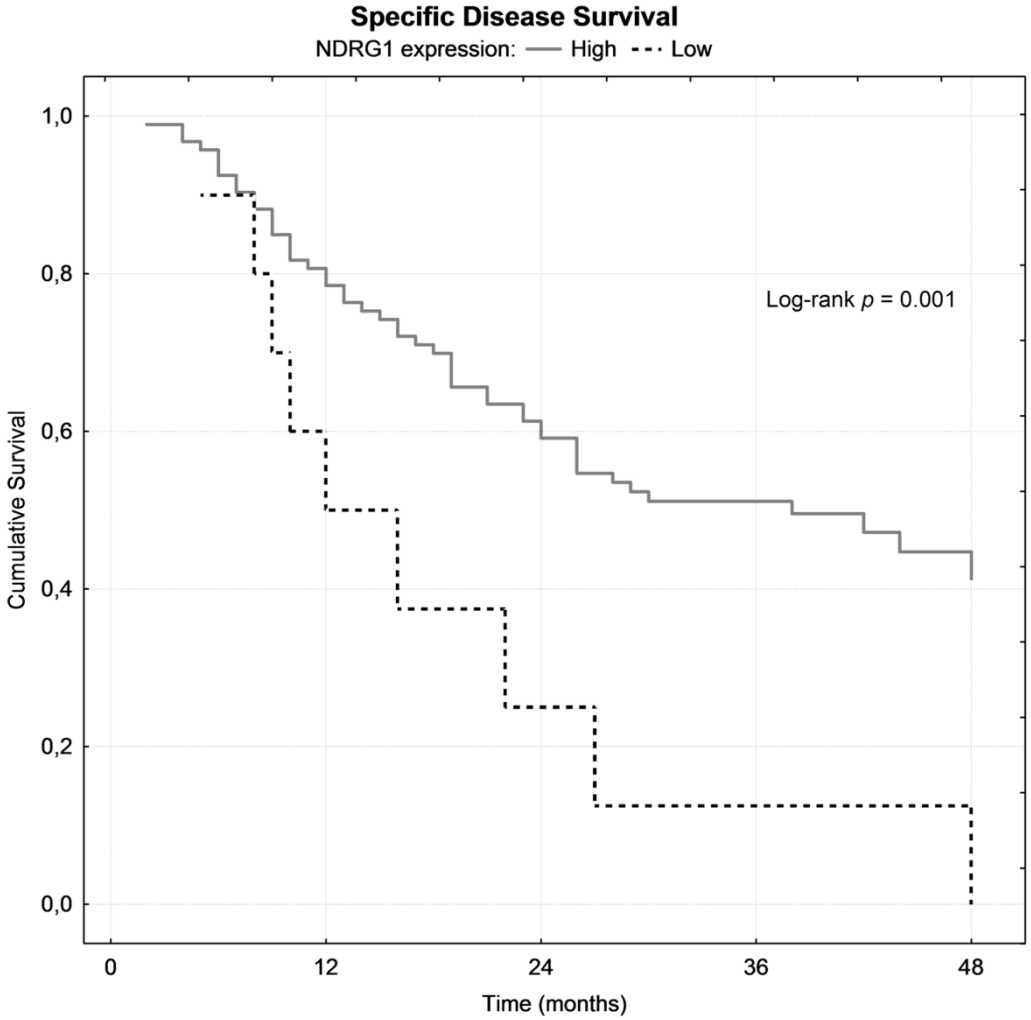
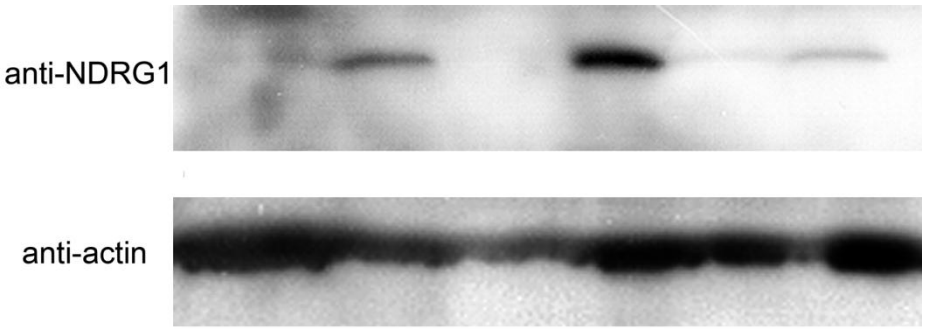


Fig. 3 Representative Western blots illustrating the NDRG1 expression in a subset of oral squamous cell carcinomas (*T*) and matched non-neoplastic surgical margins (*M*) by using anti-NDRG1. *β*-actin was used as an internal control.



TABLES

Table 1. Distribution of 120 patients with oral and oropharyngeal squamous cell carcinoma by gender, age, smoking behaviour, alcohol consumption, tumor size, lymph-node and distant metastasis.

Clinicopathological features	Number (%)
Site	
Oral cavity	93 (77.5%)
Oropharynx	27 (22.5%)
Gender	
Males	103 (85.8%)
Females	17 (14.2%)
Age (years)	
< 40	5 (4.2%)
40 – 49	33 (27.5%)
50 – 59	45 (37.5%)
60 – 69	26 (21.7%)
≥ 70	11 (9.2%)
Smoking behavior	
Never smokers	5 (4.2%)
Former smokers	22 (18.3%)
Smokers	93 (77.5%)
Alcohol consumption	
Never drinkers	18 (15.0%)
Past drinkers	34 (28.3%)
Current drinkers	68 (56.7%)
Tumor size	
T1	5 (4.2%)
T2	41 (34.2%)
T3	27 (22.5%)
T4	47 (39.2%)
Lymph-node metastasis	
Absent (N-)	44 (36.6%)
Present (N+)	76 (63.3%)
Distant Metastasis	
Absent (M-)	118 (98.3%)
Present (M+)	2 (1.7%)

Table 2 Frequency of immunoreactivity for NDRG1 in normal and primary tumor cells.

NDRG1 expression	Surgical margin (n=110)	Tumor (n=103)
	Number (%)	Number (%)
Immunoreactivity level		
Negative	110 (100%)	2 (1.9%)
Low	0 (0%)	12 (11.7%)
High	0 (0%)	89 (86.4%)
Nucleus		
Negative	110 (100%)	81 (80.2%)
Positive	0 (0%)	20 (19.8%)
Cytoplasm		
Negative	110 (100%)	20 (19.8%)
Positive	0 (0%)	81 (80.2%)

Table 3 Comparison of NDRG1 immuno-expression in relation to histopathological characteristics of 103 head and neck tumors.

Histopathological characteristics	NDRG1 expression (n=101)					
	Level		*p value*	Localization		*p value*
	Low	High		Nuclear	Cytoplasmic	
Tumor size (T)			0.494			0.494
T1	0	5		2	3	
T2	4	27		5	26	
T3	1	21		3	19	
T4	5	38		10	33	
Lymph node status			0.152			0.574
Absent (N-)	2	33		8	27	
Present (N+)	8	58		12	54	
Differentiation			0.496			0.315
Poorly	0	8		0	8	
Moderately	5	47		12	40	
Well	5	35		8	32	
Not available*	0	1		1	0	
Vascular infiltration			0.467			0.318
Negative	9	81		17	73	
Positive	1	9		3	7	
Not available*	0	1		0	1	
Lymphatic invasion			0.483			0.543
Negative	3	26		6	23	
Positive	7	65		14	58	
Not available*	0	0		0	0	
Perineural invasion			0.554			0.257
Negative	4	40		7	37	
Positive	6	50		13	43	
Not available*	0	1		0	1	
Inflammatory infiltration			0.484			0.470
Negative – mild	5	33		9	29	
Moderate – severe	5	57		11	51	
Not available*	0	1		0	1	
Desmoplasia			0.210			0.482
Mild	4	34		8	30	
Moderate	0	28		3	25	
Severe	1	15		2	14	
Not available*	5	14		7	12	

* Not available (not considered in the statistical calculations).

Table 4 Cox regression analysis of survival for head and neck carcinomas (n=101) evaluating the low and high NDRG1 expression, and absence (N-) and presence (N+) of lymph-node metastasis.

	Number	**Adjusted hazard ratio**	**Confidence interval (95%)**	***p value***
NDRG1 expression				
Low	10	1.00		
High	91	0.38	0.18 – 0.79	0.009
Lymph-node status				
Absent (N-)	35	1.00		
Present (N+)	66	2.45	1.21 – 4.91	0.013

4.3. Artigo 3: FGFR4 profile as a prognostic marker in squamous cell carcinoma of the mouth and oropharynx

O artigo intitulado "*FGFR4 profile as a prognostic marker in squamous cell carcinoma of the mouth and oropharynx*" foi publicado em Novembro de 2012, pela revista *Plos One*, a qual possui fator de impacto de 3.73 (JCR2012).

FGFR4 PROFILE AS A PROGNOSTIC MARKER IN SQUAMOUS CELL CARCINOMA OF THE MOUTH AND OROPHARYNX

Authors

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Running title

FGFR4 Profile as a Tumor Prognostic Marker

Keywords

FGFR4; Gly388Arg; expression; prognostic marker; HNSCC

Competing interests

Authors declare that they have no competing interests.

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ABSTRACT

Background: Fibroblast growth factor receptor 4 (FGFR4) is a member of a receptor tyrosine kinase family of enzymes involved in cell cycle control and proliferation. A common single nucleotide polymorphism (SNP) Gly388Arg variant has been associated with increased tumor cell motility and progression of breast cancer, head and neck cancer and soft tissue sarcomas. The present study evaluated the prognostic significance of FGFR4 in oral and oropharynx carcinomas, finding an association of FGFR4 expression and Gly388Arg genotype with tumor onset and prognosis.

Patients and methods: DNA from peripheral blood of 122 patients with oral and oropharyngeal squamous cell carcinomas was used to determine FGFR4 genotype by PCR-RFLP. Protein expression was assessed by immunohistochemistry (IHC) on paraffin-embedded tissue microarrays.

Results: Presence of allele Arg388 was associated with lymphatic embolization and with disease related premature death. In addition, FGFR4 low expression was related with lymph node positivity and premature relapse of disease, as well as disease related death.

Conclusion: Our results propose FGFR4 profile, measured by the Gly388Arg genotype and expression, as a novel marker of prognosis in squamous cell carcinoma of the mouth and oropharynx.

INTRODUCTION

The fibroblast growth factor receptor (FGFRs) family comprises structurally related tyrosine kinase receptors (FGFR1-4) involved in signaling via interactions with fibroblast growth factors (FGFs), playing an important role in a wide range of biological processes, including differentiation, proliferation, cell motility and angiogenesis [1,2]. Most FGFs have mitogenic activity in a variety of systems, including cell growth, differentiation and migration [1]. The proliferative capacity of FGFs is a function of FGFRs, to which they bind and through which they signal.

Deregulation in FGF/FGFR signaling has been implicated in human malignant diseases [3-6]. Functional studies demonstrated that FGFR4 interferes in signaling events leading to normal cell adhesiveness and corresponding invasive properties of pituitary tumors [7]. Although the molecular basis of this function is still a matter of intense research, FGFR4 seems to play a role in a broader range of human cancers [7-9].

A single nucleotide polymorphism (SNP) in exon 9 results in an amino acid change (substitution of a glycine residue for an arginine - Gly388Arg) within FGFR4 transmembrane domain and a positive correlation with prognostic parameters in several human cancers, including breast, colon, lung, prostate and head and neck cancers [7,9-14]. Nevertheless, the association between the Gly388Arg genotype and cancer prognosis is not yet clear [15-18], especially in head and neck squamous cell carcinomas (HNSCC).

HNSCC ranks among the top ten most common cancers worldwide, with a large incidence variation according to sex and geographical location [19]. No biomarkers are currently available for HNSCC patients; prognosis depends largely on the stage at presentation, with the most important prognostic factor being the presence of neck node metastases [20].

To our knowledge, there is a lack of studies suggesting the prognostic significance of FGFR4 SNP genotype in HNSCC [9,12]. Streit *et al* [9] evaluated 104 paraffin-embedded tumors and concluded that high expression of FGFR4 together with the Arg388 allele is associated with poor clinical outcome. In comparison, da Costa Andrade *et al* [12] presented results claiming an association between the FGFR4 Arg388 allele and shortened survival in

75 HNSCC patients. Given the small number of patients with tumors of different primary sites evaluated in these studies and the controversial involvement of FGFR family in human cancers, we decided to further investigate the impact the Gly388Arg polymorphism in HNSCC.

The present study evaluated the prognostic significance of FGFR4 expression and the Gly388Arg genotype in oral and oropharynx carcinomas in regard to tumor onset and prognosis. Possible correlations with clinicopathological and prognosis parameters were also analyzed.

MATERIALS AND METHODS

Ethics

This study was approved by the Committee of Ethics in Research of the Heliopolis Hospital on 07/12/2005 (CEP # 402) and an informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from 9 institutions in São Paulo State, Brazil, whose aim is to develop clinical, genetic and epidemiological analysis of HNSCC. In this study, 122 DNA and 75 tumoral tissue samples were obtained and used for polymorphism Gly388Arg genotyping and immunohistochemical analysis of the FGFR4 gene, respectively, within a total of 125 patients with oral and oropharyngeal squamous cell carcinomas, surgically treated at the Head and Neck Surgery Department of Heliópolis Hospital and Arnaldo Vieira de Carvalho Câncer Hospital, São Paulo, Brazil, during the period of January/2002 to December/2007. The clinical follow-up was at least 48 months after surgery. Previous surgical treatment, distant metastasis, no removal of cervical lymph nodes and positive surgical margins were exclusion criteria. Histopathological slides were reviewed by a senior pathologists to confirm the diagnosis and select appropriate areas

for Immunohistochemical analysis. Tumors were classified according to the TNM system [21]. Clinical, epidemiological and pathological characteristics of tumors are described in Table 1.

Genotyping

Genomic DNA was extracted from peripheral blood samples of 122 patients as previously described [22]. Genotypes were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). *FGFR4* exon 9 was amplified using primers described by Bange *et al*, [8] and analyzed for Gly388Arg polimorfism (rs351855). Selected primers were 5´ - GAC CGC AGC AGC GCC CGA GGC CAG - 3´ and 5´ - AGA GGG AAG AGG GAG AGC TTC TG - 3' (Life Technologies, Inc®, São Paulo, SP, Brazil), which produce a 168-base pair (bp) fragment. PCR conditions were: a 25-μL reaction mixture containing 200ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM of each deoxyribonucleoside 5´ triphosphates, 1.5 mM de MgCl2, 1 U Taq DNA polimerase (Life Technologies, Inc®, Rockville, MD, USA) and 25 pmol of each primer. PCR initiated with a melting step of 5 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C. PCR products were digested overnight with *BstNI* following the manufacturer's instructions (New England Biolabs®, Berverly, MA, USA). Restriction fragments were resolved on a 12% non-denaturing polyacrylamide gel. SNP Arg388 in *FGFR4* gene was characterized by two distinctive fragments of 82 and 27 bp, whereas the *FGFR4* Gly388 wild-type allele was identified by a single fragments of 109bp.

Tissue microarray

Tissue microarrays were made using buffered formalin-fixed paraffin-embedded tissue sections from 75 primary oral and oropharyngeal squamous cell carcinomas treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, SP, were used for immunohistochemistry (IHC) analysis. Histological characterization of all samples was done by Hematoxylin and Eosin staining, followed by immunohistochemistry analysis of tissue

microarrays (TMA). Two 1mm cylinders were used to represent each sample in the TMA slide (Beecher Instruments®, Silver Spring, MD, USA).

Immunohistochemistry

Anti-FGFR4 monoclonal antibody (Santa Cruz Biotechnology®, USA) was used in the IHC reaction, at a 1:400 dilution [23-25]. Positive (lung control) and negative controls (absence of primary or secondary antibody) were used for reaction quality control. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained tumor cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of FGFR4 immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 10-50% and 3 when ≥50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2) and strong (3). Both scores were multiplied [26,27] and the resulting score was used to categorize FGFR4 expression as negative (≤3), low (>3 and <7) and high (>7).

Statistical Analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $p<0.05$). Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI≥ 95%). Survival was calculated by the number of months between surgery and death for each patient or the last appointment in case the patient was alive. In order to calculate disease-free survival, the time endpoint was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon p-value and the Cox Proportional Hazards to adjust p-values and obtain hazard ratio (HR). Statistical calculations were performed using the Epi Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares. Genotype correlation with certain biological variables such as age and gender were not analyzed because we could not find biological justifications for these analyses.

RESULTS

FGFR4 Gly388Arg Genotype

Regarding the SNP Gly388Arg, 66 (54.1%) cases were genotyped as Gly/Gly (wild type allele), 47 (38.5%) as Gly/Arg and 26 (7.4%) as Arg/Arg. Allele and genotype frequencies were in Hardy-Weinberg equilibrium.

The Gly388Arg polymorphism did not show a significant association with tumor size (p=0.993), positive lymph nodes (p=0.262) and differentiation grade (p=0.700), but was significantly associated with lymphatic embolization (p=0.022, Table 1). Multivariate analysis showed that presence of at least one allele Arg388 is an independent marker for lymphatic embolization (OR=3.88, CI=1.14-13.13, Table 2).

The Gly388Arg polymorphism was significantly associated with disease specific death (p=0.008, Table 1) and multivariate analysis showed that presence of Arg388 allele is an independent death risk factor, increasing risk 6 times when compared to absence of this allele (OR=6.88, CI=1.64-28.87, Table 2). Nevertheless, the Gly388Arg polymorphism was not correlated with disease relapse (p=0.110, Table 1).

Although disease-free survival did not show a significant association with *FGFR4* polymorphisms (p=0.130), presence of the Arg388 allele was associated with disease specific survival (p=0.020). According to a 36 month after surgery follow up, approximately 25% of cases with the Gly/Gly genotype died of disease specific causes, as compared to approximately 55% of deaths in patients with the Arg388 allele (Figure 1b). Multivariate analysis revealed that the presence of Arg388 allele is an independent marker of disease specific death, with a 3 fold increased risk when compared with absence of this allele (HR=3.26, CI=1.40-7.58, Table 3).

FGFR4 expression

FGFR4 expression was detected in 75 tumors, being classified as high in 26 (34.7%) samples and low in 49 (65.3%) (Figure 2a and 2b, respectively. No samples were negative for FGFR4 expression. FGFR4 expression did not show a significant association with tumor

characteristics such as size (p=0.051) and differentiation grade (p=0.062), but was significantly associated with positive lymph nodes (p=0.036, Table 1). Multivariate analysis showed that low FGFR4 expression is an independent marker for lymph node positivity (OR=3.81, CI=1.12-12.98, Table 2). FGFR4 expression did significantly correlate with disease relapse (p=0.037) and disease specific death (p=0.013, Table 1). Multivariate analysis showed that FGFR4 low expression is an independent marker of disease relapse and disease specific death, representing an increased risk of over 6 times for both, in relation to high expression (respectively, OR=6.73, CI=1.63-27.85 and OR=6.86, CI=1.45-32.40, Table 2).

Disease-free and disease specific survival were significantly correlated with FGFR4 expression (p=0.044 and p=0.034, respectively). According to a 24 month after surgery follow up, approximately 60% of cases with low expression died of disease specific causes, as compared to approximately 30% of deaths in patients with high expression of FGFR4 (Figure 1c). Additionally, according to a 36 month after surgery follow up, approximately 50% of cases with low expression presented disease relapse, as compared to approximately 20% of recurrence in patients with high expression of FGFR4 (Figure 1d). Multivariate analysis revealed that a low expression of FGFR4 is an independent marker for a faster disease relapse and disease specific death, with a 3 fold increased risk when compared to high expression (respectively, HR=3.26, CI=1.44-7.37 and HR=3.26, CI=1.21-8.74, Table 3).

FGFR4 Risk Profiles

In an attempt to combine genotype and expression results, we categorized the FGFR4 profile in three classes: low risk (high expression and absence of Arg388 allele); intermediate risk (high expression and presence of Arg388 allele or low expression and absence of Arg388 allele) and high risk (low expression and presence of Arg388 allele). Frequencies of each FGFR4 profile were 11 (15.5%), 43 (60.6%) and 17 (23.9%), respectively for low, intermediate and high risk.

Disease-free and disease specific survival were significantly correlated with FGFR4 profiles (p=0.002 and p<0.001, respectively). According to a 24 month after surgery follow up, all cases classified as high risk had relapsed and approximately 80% died of disease

specific causes, as compared to approximately 30% of recurrence and no deaths of patients classified as low risk (Figure 1e and 1f). Multivariate analysis revealed that the high risk category is an independent marker for a faster disease relapse and disease specific death, with a 4.5 and 13 fold increased risk, respectively, when compared to the low risk profile (HR=4.50, CI=1.37-14.82 and HR=12.90, CI=1.54-107.69).

DISCUSSION AND CONCLUSIONS

FGFR4 belongs to the family of fibroblast growth factor receptors (FGFR1-4), transmembrane proteins with tyrosine kinase activity. Multiple signal transduction cascades are initiated after binding of FGF ligand to the extracellular domain of the receptor, ultimately resulting in gene expression changes [1,2]. FGFRs have been shown to play important roles in several processes of embryonic development and tissue homeostasis. Their abnormal expression or mutation can cause diverse pathologies, ranging from morphogenetic disorders to cancer [28]. This is a group of proteins of considerable interest in cancer biology, because they regulate essential processes, including mitogenic and angiogenic activity, having important roles in cell differentiation, development, proliferative signaling and motility [2,29,30].

Several studies have examined the role of FGFR4 in carcinogenesis, providing evidences for the complexity of FGF/FGFR signaling pathways in different tumor types [7,31-33].

Although the presence of *FGFR4* Arg388 allele has been shown to indicate a poor prognosis in several tumors [8,10,11,34], the mechanism by which it affects cancer progression remains unclear. This might be related to signaling cascades that control cell-matrix adhesion and angiogenesis [35].

Although some mechanisms have been described in the literature, the influence of Gly388Arg polymorphism in tumor aggressiveness may differ in specific tumors.

Our study revealed that low FGFR4 expression in the presence of Arg388 allele is associated with worse survival in patients with oral and oropharyngeal squamous cell carcinoma.

Seitzer, *et al,* [36] verified, using an animal model, that low protein expression, even in the presence of FGFR4 Arg388 polymorphism, is related to increased pathway activity. This may be explained by the activation of alternative proteins in the signaling cascade or other cascades.

Recently, it has been reported that the presence of polymorphism Gly388Arg is associated with increased cancer risk and progression of pituitary tumors through recruitment of STAT3 signaling cascade. Activation of this cascade can result in deregulation of cell proliferation and apoptosis, leading to tumor progression [37]. Signaling hiperactivation by specific mutations depends on their resistance to negative feedback loops [38]. In addition, several ubiquitylation proteins bind directly to RTKs altering receptor activation [39]. RTK Ubiquitylation may promote receptor degradation creating an important negative feedback mechanism [40,41].

FGFR4 388Arg has not been consider an oncogene per se, but rather collaborate with oncogenes involved in cell motility and invasiveness [36].

Our findings may have important therapeutic implications, because inhibition of one intracellular pathway may lead to activation of parallel signaling pathways, thereby decreasing the effectiveness of single-agent targeted therapies [42]. In support of our hypothesis, the Arg388 allele was associated with resistance to adjuvant therapy in breast cancer [43].

Ansell *et al,* [44], were the first researchers to report that the Gly388 allele showed a significantly higher risk of developing cancer, proposing the Gly388 allele as a risk allele for head and neck cancer.

In contrast, Streit *et al,* [9] reported that in head and neck SCC, expression of Gly388 FGFR4 had no impact on disease progression. In another study, da Costa Andrade *et al,* [12] observed that the presence of at least one Arg allele was significantly correlated with reduced

overall survival and an increased mortality risk of 2.2. In a recent study, Tanuma *et al*, [35] reported that *FGFR4* Arg388 allele was strongly associated with poor prognosis.

In the present study, we have shown that allele Arg388 is associated with lymphatic embolization and premature disease related death. Furthermore, low expression of FGFR4 is related to lymph node positivity and premature disease relapse and death in patients with SCC of the mouth and oropharynx.

Based on these results, we have classified patients with low FGFR4 expression/Arg388 as high risk for relapse and death. In contrast, high FGFR4 expression/Arg388-negative patients were considered at low risk. In conclusion, we propose FGFR4 profile as a novel prognostic marker in SCC of the mouth and oropharynx.

APPENDIX

The GENCAPO (Head and Neck Genome) Project members are the following: Amar A[8], Arap SS[6], Araújo NSS[6], Araújo-Filho V[6], Bandeira CM[4], Braconi MA[4], Brandão LG[6], Brandão RM[11], Canto AL[4], Carmona-Raphe J[2], Carvalho MB[8], Cerione M[5], Cernea CR[6], Chagas MJ[4], Chedid H[8], Cicco R[5], Cominato ML[5], Correa PMS[4], Correia LA[8], Costa A[12], Cunha BR[2], Curioni OA[8], Cury PM[7], Dias THG[3], Dias-Neto E[3], Durazzo M[6], Ferraz AR[6], Figueiredo DLA[9], Figueiredo RO[12], Fortes CS[12], Franzi SA[8], Frizzera APZ[7], Fukuyama EE[5], Gallo J[6], Gazito D[8], Góis-Filho JF[5], Guimarães PEM[6], Inamine R[12], Kaneto CM[11], Lehn CN[8], Leopoldino AM[15], López RVM[12], Macarenco R[4], Magalhães MR[8], Magalhães RP[6], Mamede RCM[9], Mendes GS[5], Meneses C[4], Mercante AMC[8], Michaluart-Junior P[6], Montenegro FLM[6], Moreira-Filho CA[1], Moyses RA[6], Nóbrega FG[4], Nóbrega MP[4], Nunes FD[13], Ojopi EPB[3], Okamoto OK[14], Paiva R[5], Pinheiro DG[11], Polachini GM[2], Ramos O[6], Rapoport A[8], Rodini CO[13], Rodrigues AN[12], Rodrigues RV[2], Rodrigues-Lisoni FC[2], Rossi L[8], Santos ARD[11], Santos M[8], Serafini LN[10], Settani F[5], Severino P[1], Silva AMA[18], Silva C[6], Silva FAM[15], Silva IT[11], Silva Jr WA[11], Silva MJ[5], Silva-Filho GB[6], Silveira NJF[16], Smith RB[6], Souza SCOM[13], Souza TB[8], Stabenow E[6], Tajara EH[2], Takamori JT[8], Tarlá MVC[11], Tavares MR[6], Turcano R[6], Valentim PJ[5], Vidotto A[2], Volpi EM[6], Wünsch-Filho V[12], Xavier FCA[13], Yamagushi F[5], Zago MA[17]. **Affiliations:** [1]Instituto de Ensino e Pesquisa Albert Einstein, São Paulo;

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FIGURE LEGENDS

Figure 1. Survival plots. a. and b.: Disease-free survival and disease specific survival according to FGFR4 Gly388Arg polymorphism; **c. and d.:** Disease-free survival and disease specific survival according to FGFR4 expression; **e. and f.:** Disease-free survival and disease specific survival according to FGFR4 profile.

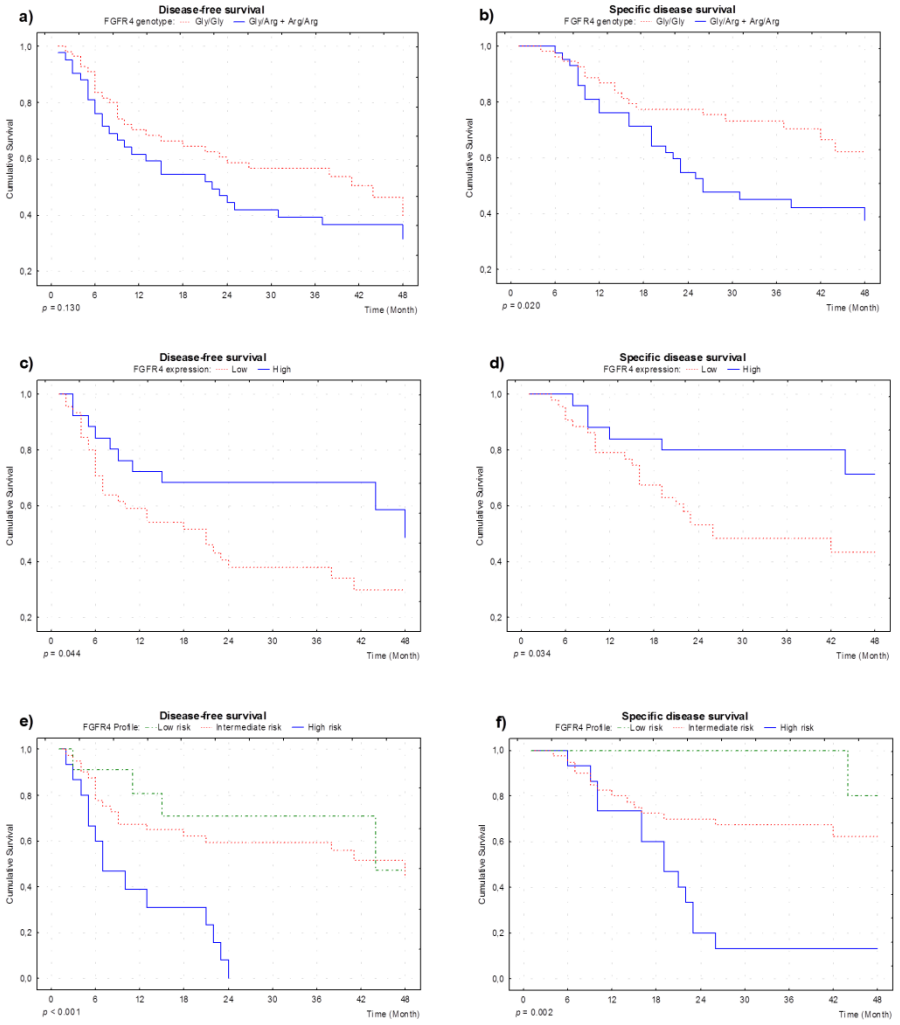
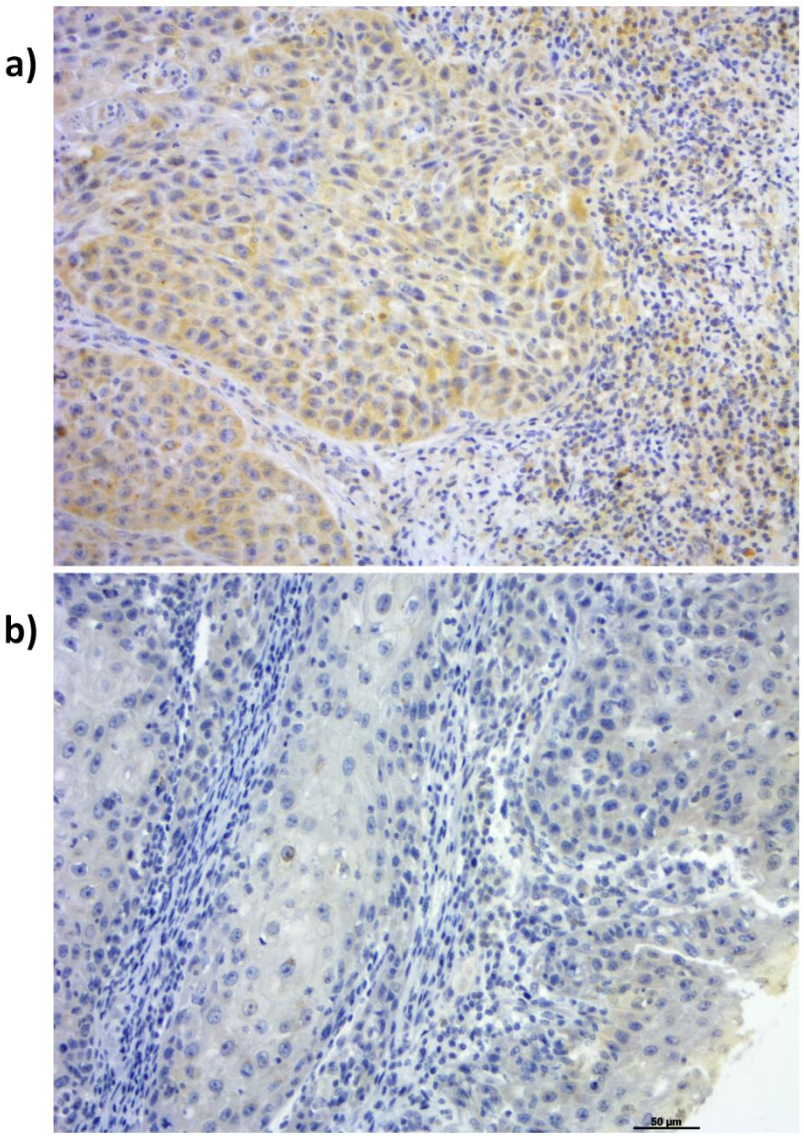


Figure 2. **Immunohistochemical analysis of tumors.** (a) strong FGFR4 expression; (b) weak FGFR4 expression. Magnification was 400x.



TABLES

Table 1. Epidemiological, clinical and pathological tumor features and their association with Gly388Arg polymorphism and FGFR4 expression.

Epidemiological, clinical and pathological features	FGFR4									
	Genotype Gly388Arg					Expression level				
	Total		Gly/Gly	Gly/Arg + Arg/Arg	*p*	Total		Low	High	*p*
	No.	(%)				No.	(%)			
Gender										
Male	106	(86.9)	–	–	–	62	(82.7)	–	–	–
Female	16	(13.1)	–	–	–	13	(17.3)	–	–	–
Age, yr										
median 54, df ±10.2										
Smoker	98	(80.3)	–	–	–	54	(72.0)	–	–	–
Alcoholic	74	(60.7)	–	–	–	42	(56.0)	–	–	–
Treatment										
Only operated	43	(35.2)	–	–	–	34	(45.3)	–	–	–
Operated + irradiated	79	(64.8)	–	–	–	41	(54.7)	–	–	–
Tumor sities										
Oral cavity	87	(71.3)	–	–	–	60	(80.0)	–	–	–
Oropharynx	35	(28.7)	–	–	–	15	(20.0)	–	–	–
Tumor size (T)										
T1+T2	48	(39.3)	26	22	0.993	29	(38.7)	18	11	0.051
T3	31	(25.4)	17	14		19	(25.3)	9	10	
T4	43	(35.3)	23	20		27	(36.0)	22	5	
Lymph nodes										
Absent	59	(48.4)	35	24	0.262	31	(41.3)	16	15	0.036
Present	63	(51.6)	31	32		44	(58.7)	33	11	
Differentiation										
Well	47	(38.5)	24	23	0.700	32	(42.7)	24	8	0.062
Moderately	65	(53.3)	37	28		35	(46.7)	18	17	
Poorly	9	(7.4)	4	5		7	(9.3)	6	1	
Not available [a]	1	(0.8)				1	(1.3)			
Lymphatic embolization										
Negative	54	(44.3)	35	19	0.022	26	(34.7)	21	5	0.034
Positive	66	(54.1)	29	37		49	(65.3)	28	21	
Not available [a]	2	(1.6)				0	(0.0)			
Perineural invasion										
Negative	63	(51.6)	31	32	0.386	39	(52.0)	24	15	0.526
Positive	56	(45.9)	32	24		35	(46.7)	24	11	
Not available [a]	3	(2.5)				1	(1.3)			
Disease specific death										
No	55	(45.1)	37	18	0.008	40	(53.3)	21	19	0.013
Yes	44	(36.0)	18	26		31	(41.4)	25	6	
Not available [a]	23	(18.9)				4	(5.3)			
Disease relapse										
No	44	(36.1)	29	15	0.110	33	(44.0)	17	16	0.037
Yes	56	(45.9)	28	28		40	(53.3)	30	10	
Not available [a]	22	(18.0)				2	(2.7)			
Total	**122**	**(100.0)**	**66**	**56**		**75**	**(100.0)**	**49**	**26**	

[a] Not available (not considered in the statistical calculations).

Table 2. Multivariate analysis of the relationship between clinical and pathological tumor features with gene polymorphism and FGFR4 expression.

Variables	Multivariate analysis							
	Lymphatic embolization		Lymph-nodes		Disease relapse		Disease specific death	
	OR (95% CI) [a]	*P* value [b]	OR (95% CI) [a]	*P* value [b]	OR (95% CI) [a]	*P* value [b]	OR (95% CI) [a]	*P* value [b]
FGFR4 expression								
High	1		1		1		1	
Low	0.46 (0.13-1.68)	0.245	3.81 (1.12-12.98)	0.032	6.73 (1.63-27.85)	0.009	6.86 (1.45-32.40)	0.015
FGFR4 genotype Gly388Arg								
Gly/Gly	1		1		1		1	
Gly/Arg+Arg/Arg	3.88 (1.14-13.13)	0.029	1.88 (0.60-5.83)	0.276	3.57 (0.99-12.91)	0.052	6.88 (1.64-28.87)	0.008
Tumor size (T)								
T1+T2	1		1		1		1	
T3	2.00 (0.45-8.84)	0.358	1.38 (0.36-5.22)	0.640	3.13 (0.67-14.57)	0.147	2.67 (0.52-13.78)	0.241
T4	1.12 (0.30-4.23)	0.859	3.16 (0.86-11.59)	0.083	1.11 (0.28-4.33)	0.885	2.31 (0.57-9.39)	0.242
Differentiation								
Well	1		1		–	–	–	–
Moderately	2.77 (0.83-9.18)	0.094	2.98 (0.90-9.88)	0.075	–	–	–	–
Poorly	5.70 (0.47-69.08)	0.171	3.91 (0.35-43.15)	0.266	–	–	–	–
Lymph nodes								
Absent	–	–	–	–	1		1	
Present	–	–	–	–	7.69 (1.21-49.00)	0.031	9.44 (1.52-58.65)	0.016
Irradiated								
No	–	–	–	–	1		1	
Yes	–	–	–	–	0.07 (0.01-0.50)	0.008	0.39 (0.07-2.18)	0.286

[a, b] Values adjusted by multivariate logistic regression.

For Gly388Arg and FGFR4 expression correlation with lymphatic embolization and lymph node status, tumor size and differentiation status were considered in the multivariate analysis. For disease relapse and disease specific death, tumor size, lymph node status and radiotherapy treatment were considered.

Table 3. Multivariate analysis of disease specific survival.

Variables	Cox proportional			
	Disease-free survival		Disease-specific survival	
	HR (95% CI) [a]	*P* value [b]	HR (95% CI) [a]	*P* value [b]
FGFR4 expression				
High	1		1	
Low	3.26 (1.44-7.37)	0.005	3.26 (1.21-8.74)	0.019
FGFR4 genotype Gly388Arg				
Gly/Gly	1		1	
Gly/Arg+Arg/Arg	1.77 (0.85-3.67)	0.124	3.26 (1.40-7.58)	0.006
Tumor size (T)				
T1+T2	1		1	
T3	3.53 (1.46-8.52)	0.005	3.35 (1.13-9.92)	0.029
T4	1.99 (0.85-4.69)	0.115	1.65 (0.64-4.26)	0.304
Lymph nodes				
Absent	1		1	
Present	2.62 (1.05-6.53)	0.039	4.80 (1.56-14.73)	0.006
Irradiated				
No	1		1	
Yes	0.22 (0.09-6.53)	0.002	0.48 (0.18-1.27)	0.139

[a, b] Values adjusted by Cox proportional hazards.

Tumor size, lymph node status and radiotherapy treatment were considered in the multivariate analysis.

4.4. Artigo 4: FAS/FASL expression profile as a prognostic marker in squamous cell carcinoma of the oral cavity

O artigo intitulado "*FAS/FASL expression profile as a prognostic marker in squamous cell carcinoma of the oral cavity*" foi publicado em Julho de 2013, pela revista *Plos One*, a qual possui fator de impacto de 3.73 (JCR2012).

FAS/FASL EXPRESSION PROFILE AS A PROGNOSTIC MARKER IN SQUAMOUS CELL CARCINOMA OF THE ORAL CAVITY

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Running title

FAS/FASL Expression as a Marker in Oral Cancer.

Keywords

FAS; FASL; expression profile; prognostic marker; oral cancer.

Competing interests

Authors declare that they have no competing interests.

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ABSTRACT

FAS/FASL altered expression may cause tumor protecting immunomodulation, with a direct impact on patient prognosis. FAS expression was studied in 60 squamous cell carcinomas of the oral cavity. FAS expression did not show a significant association with tumor histopathological characteristics, but was significantly associated with lymph node positivity. FAS expression was significantly associated with disease specific death and negative FAS expression was an independent risk factor, increasing risk 4 times when compared to positive expression. When FAS and FASL expression results were combined, we were able to define high, intermediate and low risk profiles. Disease-free and disease-specific survival were significantly correlated with FAS/FASL expression profiles. The high risk category was an independent marker for earlier disease relapse and disease-specific death, with approximately 4- and 6-fold increased risk, respectively, when compared to the low risk profile. Risk profiles based on FAS/FASL expression showed that high risk was significantly associated with increased disease relapse and death, as well as shorter disease-free or disease-specific survival. This categorization, added to patient clinical data, may facilitate the choice of therapy, minimizing treatment failure and increasing disease control.

INTRODUCTION

Head and neck cancer (HNC) is a significant cause of mortality and morbidity worldwide, presenting approximately 600,000 new cases yearly [1], whereas tumors of the oral cavity contribute with 389,000 new cases per year, with a mortality rate of 50% [2].

Currently, the most important HNC prognostic factor is the presence of regional lymph node metastases, which correlates with a 50% reduction in life expectancy [2-4], however, micrometastases may not be detected by routine histology [5].

Several factors are responsible for the modulation of tumoral growth and patient prognosis. Throughout the years, factors that alter proliferation and apoptosis have received a lot of attention. It is believed that disequilibrium between proliferation and apoptosis may be the key factor in tumor development and prognosis [6].

Programmed cell death plays a critical role in the development and homeostasis of multicelullar organisms [6]. This complex process involves several genes, as well as mutations and polymorphisms that may lead to deficient death signaling and potentiation of tumor aggressiveness. Some tumor cells have acquired the ability to overcome apoptosis or to induce apoptosis of tumor-specific lymphocytes, favoring tumor progression [7]. Apoptosis resistance is a capacity shared by most malignancies. Subversion of apoptotic pathways is a major mechanism in cancer devopment, being also related with tumor aggressiveness, histological differentiation and prognosis [8,9].

FAS (CD95), a member of the TNF family, is a transmembrane protein with cystein rich extracellular domains and a death cytoplasmatic domain, common to all family members and essential in the translation of the death stimulus [10,11]. Immediately after the receptor stimulation by the FASL ligand (CD95L), the apoptotic signal is transmitted through the adapter FADD (*FAS Associated Death Domain*), which converts caspase 8 zymogen into its active form, triggering the apoptosis start. Activation of this cascade will culminate into DNA fragmentation, causing radical morphological and biochemical intracellular changes [11-12].

FAS/FASL altered expression may cause tumor protecting immunomodulation, with a direct impact on patient prognosis [13]. In a previous study, microarray experiments compared gene expression between more aggressive oral tumors (tumors with premature metastasis; T1/T2, N+) and more benign tumors (advanced tumors without metastasis; T3/T4, N0). These results generated a list of genes with differential expression, where FAS and FASL were among the least expressed in more benign tumors, suggesting a role in tumor apoptosis resistance [14]. Owing to these results, the present study aimed to correlate FAS/FASL tumor expression with clinical variables, tumor histology and prognosis of squamous cell carcinoma of the oral cavity.

MATERIALS AND METHODS

Ethics

This study was approved by the Research Ethics Committee of the Heliopolis Hospital on 08/12/2008 (CEP no 637) and an informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from 9 institutions in São Paulo State, Brazil, whose aim is to develop clinical, genetic and epidemiological analysis of HNSCC. In this study, 60 tumoral tissue samples were obtained and used for immunohistochemical analysis of the FAS and FASL gene, within a total of 60 patients with oral squamous cell carcinomas, surgically treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, Brazil, during the period of January/2002 to December/2008. The clinical follow-up was at least 48 months after surgery. Previous surgical or chemotherapic treatment, distant metastasis, no removal of cervical lymph nodes and positive surgical margins were exclusion criteria. Histopathological slides were reviewed by a senior pathologist to confirm the

diagnosis and select appropriate areas for immunohistochemical analysis. Tumors were classified according to the TNM system (3rd edition) [15]. Clinical, epidemiological and pathological tumor characteristics are described in Table 1 and 2.

Tissue microarray

Formalin-fixed, paraffin-embedded tissue sections of 60 primary oral squamous cell carcinomas treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, Brazil, were used for immunohistochemistry (IHC) analysis. Histological characterization of all samples was done by hematoxylin and eosin staining, followed by immunohistochemistry analysis of tissue microarrays (TMA). Two 1mm cylinders were used to represent each sample in the TMA slide (Beecher Instruments®, Silver Spring, MD, USA).

Immunohistochemistry

Anti-FAS monoclonal antibody and anti-FASL monoclonal antibody (Santa Cruz Biotechnology®, USA) were used in the IHC reaction, at a 1:400 dilution [16-18]. Positive and negative controls were used. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 10-50% of cells were positive and 3 when ≥50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2) and strong (3). Both scores were multiplied [19,20] and the resulting score was used to categorize FAS and FASL expression as positive (>3, Figure 1a and 1b, respectively) and negative (≤3, Figure 1c).

Statistical Analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $p<0.05$). Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI 95%). Survival was calculated by the number of months between surgery and death for each patient or the last appointment in case the patient was alive. In order to calculate disease-free survival, the time endpoint was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon p-value and the Cox Proportional Hazards to adjust p-values and obtain hazard ratio (HR). Statistical calculations were performed using the Epi Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares.

RESULTS

FAS expression

FAS expression was studied in 60 tumors, of which 28 were positive (46.7%) and 32 were negative (53.3%). FAS expression did not show a significant association with tumor characteristics such as size ($p=0.233$) and differentiation grade ($p=0.441$), but was significantly associated with positive lymph nodes ($p=0.004$, Table 2). Multivariate analysis showed that negative FAS expression was an independent marker for lymph node positivity (OR=5.02, CI=1.34-18.75, Table 3).

FAS expression was significantly associated with disease specific death ($p<0.001$, Table 2) and multivariate analysis showed that negative expression was an independent death risk factor, increasing risk 4 times when compared to positive expression (OR=4.59, CI=1.01-21.51, Table 3). Nonetheless, FAS expression was not correlated with disease relapse ($p=0.080$, Table 2).

Disease-free and disease-specific survival were significantly correlated with FAS expression ($p=0.025$ and $p<0.001$, respectively). According to a 24 month after surgery follow up, approximately 70% of cases with negative expression presented

disease relapse, as compared to approximately 25% of recurrence in patients with positive expression of FAS (Figure 2a). Additionally, according to a 36 month after surgery follow up, approximately 65% of cases with negative expression died of disease specific causes, as compared to 15% of deaths in patients with positive expression of FAS (Figure 2b). Multivariate analysis revealed that a negative expression of FAS was an independent marker for earlier disease specific death, showing a 3-fold increased risk when compared to positive expression (HR=3.73, CI=1.16-11.95, Table 3), but the same association was not found for disease relapse (HR=1.66, CI=0.69-3.97, Table 3).

FASL expression

Regarding FASL, 33 (55.0%) tumors showed positive expression, whereas 27 (45.0%) were negative. FASL expression was significantly associated with differentiation grade (p=0.003), but was not associated with tumor size (p=0.297) or positive lymph nodes (p=0.548, Table 2).

FASL expression did significantly correlate with disease relapse (p=0.007) and disease specific death (p=0.006, Table 2). Multivariate analysis showed that negative FASL was an independent marker of disease relapse and disease specific death, representing an increased risk of over 6 times for each, when compared to a positive expression (respectively, OR=5.51, CI=1.32-23.04 and OR=6.06, CI=1.05-35.06; Table 3).

In contrast, disease-free and disease-specific survival were not associated with FASL expression (p=0.143 and p=0.097, respectively, Figure 2c and 2d).

FAS/FASL Profiles

In an attempt to combine FAS and FASL expression results, we categorized the FAS/FASL profile in three classes: low risk (positive FAS and FASL expression); intermediate risk (negative expression of one marker) and high risk (negative expression

of both markers). Frequencies of each FAS/FASL profiles were 20 (33.3%), 21 (35.0%) and 19 (31.7%), respectively for low, intermediate and high risk.

Disease-free and disease-specific survival were significantly correlated with FAS/FASL profiles (p=0.038 and p=0.008, respectively). On a 24 month after surgery follow up, 80% of cases classified as high risk had relapsed and approximately 70% died of disease-specific causes, compared to approximately 30% of relapse and 15% of death in patients classified as low risk (Figure 3a and 3b). Multivariate analysis revealed that the high risk category is an independent marker for earlier disease relapse and disease-specific death, with approximately 4- and 6-fold increased risk, respectively, when compared to the low risk profile (HR=3.80, CI=1.19-12.52 and HR=6.43, CI=1.45-28.55).

DISCUSSION AND CONCLUSIONS

Apoptosis is a physiological process of cell number control, which plays an important role in cellular homeostasis and embryonic development [21-24]. Cell population is defined by a balance between proliferation and survival and disruption of this balance can lead to cancer growth [25-32].

The extrinsic apoptosis pathway can be triggered by enzymes of the TNF family, including FAS and FASL. FASL positive T-cells can eliminate FAS positive tumoral cells by inducing apoptosis [10,12]. Therefore, reduction or loss of FAS expression may result in decreased sensitivity of tumoral cells to cytotoxic activity, impairing apoptosis.

FAS expression has been previously associated with tumor apoptosis in the stomach [13], esophagus [33] and liver [34]. In addition, FAS/FASL diminished expression correlates with worse prognosis in lung [35] esophageal [36], larynx [37], colorectal [38] and gastric [39] tumors.

In agreement with the literature, our results show that negative FAS expression correlates with lymph node metastasis (5 times increased risk). When compared with positive expression, negative expression was significantly associated with cancer related

deaths and shorter disease-free and disease-specific survival. Multivariate analysis confirmed that negative FAS expression was an independent risk factor for death and disease-specific survival reduction, increasing risk approximately 5 times for each. Our results also showed that negative FASL expression was associated with increased disease relapse and disease-related deaths. Multivariate analysis confirmed that FASL negative expression was an independent risk factor for disease relapse and death, increasing risk up to 6 times when compared to positive expression. However, FASL expression was not related to worse disease-free survival or disease-specific survival.

In contrast with our results, other studies have revealed higher FASL expression as a marker of worse prognosis in esophageal [36] and lung [40-42] tumors. Their hypothesis relies on tumor FASL expression as a T-cell apoptosis inducer, resulting in lower tumor attack by the immune system [43-45]. However, our results support the hypothesis that the immune system response is already compromised in oral cancer, most likely because it is a tobacco/alcohol associated disease[46]. As previously reported, chronic alcohol consumption impairs Natural Killer cell (NK) activity and decreases NK cell number, therefore affecting their ability to destroy tumor cells [47]. In addition, several studies have reported a similar decrease in number and activity of NK cells in smokers [48,49], in which cases a lower production of interferon-c and TNF-a cytokines is observed [50]. Based on these facts, our hypothesis predicts that the oral immune response is attenuated in patients with chronic tobacco and alcohol consumption, therefore in these individuals, a lack of FASL may represent a loss of the extrinsic apoptosis signal in tumor cells, conferring a worse prognosis.

In summary, our results correlate a negative FAS/FASL expression with worse prognosis in oral squamous cell carcinoma patients, suggesting that these proteins play important roles in oral cancer cell apoptosis.

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FIGURE LEGENDS

Figure 1. Immunohistochemical. a. Positive FAS expression; **b.** Positive FASL expression; **c.** Negative immunostaining. Magnification was 400x.

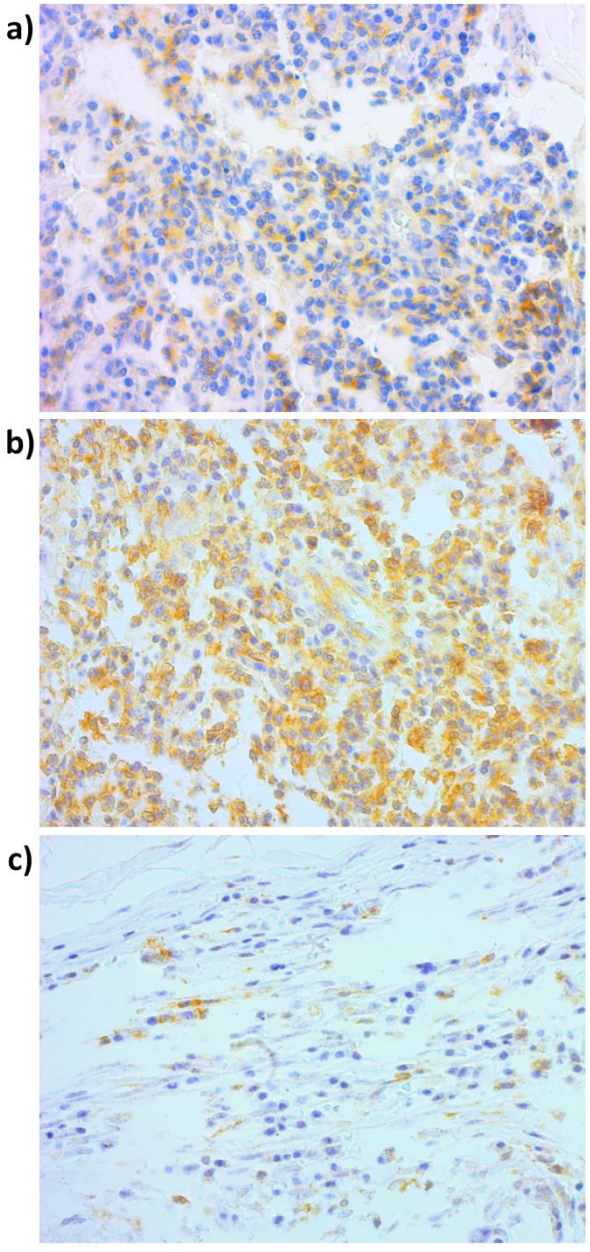


Figure 2. Survival plots. a. and b.: Disease-free survival and disease-specific survival according to FAS expression; **c. and d.:** Disease-free survival and disease-specific survival according to FASL expression.

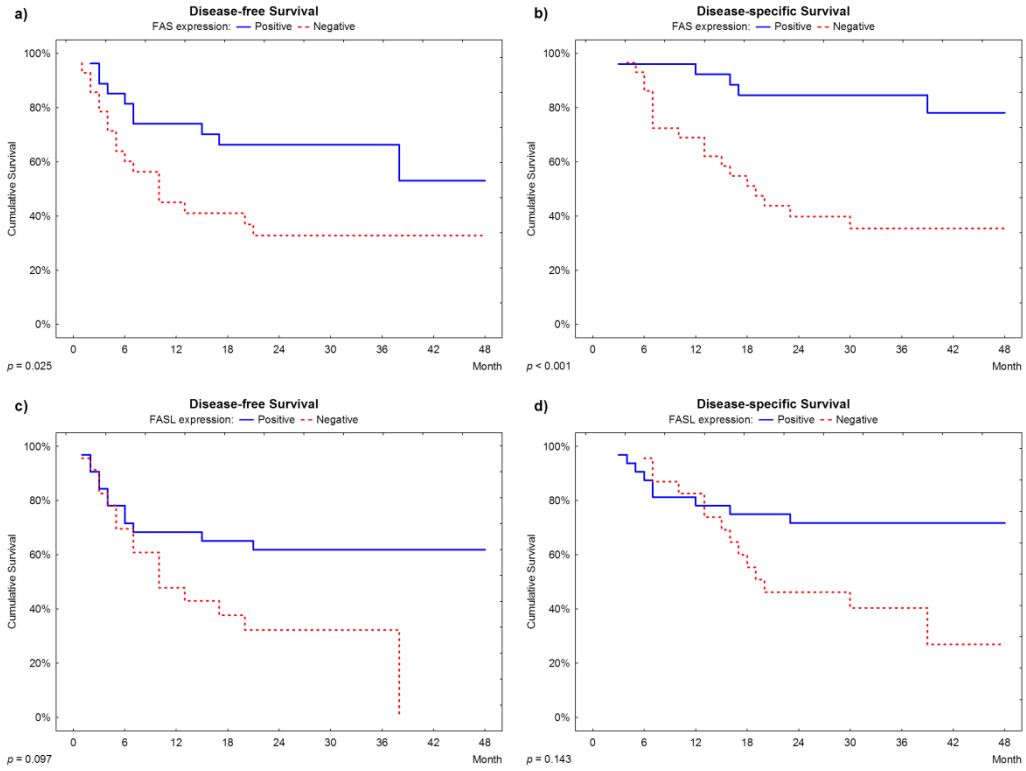
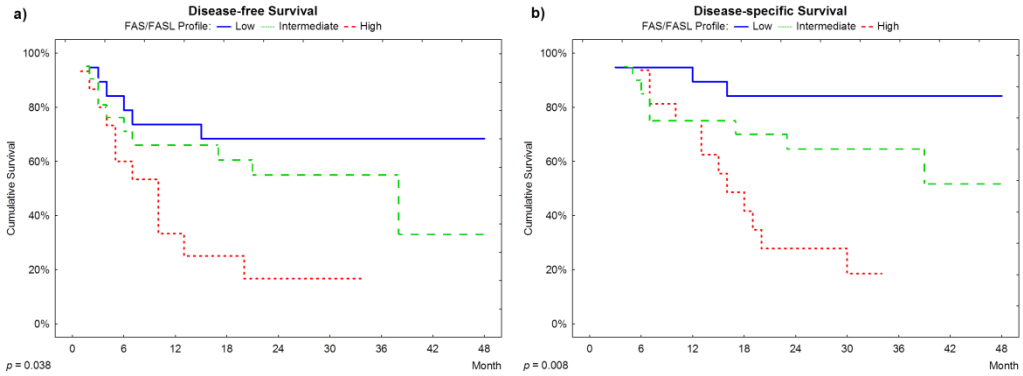


Figure 3. Survival plots. a. and b.: Disease-free survival and disease-specific survival according to FAS/FASL profile.



TABLES

Table 1. Epidemiological features.

Epidemiological features	Total	
	No.	**(%)**
Gender		
Female	8	(13.3)
Male	52	(86.7)
Age, yr		
Median 55, df ±10.7		
Tobacco and Alcohol habits		
Smoker and alcoholic	50	(83.3)
Only smoker	7	(11.7)
Only alcoholic	1	(1.7)
Tumor sub-sities		
Tongue	22	(36.7)
Gum	12	(20.0)
Floor mouth	21	(35.0)
Retromolar area	5	(8.3)
Treatment		
Only operated	60	(100.0)
Operated + irradiated	31	(51.7)
Total	**60**	**(100.0)**

Table 2. Epidemiological, clinical and pathological tumor features and their association with FAS and FASL expression.

Clinical and pathological features	Total		FAS expression		*p*	FASL expression		*p*
			Negative	Positive		Negative	Positive	
	No.	(%)	No.	No.		No.	No.	
Stage								
2	17	(28.3)	6	11	0.025	7	10	0.177
3	17	(28.3)	7	10		5	12	
4	26	(43.4)	19	7		15	11	
Tumor size (T) ¥								
T1+T2	24	(40.0)	11	13	0.233	8	16	0.297
T3	12	(20.0)	5	7		7	5	
T4	24	(40.0)	16	8		12	12	
Lymph nodes (N) ¥								
Absent	27	(45.0)	9	18	0.004	11	16	0.548
Present	33	(55.0)	23	10		16	17	
Diferentiation								
Well	26	(43.4)	16	10	0.441	18	8	0.003
Moderately	29	(48.3)	13	16		7	22	
Poorly	5	(8.3)	3	2		2	3	
Disease specific death								
No	32	(53.3)	11	21	< 0.001	9	23	0.006
Yes	25	(41.7)	20	5		16	9	
Not available*	3	(5.0)					1	
Disease relapse								
No	26	(43.4)	10	16	0.080	6	20	0.007
Yes	29	(48.3)	18	11		17	12	
Not available*	5	(8.3)						
Total	**60**	**(100.0)**	**32**	**28**		**27**	**33**	

¥ TNM classification 3rd edition

* Not available (not considered in the statistical calculations).

Table 3. Multivariate analysis of the relationship between clinical, pathological tumor features and survival with FAS and FASL expression.

Variables	Logistic regression						Cox proportional hazard			
	Lymph-nodes		Disease relapse		Disease specific death		Disease-free survival		Disease-specific survival	
	OR (95% CI)[¥]	*p*[¥]	OR (95% CI)[¥]	*p*[¥]	OR (95% CI)[¥]	*p*[¥]	HR (95% CI)[§]	*p*[§]	HR (95% CI)[§]	*p*[§]
FAS expression										
Positive	1		1		1		1		1	
Negative	5.02 (1.34-18.75)	0.017	1.49 (0.39-5.78)	0.561	4.59 (1.01-21.51)	0.050	1.66 (0.69-3.97)	0.257	3.73 (1.16-11.95)	0.027
FASL expression										
Positive	1		1		1		1		1	
Negative	1.22 (0.30-5.00)	0.780	5.51 (1.32-23.04)	0.019	6.06 (1.05-35.06)	0.044	2.58 (1.03-6.46)	0.044	2.14 (0.73-6.30)	0.166
Tumor size (T)										
T1+T2	1		1		1		1		1	
T3	1.62 (0.30-8.67)	0.576	1.63 (0.29-9.25)	0.581	2.32 (0.33-16.20)	0.395	2.31 (0.73-7.35)	0.156	3.00 (0.76-11.91)	0.118
T4	4.44 (1.08-18.20)	0.038	2.68 (0.62-11.55)	0.186	2.76 (0.51-14.84)	0.236	2.05 (0.77-5.50)	0.152	1.97 (0.63-6.22)	0.245
Differentiation										
Well	1		1		1		1		1	
Moderately	3.56 (0.81-15.63)	0.092	1.09 (0.24-4.96)	0.909	1.66 (0.26-10.44)	0.589	1.57 (0.57-4.35)	0.385	1.84 (0.56-6.05)	0.318
Poorly	6.07 (0.45-81.73)	0.174	0.28 (0.03-2.97)	0.291	7.19 (0.37-139.86)	0.193	0.54 (0.11-2.79)	0.465	1.94 (0.41-9.17)	0.405
Lymph-nodes										
Absent	–	–	1		1		1		1	
Present	–	–	4.07 (0.48-34.40)	0.197	13.55 (0.94-195.73)	0.056	2.28 (0.62-8.33)	0.214	3.49 (0.78-15.65)	0.102
Irradiated										
No	–	–	1		1		1		1	
Yes	–	–	0.17 (0.02-1.27)	0.085	0.30 (0.02-3.68)	0.344	0.30 (0.09-0.97)	0.044	0.52 (0.17-1.56)	0.241

OR – Odds ratio; HR – Hazard ratio; CI – Confidence interval

[¥] Values adjusted by multivariate logistic regression.

[§] Values adjusted by Cox proportional hazards.

4.5. Manuscrito 1: LEPR expression and Gln223Arg polymorphism as a prognostic marker in squamous cell carcinoma of the oral cavity and oropharynx

O manuscrito intitulado "*LEPR expression and Gln223Arg polymorphism as a prognostic marker in squamous cell carcinoma of the oral cavity and oropharynx*" foi submetido para a revista *Molecular Biology Reports*, a qual possui fator de impacto de 2.506 (JCR 2012).

LEPR EXPRESSION AND GLN223ARG POLYMORPHISM AS A PROGNOSTIC MARKER IN SQUAMOUS CELL CARCINOMA OF THE ORAL CAVITY AND OROPHARYNX

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Running title

LEPR as a tumor prognostic marker.

Keywords

LEPR; expression; Gln223Arg; prognostic marker; HNSCC.

Competing interests

Authors declare that they have no competing interests.

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ABSTRACT

The Leptin gene product is released into the blood stream, passes through the blood-brain barrier and finds its receptor (LEPR) in the central nervous system. This hormone regulates food intake, hematopoiesis, inflammation, immunity, differentiation and cell proliferation. The LEPR Gln223Arg polymorphism has been reported to alter receptor function and expression. They have both been related with prognostic in several tumor types. Furthermore, several studies have shown a relationship between the Glin223Arg polymorphism and tumor development. Nonetheless, its role in oral and oropharyngeal squamous cell carcinoma is now well understood. In this study, 315 DNA samples were used for LEPR Gln223Arg genotyping and 87 primary oral and oropharyngeal squamous cell carcinomas were used for immunohistochemistry (IHC) analysis, such that a relationship between these and tumor development and prognosis could be established. Homozygous LEPR Arg223 was related with a reduction in oral and oropharyngeal cancer risk by 2 fold. In contrast, presence of the Arg223 allele in tumors was associated with worse disease-free and disease-specific survival. Low LEPR expression was an independent risk factor, increasing 4 times the risk for lymph node metastasis. In conclusion, the Gln223Arg polymorphism and LEPR expression may be valuable markers for oral and oropharyngeal cancer, suggesting LEPR as a potential target for future therapies.

INTRODUCTION

Head and neck cancer (HNC) is a significant cause of mortality and morbidity worldwide, presenting approximately 600,000 new cases yearly [1], whereas tumors of the oral cavity contribute with 389,000 new cases per year, with a mortality rate of 50% [2,3]. HNC has a multifactor etiology involving smoking and drinking habits, HPV (Human Pappillomavirus) infection and genetic factors [4].

Leptin is a hormone released into the blood stream and transfered through the blood-brain barrier, reaching its receptor LEPR (also known as OBR) located at the plasma membrane of hipothalamus cells. This hormone regulates food intake, hematopoiesis, inflammation, immunity, cell differentiation and proliferation [5-7].

LEPR is a member of a family of citokine class 1 receptors [8]. Upon ligand binding, LEPR undergoes structural changes, which tethers *Janus Kinase* (JAK) molecules, enabling cross phosporilation. This step is needed for phosphorilation of S*ignal Transducer and Activator of Transcription* (STAT) molecules at the cytoplasmic region of the receptor. STAT3 autodimerizes after phosphorilation of JAK2 and translocates into the nucleus, where it is going to activate transcription at specific gene promoters. Among the various genes induced by STAT3, there are enzymes involved in the development of many tumors [6,9-11].

Several LEPR polymorphisms have been described in humans. An A/G transition at nucleotide 668 converts a glutamine to an arginine at codon 223 (Gln223Arg) of this protein. This transition results in an exchange of a neutral aminoacid for one with a positive charge, altering receptor function and signalling capacity, modifying circulating lepting levels [10]. This polymorphism is located inside the extracellular domain coding region, affecting the entire structure of the receptor [12].

Previous studies have correlated the Gln223Arg polymorphism and LEPR expression with develpment and prognosis of several tumors, such as breast, [10,13-15], lung [12], stomach [16] and prostate [17]. However, conclusive data is not yet available for HNC and studies are scarce. Therefore, the present study aimed to analyse

Gln223Arg polymorphism and LEPR expression with develpment, prognosis and survival of patients with oral and oropharyngeal cancer.

MATERIALS AND METHODS

Ethics

This study was approved by the Committee of Ethics in Research of the Heliopolis Hospital (CEP # 446) and an informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from institutions in Brazil. In this study, 315 DNA samples obtained and used for polymorphism LEPR Gln223Arg genotyping, being 186 (59.0%) individuals controls had no history of cancer and 129 (41.0%) patients with oral and oropharyngeal squamous cell carcinomas. The LEPR immunohistochemical analysis was performed on tumoral tissue samples of 87 patients with oral and oropharyngeal squamous cell carcinomas, surgically treated at the Head and Neck Surgery Department of Heliópolis Hospital and Arnaldo Vieira de Carvalho Câncer Hospital, São Paulo, Brazil, during the period of January/2002 to December/2009. The clinical follow-up was at least 48 months after surgery. Previous surgical or chemotherapic treatment, distant metastasis, no removal of cervical lymph nodes and positive surgical margins were exclusion criteria [18].

Among analyzed individuals, mean age was 54.2 years (df ±11.1), being 173 (93.0%) men and 13 (7.0%) women in the control group. For patients with head and neck cancer, mean age was 54.9 years (df ±10.7), being 110 (85.3%) men and 25 (19.4%) women (Table 1). According to the anatomical localization of the tumor, 95

(73.6%) were on the oral cavity and 34 (26.4%) on the oropharynx. Clinical-pathological characteristics of tumors are described in Table 2.

Genotyping

Genomic DNA was extracted from peripheral blood samples of 315 individuals (cancer patients and controls) as previously described [19]. Genotypes were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). LEPR exon 6 was amplified using primers described by Snoussi et al. and analyzed for Gln223Arg polimorphism (rs1137101) [5]. Selected primers were 5' – AAA CTC AAC GAC ACT CTC CTT – 3' and 5' – TGA ACT GAC ATT AGA GGT GAC –3', which produce an 80-base pair (bp) fragment. PCR conditions were: a 25μL reaction mixture containing 200ng of genomic DNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 200μM of each deoxyribonucleoside 5´ triphosphates, 1.5mM de MgCl2, 1U Taq DNA polimerase (Life Technologies Inc® , Rockville, MD, USA) and 25pmol of each primer (Biobrás® , São Paulo, SP, Brasil). PCR initiated with a melting step of 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 52°C and 45 seconds at 72°C and 5 minutes extension at 72ºC. PCR products were digested overnight with MspI (New England Biolabs® , Berverly, MA, USA) following the manufacturer's instructions. Restriction fragments were resolved on a 12% non-denaturing polyacrylamide gel. SNP Arg223 in LEPR gene was characterized by two distinctive fragments of 58 and 22-bp, whereas the LEPR Gln223 wild-type allele was identified by a single fragments of 80-bp.

Tissue microarray

Tissue microarrays were made using buffered formalin-fixed paraffin-embedded tissue sections from 87 primary oral and oropharyngeal squamous cell carcinomas treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, SP, were used for immunohistochemistry (IHC) analysis. Histological characterization of all samples was done by Hematoxylin and Eosin staining, followed by

immunohistochemistry analysis of tissue microarrays (TMA). Two 1mm cylinders were used to represent each sample in the TMA slide (Beecher Instruments® , Silver Spring, MD, USA).

Immunohistochemistry

Anti-LEPR policlonal antibody (Santa Cruz Biotechnology® , USA) was used in the IHC reaction, at a 1:50 dilution [20-22]. Positive and negative controls (absence of primary antibody) were used for reaction quality control. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained tumor cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of LEPR immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 10-50% of cells were positive and 3 when ≥50% of cells were positive. Signal intensity was scored as weak (1), moderate (2) and strong (3). Both scores were multiplied [23,24] and the resulting score was used to categorize LEPR expression as low (<4) and high (≥4).

Statistical Analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $p<0.05$). Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI≥ 95%). Survival was calculated by the number of months between surgery and death for each patient or the last appointment in case the patient was alive. In order to calculate disease-free survival, the time endpoint was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon p-value and the Cox Proportional Hazards to adjust p-values and obtain hazard ratio (HR). Statistical calculations were performed using the Epi Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares.

RESULTS

LEPR Gln223Arg Polymorphism

Regarding the LEPR Gln223Arg polymorphism frequencies, 60 (46.5%) patients with cancer were genotyped as wild type allele Gln/Gln, 61 (47.3%) as Gln/Arg and 8 (6.2%) as Arg/Arg. In the control group, 68 (36.5%) individuals were genotyped as Gln/Gln, 92 (49.5%) as Gln/Arg and 26 (14.0%) as Arg/Arg. The observed genotype frequencies of LEPR Gln223Arg polymorphism were at Hardy-Weinberg equilibrium in the controls (p=0.563). The Arg223 allele in homozygosis was more frequent in control individuals than in cases, with a significant association (p=0.045). Multivariate analysis showed that Arg/Arg genotype is an independent factor for head and neck cancer development, representing a decreased risk of approximately 2.5 times in relation to the wild-type genotype (Gln/Gln) (OR=0.40, CI=0.16-0.97, Table 1). However, the genotype Gln/Arg showed no relation to risk.

The Gln223Arg polymorphism did not show a significant association with tumor size (p=0.406), lymph node metastases (p=0.430), lymphatic invasion (p=0.824) and perineural invasion (p=0.621), but was significantly associated with differentiation grade (p=0.038, Table 2). It was also significantly associated with disease relapse (p=0.003) and disease specific death (p=0.039, Table 2). Multivariate analysis showed that presence of Arg223 allele was an independent marker of disease relapse representing an increased risk of over 3 times, when compared to the absence of this allele (OR=3.30, CI=1.37-7.93), but the same association was not found for disease specific death (OR=2.40, CI=0.95-6.08; Table 3).

Disease-free and disease specific survival were significantly correlated with Gln223Arg polymorphism (p=0.001 and p=0.012, respectively). According to a 24 month after surgery follow up, approximately 60% of cases with at least one Arg223 allele presented disease relapse, as compared to approximately 30% of recurrence in patients without the Arg223 allele (Figure 1a). Additionally, according to a 36 month after surgery follow up, approximately 60% of cases with at least one Arg223 allele died of disease specific causes, as compared to approximately 20% of deaths in patients without the Arg223 allele (Figure 1b). Multivariate analysis revealed that presence of

Arg223 allele in LEPR gene is an independent marker for early disease relapse and disease specific death, with a 2-fold increased risk when compared to absence of this allele (respectively, HR=2.38, CI=1.29-4.29 and HR=2.11, CI=1.05-4.24, Table 4).

LEPR Expression

LEPR expression was evaluated in 87 tumors, being classified as low in 40 (46.0%) and high in 47 (54.0%). LEPR expression did not show a significant association with tumor characteristics such as size (p=0.844), differentiation grade (p=0.761), lymphatic invasion (p=0.604) and perineural invasion (p=0.054), but was significantly associated with lymph nodes metastases (p=0.006, Table 2). Multivariate analysis showed that low LEPR expression is an independent marker for lymph nodes metastases (OR=3.75, CI=1.40-10.04, Table 3).

In contrast, the LEPR expression did not significantly correlate with disease relapse (p=0.149) and disease specific death (p=0.123, Table 2), as well as disease-free and disease-specific survival (p=0.407 and p=0.259, respectively, Figure 1c and 1d).

DISCUSSION AND CONCLUSIONS

Leptin is a cell growth factor hormone that has been related with tumor cell migration and invasion, as well as angiogenesis in some tumors [25]. LEPR is involved with several signalling pathways, such as JAK/STAT, Phosph*atidilinositol 3-kinase* (PI3K), Protein Kinase B (AKT) and Mitogen Activated Protein Kinase (MAPK). These pathways, under leptin-LEPR control, are strongly related with cell survival and differentiation [26].

The aminoacid exchange caused by the LEPR Gln223Arg gene polymorphism yields a weaker interaction between leptin and its receptor, and as a result of that, a reduction in cell growth signalling [27]. Our results show that Arg223 homozygous genotype was related with a 2,5 smaller risk of delevoping cancer, when compared to the Gln223 homozygous variant. Similar results were reported in prostate cancer,

suggesting that this allele increases tumor development risk [28]. Therefore, Gln223Arg may play an important role in the inhibition of cell division [29]. In contrast, in breast cancer, it appears that the Arg223 allele increases cancer risk [5], shedding uncertainty as to through which mechanism this allele interacts with oncogenesis.

In contrast, our results suggest that presence of Arg223 is related with relapse risk and with worse disease-free and disease-specific survival. Similar studies in breast [5] and prostate cancer [28], show that Arg223 bearing patients have a significant increased risk of relapse, and consequently worse prognosis. Reports that high levels of circulating leptin are related with a decreased globa survival of breast cancer patients [30] and that a weak interaction between Leptin-LEPR favours angionesis and tumor growth [31,32], support the hypothesis that presence of Arg223 may result in worse prognosis in these patients. In contrast with these results, lung [12] and colorectal cancer [11] studies present allele Gln223 as the one related with worse prognosis.

According to our results, low LEPR expression increasis the risk of lymph node metastasis by 4 fold, when compared with strong expression. Considering that weak LEPR expression has a similar effect to the Gln223Arg polymorphism, weak Leptin-LEPR interaction and increased circulating Leptin, [31,33], these effects have been previously suggested as facilitators of tumor invasion and metastasis [11,34].

In conclusion, the present work attributed a protective role to the homozygous LEPR Arg223 allele in oral and oropharyngeal cancer. Arg223 showed to be an independent marker of worse prognosis, as shown by a lower disease-free and disease-specific survival. Low LEPR expression was an independent marker of metastasis risk, showing a 4-fold increased risk of lymph node metastasis. These results suggest a tumor marker role for LEPR, as well as a potential target for molecular therapies in oral and oropharyngeal cancer patients.

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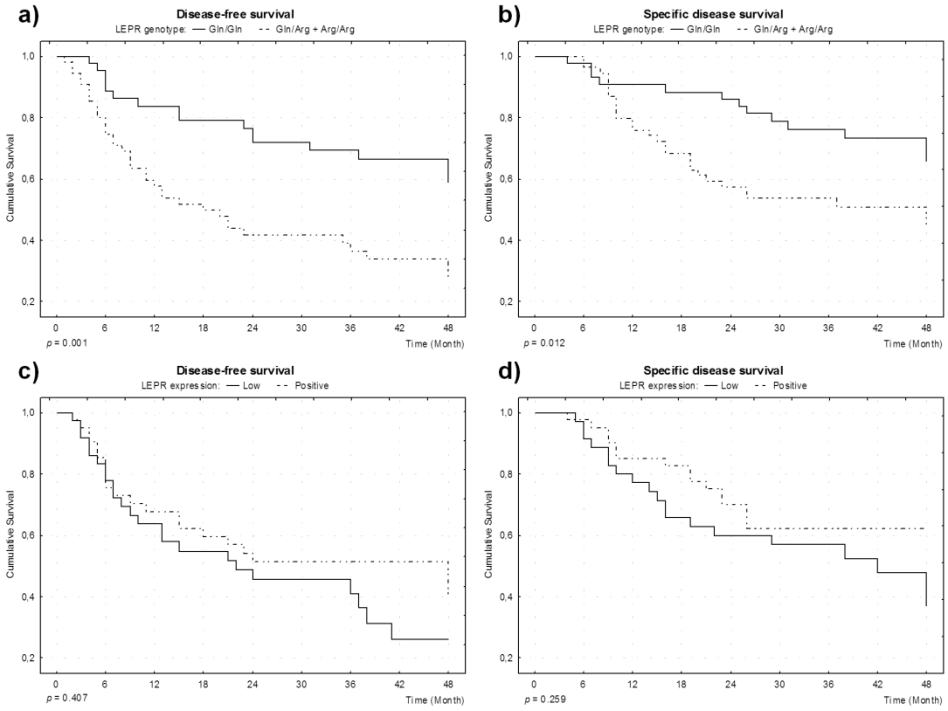
FIGURE LEGEND

Figure 1. Survival plots. a. and b.: Disease-free survival and disease specific survival according to LEPR Gln223Arg polymorphism; **c. and d.:** Disease-free survival and disease specific survival according to LEPR expression.



TABLES

Table 1. Epidemiological features and LEPR Gln223Arg polymorphism associated with oral and oropharyngeal cancer risk.

Features	Case		Control		*P*	Multivariate analysis	
	No.	**(%)**	**No.**	**(%)**		**OR (CI 95%)** [a]	***p*** [b]
Gender							
Female	25	(19.4)	13	(7.0)	0.001	1	
Male	110	(85.3)	173	(93.0)		0.12 (0.04-0.35)	<0.001
Age. yr (mean 54.5, df±10.9)							
≤ 55	72	(55.8)	105	(56.5)	0.497	–	–
> 55	63	(48.8)	81	(43.5)		–	–
Smoker							
No	59	(45.7)	103	(55.4)	0.039	1	
Yes	76	(58.9)	83	(44.6)		3.57 (1.48-8.61)	0.005
Drinking habit							
No	34	(26.4)	96	(51.6)	< 0.001	1	
Yes	101	(78.3)	90	(48.4)		2.42 (0.98-5.96)	0.055
LEPR genotype Gln223Arg							
Gln/Gln	60	(46.5)	68	(36.5)	0.045	1	
Gln/Arg	61	(47.3)	92	(49.5)		0.78 (0.47-1.28)	0.317
Arg/Arg	8	(6.2)	26	(14.0)		0.40 (0.16-0.97)	0.042
Total	**129**	**(41.0)**	**186**	**(59.0)**			

OR – Odds ratio; CI – Confidence interval.

[a, b] Values adjusted by multivariate logistic regression.

Table 2. Clinical and pathological tumor features and their association with Gln223Arg polymorphism and LEPR expression.

Features	LEPR									
	Genotype Gln223Arg					Expression level				
	Total		Gln/Gln	Gln/Arg +Arg/Arg	*p*	Total		Low	High	*p*
	No.	(%)				No.	(%)			
Tumor size (T) ¥										
T1+T2	50	(38.8)	21	29	0.406	34	(39.1)	15	19	0.844
T3	31	(24.0)	13	18		21	(24.1)	9	12	
T4	48	(37.2)	26	22		32	(36.8)	16	16	
Lymph-node (N) ¥										
Absent (N-)	64	(49.6)	32	32	0.430	33	(37.9)	9	24	0.006
Present (N+)	65	(50.4)	28	37		54	(62.1)	31	23	
Differentiation										
Well	52	(40.3)	21	31	0.038	38	(43.7)	18	20	0.761
Moderately	69	(53.5)	38	31		42	(48.3)	18	24	
Poorly	8	(6.2)	1	7		7	(8.0)	4	3	
Lymphatic invasion										
Negative	61	(47.3)	29	32	0.824	28	(32.2)	14	14	0.604
Positive	68	(52.7)	31	37		59	(67.8)	26	33	
Perineural invasion										
Negative	93	(72.1)	42	51	0.621	65	(74.7)	26	39	0.054
Positive	36	(27.9)	18	18		22	(25.3)	14	8	
Disease relapse										
No	50	(38.8)	30	20	0.003	35	(40.2)	13	22	0.149
Yes	52	(40.3)	16	36		45	(51.7)	24	21	
Not available *	27	(20.9)	14	13		7	(8.0)	3	4	
Disease specific death										
No	59	(45.7)	31	28	0.039	42	(48.3)	16	26	0.123
Yes	41	(31.8)	13	28		36	(41.4)	20	16	
Not available *	29	(22.5)	16	13		9	(10.3)	4	5	
Total	**129**	**(100.0)**	**60**	**69**		**87**	**(100.0)**	**40**	**47**	

* Not available(not considered in the statistical calculations).

¥ TNM classification 3rd edition.

Table 3. Multivariate analysis of the relationship between clinical and pathological tumor features with gene polymorphism and LEPR expression.

Features	Multivariate Analysis					
	Lymph-node (N) ¥		Disease relapse		Disease specific death	
	OR (95% CI) [a]	*p* [b]	OR (95% CI) [a]	*p* [b]	OR (95% CI) [a]	*p* [b]
LEPR expression						
High	1		–	–	–	–
Low	3.75 (1.40-10.04)	0.009	–	–	–	–
LEPR Gln223Arg						
Gln/Gln	–	–	1		1	
Gln/Arg+Arg/Arg	–	–	3.30 (1.37-7.93)	0.008	2.40 (0.95-6.08)	0.065
Tumor size (T) ¥						
T1+T2	1		1		1	
T3	1.13 (0.36-3.56)	0.833	1.04 (0.34-3.15)	0.946	1.22 (0.37-4.02)	0.740
T4	4.56 (1.43-14.62)	0.011	1.24 (0.44-3.45)	0.682	2.73 (0.96-7.80)	0.060
Differentiation						
Well	1		–	–	–	–
Moderately	2.33 (0.79-6.91)	0.126	–	–	–	–
Poorly	3.61 (0.31-42.76)	0.309	–	–	–	–
Lymph node (N) ¥						
Absent (N-)	–	–	1		1	
Present (N+)	–	–	4.67 (1.61-13.59)	0.005	6.67 (2.24-19.87)	<0.001
Irradiated						
No	–	–	1		1	
Yes	–	–	0.23 (0.08-0.68)	0.008	0.56 (0.18-1.70)	0.304

OR – Odds ratio; CI – Confidence interval.

[a, b] Values adjusted by multivariate logistic regression.

[¥] TNM classification 3rd edition.

Table 4. Multivariate analysis of disease specific survival.

Features	Cox Proportional			
	Disease-free survival		Specific disease survival	
	HR (95% CI) [a]	*p* [b]	HR (95% CI) [a]	*p* [b]
LEPR genotype Gln223Arg				
Gln/Gln	1		1	
Gln/Arg+Arg/Arg	2.38 (1.29-4.39)	0.006	2.11 (1.05-4.24)	0.036
Tumor size (T) [¥]				
T1+T2	1		1	
T3	1.22 (0.58-2.54)	0.598	1.27 (0.52-3.11)	0.604
T4	1.37 (0.71-2.63)	0.343	1.62 (0.80-3.29)	0.184
Lymph-node (N) [¥]				
Absent	1		1	
Present	2.52 (1.27-4.99)	0.008	3.28 (1.44-7.47)	0.005
Irradiated				
No	1		1	
Yes	0.39 (0.20-0.74)	0.004	0.81 (0.37-1.76)	0.592

HR – Hazard ratio; CI – Confidence interval.

[a, b] Values adjusted by Cox proportional hazards.

[¥] TNM classification 3rd edition.

4.6. Manuscrito 2: JMJD1A expression as a prognostic marker in squamous cell carcinoma of the oral cavity

O manuscrito intitulado "*JMJD1A expression as a prognostic marker in squamous cell carcinoma of the oral cavity*" foi submetido para a revista *Plos One*, a qual possui fator de impacto de 3.73 (JCR 2012).

JMJD1A EXPRESSION AS A PROGNOSTIC MARKER IN ORAL AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

Authors

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Running title

JMJD1A expression in oral and oropharyngeal cancer

Keywords

JMJD1A expression; lymph-node; prognostic marker; head neck cancer

Competing interests

Authors declare that they have no competing interests.

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ABSTRACT

JMJD1A is a histone demethylase, which plays an important role in DNA epigenetic regulation, being able to alter gene expression, DNA replication and repair, as well as cell differentiation. Moreover, JMJD1A protein has been related with development risk and prognosis of several tumor types. Nonetheless, it role in head and neck cancer has yet to be clarified. Therefore, we aimed to evaluate JMJD1A expression in 80 paraffinized oral and oropharyngeal squamous cell carcinoma samples through immunohistochemistry analysis. Our results show that nuclear and cytoplasm expression were related with risk for lymph node metastasis, in a way that strong expression increases risk by 4 to 11 times respectively. In addition, strong expression was associated with disease relapse and worse disease specific survival. In conclusion, we suggest propose JMJD1A as a promising prognostic marker for oral and oropharyngeal squamous cell carcinoma patients.

INTRODUCTION

Head and neck cancer (HNC) is a significant cause of mortality and morbidity worldwide, presenting approximately 600,000 new cases yearly [1], whereas tumors of the oral cavity (OSCC) contribute with 389,000 new cases per year, with a mortality rate of 50% [2,3]. The main prognostic factors for HNC is lymph node metastasis, decreasing by 50% the patient survival chance. [4].

HNC is a complex disease, caused by multiple factors such as smoking and drinking habits, HPV infection, dietary and genetic factors [5].

Tumor cell response to hypoxia involves activation of over 100 genes [6]. Currently, little is known about the epigenetic relation resulting from HIF system transcription activation [7]. However, such changes probably include epigenetic histone modifications [8].

The protein Jumonji Domain-Containing 1A (JMJD1A, JHDM2A or KDM3A) is regulated by HIF1a under hypoxic conditions. JMJD1A gene is activated via its hypoxia response elements in the promoter region, promoting demethylation of genes that will help cell adaptation in low oxygen environments [9]. Demethylation occurs at lysin and arginine residues in an oxygen-dependent reaction that needs Fe (II) ions and α-ketoglutarate as cofactors [10]. This can alter tumor cell behavior due to chromatin structural changes, gene expression and DNA repair [8,11].

In embryonic stem cells, JMJD1A helps maintain pluripotency, inhibiting demethylation of differentiation gene promoters [12]. JMJD1A has been associated with development and prognosis of several tumor types, such as colorectal [13], nasopharyngeal [14], hepatocellular [15] and renal [16] tumors. Its role in HNC is still a matter of debate.

Therefore, we have aimed to study the association of JMJD1A with clinicopathological features and prognosis of patients with HNC or the oral and oropharyngeal cavities.

MATERIALS AND METHODS

Ethics

This study was approved by the Committee of Ethics in Research of the Heliópolis Hospital (CEP # 619) and a written informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from 9 institutions in São Paulo State, Brazil, whose aim is to develop clinical, genetic and epidemiological analysis of HNSCC. In this study, 80 tumoral tissue samples were obtained and used for immunohistochemical analysis of the JMJD1A, within a total of 80 patients with oral and oropharyngeal squamous cell carcinomas, surgically treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, Brazil, during the period of January/2002 to December/2008. The clinical follow-up was at least 24 months after surgery. Previous surgical or chemotherapic treatment, distant metastasis, no removal of cervical lymph nodes and positive surgical margins were exclusion criteria. Histopathological slides were reviewed by a senior pathologist to confirm the diagnosis and select appropriate areas for immunohistochemical analysis. Tumors were classified according to the TNM system (3^{rd} edition) [17].

Among the analyzed individuals, the mean age was 54.4 years (df ±10.4) being 68 (85%) men and 12 (15%) women. According to tumor anatomical sites, 60 (75%) were in the oral and 20 (25%) in the oropharyngeal cavity (Table 1).

Tissue microarray

Tissue microarrays were made using buffered formalin-fixed paraffin-embedded tissue sections from 80 primary oral and oropharyngeal squamous cell carcinomas

treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, SP, were used for immunohistochemistry (IHC) analysis. Histological characterization of all samples was done by Hematoxylin and Eosin staining, followed by immunohistochemistry analysis of tissue microarrays (TMA). Two 1mm cylinders were used to represent each sample in the TMA slide (Beecher Instruments®, Silver Spring, MD, USA).

Immunohistochemistry

Anti-JMJD1A monoclonal antibody (Abcam®) was used in the IHC reaction, at a 1:400 dilution [18-20]. Positive and negative controls (absence of primary antibody) were used for reaction quality control. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained tumor cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of JMJD1A immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 11-50% of cells were positive and 3 when >50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2) and strong (3). Both scores were multiplied [21,22] and the resulting score was used to categorize JMJD1A expression as negative (0), positive low (1-4) and positive high (≥4).

Statistical Analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $p<0.05$). Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI ≥95%). Survival was calculated by the number of months between surgery and death for each patient or the last appointment in case the patient was alive. In order to calculate disease-free survival, the time endpoint was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon p-value and the Cox Proportional Hazards to adjust p-values and obtain hazard ratio (HR). Statistical

calculations were performed using the Epi Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares.

RESULTS

JMJD1A nuclear expression positivity was studied in 80 tumors, of which 27 were negative (33.8%), 31 were weakly positive (38.8%) and 22 were strongly positive (27.5%). Regarding JMJD1A cytoplasmic expression, 11 were negative (13.8%), 63 (78.8%) were weakly positive and only 6 (7.5%) were strongly positive. Differential expression by intracellular localization was significantly different ($p < 0.001$, Table 2).

JMJD1A cytoplasmic expression

Positive JMJD1A cytoplasmic expression did not show a significant association with differentiation grade ($p=0.342$), disease relapse ($p=0.414$) and disease specific death ($p=0.434$), but the expression was significantly associated with tumor size ($p=0.023$) and lymph-node status ($p<0.001$, Table 3). Multivariate analysis showed that positive JMJD1A cytoplasmic expression was an independent marker for lymph-node positivity, yielding an approximately 11-fold increased risk (OR=11.46, CI=1.10-119.38, Table 4). In addition, cytoplasmic expression levels did not show a significant relation with tumor characteristics such as size ($p=0.432$), differentiation grade ($p=0.590$), lymph node status ($p=0.341$), disease relapse ($p=0.081$) and disease specific death ($p=0.178$, Table 4).

Cytoplasmic expression positivity was not significantly associated with disease free and disease specific survival ($p=0.505$ and $p=0.670$, respectively), neither was expression levels ($p=0.150$ and $p=0.159$, respectively; data not show).

JMJD1A nuclear expression

Positive JMJD1A nuclear expression did not show a significant association with tumor characteristics such as size (p=0.348), differentiation grade (p=0.972), disease relapse (p=0.408) and disease specific death (p=0.830), but it was significantly associated with lymph-node status (p<0.001, Table 3). Multivariate analysis showed that positive JMJD1A nuclear expression was an independent marker for lymph-node positivity, yielding an approximately 4-fold increased risk (OR=3.76, CI=1.21-11.74, Table 4).

JMJD1A nuclear expression levels did not show a significant relation with tumor size (p=0.762), differentiation grade (p=0.888), lymph node status (p=0.992) and disease specific death (p=0.061), but it was significantly associated with disease relapse (p<0.040, Table 3). Multivariate analysis showed that high expression was an independent marker of disease relapse representing an increased risk over of 5 times, when compared to nuclear JMJD1A low expression (OR=5.09, CI=1.11-23.38, Table 5).

Disease free and disease specific survival did not show association with JMJD1A nuclear expression positivity (p=0.902 and p=0.959, respectively, data not show). JMJD1A nuclear expression levels did not show an association with disease free survival either (p=0.172, data not show), but it was significantly associated with disease specific survival (p=0.039). According to a 36 month after surgery follow up, approximately 60% of cases with high expression died of the disease, as compared to approximately 35% of death in patients with low nuclear expression (Figure 1). Multivariate analysis revealed that JMJD1A high nuclear expression is an independent marker for faster disease specific death, with a 2.5 fold increased risk when compared to low expression (HR=2.56, CI=1.21-5.41, Table 5).

DISCUSSION AND CONCLUSIONS

JMJD1A protein promotes demethylation of histones, especialy at lysin-9 of bi-methylated histone H3 (H3K9me2) or mono-methylated (H3K9me1) [10]. Histone

demethylation alters chromatin structure resulting in gene expression changes, DNA repair, replication [11], as well as cell differentiation [12]. JMJD1A expression has been related to development and prognosis of diverse tumor types [13-16].

Our results suggest that positive JMJD1A nuclear expression increases lymph node metastasis risk by 4-fold, whereas cytoplasmic expression increases risk by over 11-fold. Colorectal cancer studies have correlated high JMJD1A expression with an augmented risk for lymph node positivity by over 6-fold [13].

We also show that cytoplasmic JMJD1A expression is related with tumor size, which could be due to activation of cell cycle regulating genes, such as Cyclin A1(CCNA1) [23], adrenomedullin (ADM) [11] and Cyclin D1 (CCND1) [24].

JMJD1A gene expression results in activation of homeobox A1 (HOXA1) gene, which induces CCND1 gene expression, increasing cell cycling. Over activation of CCND1 may result in an uncontrolled proliferation state and tumor metastasis. IN colorectal cancer, under hypoxic conditions, JMJD1A expression has been related to ADM protein expression and tumor cell growth [11].

Hypoxic and starved tumor cells may show a more aggressive behavior, due to profound metabolic changes [15,25], which favors the hypothesis that large and hypoxic tumor cells may migrate to other regions in search of more favorable conditions (metastasis). Our results show that strong nuclear JMJD1A expression correlates with a 5-fold increased risk of disease relapse. In addition, strong nuclear expression was related with worse disease specific survival. Similar results were described for hepatocellular carcinoma, in which strong expression was related with higher relapse risk [26].

Therefore, JMJD1A may be considered a marker of worse prognosis, for been related with epigenetic activation of various genes associated with tumor growth and treatment resistance [13,25,27].

Moreover, JMJD1A is related with non-differentiation of stem cells [12]. Indifferentiated cell are responsible for tumor initiation, progression and aggressiveness

[28]. In conclusion, the present study proposes JMJD1A as a promising prognostic marker for oral and oropharyngeal squamous cell carcinomas.

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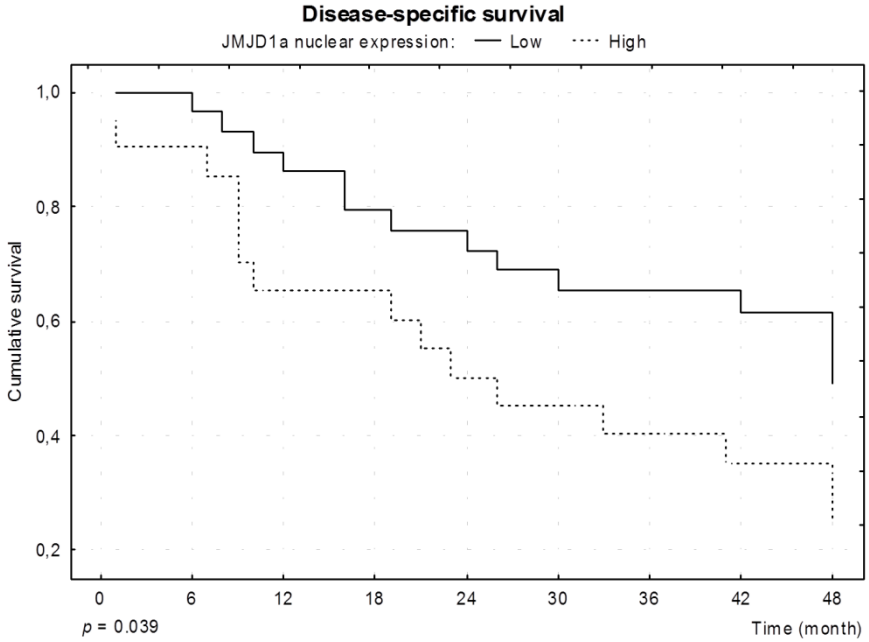
FIGURE LEGEND

Figure 1. Survival plots. Disease specific survival according to JMJD1A nuclear expression.



TABLES

Table 1. Epidemiological features.

Features	Total	
	No.	(%)
Gender		
Female	12	(15.0)
Male	68	(85.0)
Age, yr		
median 54.4, df±10,4		
Smoker	57	(71.3)
Alcohol user	44	(55.0)
Tumor sites		
Oral cavity	60	(75.0)
Oropharingeal	20	(25.0)
Tumor stage		
I, II	19	(23.8)
III	20	(25.0)
IV	41	(51.3)
Total	**80**	**(100.0)**

Table 2. JMJD1A expression in primary tumor cells, according to cell localization.

JMJD1A expression	Cellular localization				*p*
	Nuclear		Cytoplasmic		
	No.	(%)	No.	(%)	
Negative	27	(33.8)	11	(13.8)	<0.001
Low	31	(38.8)	63	(78.8)	
High	22	(27.5)	6	(7.5)	
Total	**80**	**(100.0)**	**80**	**(100.0)**	

Table 3. Clinical and pathological tumor features and their association with JMJD1A expression, according to cell localization.

Features	JMJD1A expression																	
	Nuclear									Cytoplasmic								
	Negative		Positive		*p*	Low		High		*p*	Negative		Positive		*p*	Low		High		*p*
	No.	(%)	No.	(%)		No.	(%)	No.	(%)		No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Tumor size (T) ¥																				
T1, T2	14	(51.9)	19	(35.8)	0.348	12	(38.7)	7	(31.8)	0.762	6	(54.5)	27	(39.1)	0.023	24	(38.1)	3	(50.0)	0.432
T3	6	(22.2)	13	(24.5)		8	(25.8)	5	(22.7)		5	(45.5)	14	(20.3)		12	(19.0)	2	(33.3)	
T4	7	(25.9)	21	(39.6)		11	(35.5)	10	(45.5)		0	(0.0)	28	(40.6)		27	(42.9)	1	(16.7)	
Lymph node (N) ¥																				
Negative	18	(66.7)	15	(28.3)	<0.001	9	(29.0)	6	(27.3)	0.888	10	(90.9)	23	(33.3)	<0.001	22	(34.9)	1	(16.7)	0.341
Positive	9	(33.3)	38	(71.7)		22	(71.0)	16	(72.7)		1	(9.1)	46	(66.7)		41	(65.1)	5	(83.3)	
Differentiation																				
Well	11	(40.7)	22	(41.5)	0.972	13	(41.9)	9	(40.9)	0.992	6	(54.5)	27	(39.1)	0.619	25	(39.7)	2	(33.3)	0.590
Moderate	13	(48.1)	26	(49.1)		15	(48.4)	11	(50.0)		4	(36.4)	35	(50.7)		31	(49.2)	4	(66.7)	
Poorly	3	(11.1)	5	(9.4)		3	(9.7)	2	(9.1)		1	(9.1)	7	(10.1)		7	(11.1)	0	(0.0)	
Disease relapse																				
No	11	(40.7)	16	(30.2)	0.408	13	(41.9)	3	(13.6)	0.040	5	(45.5)	22	(31.9)	0.414	22	(34.9)	0	(0.0)	0.081
Yes	16	(59.3)	35	(66.0)		18	(58.1)	17	(77.3)		6	(54.5)	45	(65.2)		39	(61.9)	6	(100.0)	
Not available§	0	(0.0)	2	(3.8)		0	(0.0)	2	(9.1)		0	(0.0)	2	(2.9)		2	(3.2)	0	(0.0)	
Disease specific death																				
No	13	(48.1)	29	(54.7)	0.830	14	(45.2)	15	(68.2)	0.061	6	(54.5)	36	(52.2)	0.434	31	(49.2)	5	(83.3)	0.178
Yes	10	(37.0)	20	(37.7)		15	(48.4)	5	(22.7)		3	(27.3)	27	(39.1)		26	(41.3)	1	(16.7)	
Not available§	4	(14.8)	4	(7.5)		2	(6.5)	2	(9.1)		2	(18.2)	6	(8.7)		6	(9.5)	0	(0.0)	
Total	**27**	**(33.8)**	**53**	**(66.3)**		**31**	**(58.5)**	**22**	**(41.5)**		**11**	**(13.8)**	**69**	**(86.3)**		**63**	**(91.3)**	**6**	**(8.7)**	

[¥] TNM classification 3rd edition.

[§] Not available (not considered in the statistical calculations).

Table 4. Multivariate analysis of the relationship between lymph node status and JMJD1A expression.

Features	Multivariate Analysis	
	Lymph nodes (N) [¥]	
	OR (CI 95%)	*p*
JMJD1A Nuclear expression		
Negative	1	
Positive	3.76 (1.21-11.74)	0.022
JMJD1A Cytoplasmic expression		
Negative	1	
Positive	11.46 (1.10-119.38)	0.041
Tumor size (T) [¥]		
T1, T2	1	
T3	2.93 (0.68-12.64)	0.151
T4	3.77 (1.06-13.41)	0.041
Differentiation		
Well	1	
Moderate	1.76 (0.56-5.59)	0.337
Poorly	3.11 (0.34-28.06)	0.312

[¥] TNM classification 3rd edition.

Table 5. Multivariate analysis of the relationship between JMJD1A expression and prognosis.

Variable	Multivariate Analysis		Cox Proportional	
	Disease relapse		Disease specific survival	
	OR (CI 95%)	*P*	HR (CI 95%)	*p*
JMJD1A Nuclear expression				
Low	1		1	
High	5.09 (1.11-23.38)	0.036	2.56 (1.21-5.41)	0.014
Stage tumor				
I, II	1		1	
III	2.50 (0.35-17.91)	0.362	3.24 (0.82-12.82)	0.094
IV	3.37 (0.60-19.11)	0.170	4.42 (1.27-15.44)	0.020