Rodrigo Vieira Rodrigues

**IDENTIFICAÇÃO E CARACTERIZAÇÃO DE
MARCADORES MOLECULARES EM CARCINOMAS
EPIDERMÓIDES DE CABEÇA E PESCOÇO**

IDENTIFICATION AND CHARACTERIZATION OF
MOLECULAR MARKERS IN HEAD AND NECK
SQUAMOUS CELL CARCINOMA

São Paulo

2011

Rodrigo Vieira Rodrigues

Versão Corrigida

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Tese apresentada ao Instituto de
Biociências da Universidade de São Paulo,
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Orientadora: Eloiza Helena Tajara da Silva

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Aos pacientes e seus familiares,
por sua coragem e determinação
na luta contra o câncer.

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CAPÍTULO 1

INTRODUÇÃO GERAL

CARCINOMA EPIDERMÓIDE DE CABEÇA E PESCOÇO

O carcinoma epidermóide de cabeça e pescoço (CECP) é uma doença heterogênea de origem epitelial que atinge a cavidade oral, a faringe e a laringe (McMahon and Chen 2003), sendo seu principal fator etiológico a exposição crônica ao álcool e ao tabaco (Goldenberg, Lee et al. 2004; Hashibe, Brennan et al. 2009). Embora seja bem conhecido o fato de que há uma elevação da sua frequência com a idade (Llewellyn, Johnson et al. 2001; Reichart 2001), alguns estudos relatam um aumento de incidência em homens jovens (Patel, Carpenter et al. 2011), mesmo entre aqueles que nunca fumaram ou consumiram álcool. Esses dados indicam que as causas da doença ainda não são bem conhecidas (Llewellyn, Johnson et al. 2001).

No Brasil, foram estimados cerca de 14 mil novos casos em 2010, considerando somente os de cavidade oral (Brasil. Ministério da Saúde 2009), o que representa a maior taxa de incidência desse subgrupo em países com renda média-alta (de Camargo Cancela, Voti et al. 2010). Mundialmente, mais de 500.000 novos casos de carcinomas de CECP são diagnosticados por ano e a incidência varia em diferentes países, provavelmente como resultado de exposição a fatores ambientais distintos (Parkin, Bray et al. 2005). Por exemplo, na França são encontradas taxas mais altas de carcinoma de cavidade oral em comparação aos Estados Unidos e ao Brasil (Moriniere 2006; Jemal, Siegel et al. 2007; Brasil. Ministério da Saúde 2009).

Apesar do uso de tabaco e do consumo de álcool serem os principais fatores de risco em CECP (Goldenberg, Lee et al. 2004; Hashibe, Brennan et al. 2009), outros agentes etiológicos estão relacionados com diferentes etapas da tumorigênese de cabeça e pescoço, como ocupação profissional, características hereditárias, deficiência imunológica e infecção viral (Gallo 1996). Por exemplo, diversos trabalhos indicam o envolvimento do papilomavírus humano (HPV) na etiologia dos CECPs (D'Souza, Kreimer et al. 2007; IARC 2007; Ang, Harris et al. 2010; Jung, Briolat et al. 2010), especialmente nos tumores de orofaringe (Syrjanen 2005; Smeets, Brakenhoff et al. 2009). Alguns polimorfismos em enzimas que metabolizam tabaco e álcool (Hashibe, Boffetta et al. 2006), história familiar (Suarez, Rodrigo et al. 2006; Negri, Boffetta et al.

2009) e baixa ingestão de frutas e vegetais (Freedman, Park et al. 2008; Sapkota, Hsu et al. 2008) também são fatores de risco conhecidos.

Embora tenham ocorrido avanços significativos no tratamento dessa doença, levando à diminuição da morbidade, a taxa de sobrevida em cinco anos permaneceu praticamente inalterada ao longo das últimas décadas. A evolução clínica e a resposta à terapia dos carcinomas epidermóides de cabeça e pescoço são bastante variáveis, mesmo nos casos histologicamente semelhantes (Lingen, Sturgis et al. 2001; Rodrigo, Suarez et al. 2001). Aqueles pacientes com tumores em estágios iniciais geralmente apresentam poucos sintomas, o que costuma atrasar o diagnóstico e reduzir a expectativa de vida. Muitas vezes, o câncer é diagnosticado e tratado apenas quando suas células já invadiram tecidos adjacentes (Wulfkühle, Paweletz et al. 2003).

Atualmente, as decisões sobre tratamento e prognóstico têm como base a avaliação histopatológica dos tumores e de suas margens cirúrgicas, a presença de metástases em linfonodos e o tamanho do tumor. Contudo, essas variáveis são frequentemente insuficientes para predizer o desfecho clínico. Por exemplo, o status das margens cirúrgicas é utilizado na avaliação do prognóstico, tanto para recorrência como para metástases à distância (Martone, Gillio-Tos et al. 2007). Entretanto, alguns pacientes com margens negativas manifestam lesões múltiplas no mesmo sítio anatômico ou em outros sítios do trato aerodigestivo superior (Poh, Zhang et al. 2006; Roblyer, Kurachi et al. 2009). Estes achados corroboram a hipótese de cancerização de campo proposta por Slaughter e colaboradores (1953) (Slaughter, Southwick et al. 1953), segundo a qual o epitélio ao redor do tumor e de sítios anatômicos vizinhos apresenta alterações que o tornam mais suscetível ao aparecimento de novos tumores. Isto ocorre devido à exposição crônica a insultos carcinogênicos em toda a mucosa do trato aerodigestivo (Lopez-Blanc, Collet et al. 2009).

Essas alterações do tecido são, provavelmente, decorrentes de modificações genéticas e epigenéticas que afetam vias metabólicas importantes e podem ser exploradas tanto para o diagnóstico quanto para o prognóstico de doenças (Leemans, Braakhuis et al. 2011). Califano e colaboradores (1996) sugeriram um modelo de progressão tumoral em CECP envolvendo modificações moleculares durante as várias etapas do desenvolvimento neoplásico (Califano, van der Riet et al. 1996). Nesse modelo, a inativação de genes supressores de tumor e a ativação de proto-oncogenes surgem sequencialmente, desde lesões hiperplásicas benignas até carcinoma invasivo. Alguns estudos moleculares têm continuamente acrescentado novos elementos ao

modelo de Califano, incluindo mudanças epigenéticas e do microambiente tumoral (Argiris, Karamouzis et al. 2008).

O estabelecimento do padrão de metilação apropriado do DNA é essencial para o desenvolvimento e o funcionamento celular normal. Qualquer anormalidade nesse processo pode levar ao surgimento de várias doenças, incluindo o câncer. A inativação epigenética de genes relacionados com o controle normal do crescimento celular é um evento frequente em câncer é tão importante quanto a inativação por mutação (Esteller and Herman 2002; Park, Claus et al. 2011). A repressão transcricional epigenética tem sido demonstrada em uma ampla variedade de tipos tumorais e ocorre em genes de reparo do DNA, supressores de tumor, reguladores do ciclo celular e em genes envolvidos em processos de invasão e metástase (Garinis, Patrinos et al. 2002; Jaenisch and Bird 2003; Hill, Ricketts et al. 2011; Paluszczak, Misiak et al. 2011).

Nos últimos anos, muitos pesquisadores têm concentrado seus esforços na busca de marcadores moleculares que possam ser utilizados como alvos terapêuticos ou que auxiliem no diagnóstico precoce desse grupo de neoplasias, no seu prognóstico e na identificação de populações de risco (Hunter, Parkinson et al. 2005; Karamouzis, Grandis et al. 2007; Argiris, Karamouzis et al. 2008). Nesse contexto, várias técnicas de análise de expressão gênica de amplo alcance foram desenvolvidas. Entre essas técnicas, estão os microarranjos de cDNA, a análise seriada de expressão gênica (SAGE) e, mais recentemente, as técnicas de sequenciamento em larga escala, que têm fornecido uma lista crescente de biomarcadores potenciais.

Tais avanços tecnológicos, principalmente no que se refere às novas metodologias de sequenciamento, bem como a novos métodos de isolamento de complexos de DNA, RNA e proteínas, têm possibilitado a melhor compreensão de processos moleculares como *splicing* alternativo e regulação da transcrição gênica por RNAs não codificantes (Licatalosi, Mele et al. 2008; Sultan, Schulz et al. 2008; Yang, Tschaplinski et al. 2011). Na verdade, os dados têm mostrado que o dogma central da biologia molecular, que coloca os RNAs apenas como intermediários entre as moléculas de DNA e proteína, está caindo por terra.

Vários estudos recentes têm fornecido evidências de que mais de 90% dos genes humanos sofrem *splicing* alternativo (Pan, Shai et al. 2008; Wang, Sandberg et al. 2008). Os mecanismos de *splicing* alternativo e *trans-splicing* têm revelado a versatilidade do genoma e a importância da unidade de transcrição como fonte de informação genética (Costa, 2010). Tais mecanismos podem ser regulados

diferencialmente dependendo do tipo celular, do estágio do desenvolvimento ou por diferentes padrões de sinalização (Cooper, Wan et al. 2009) e, quando alterados, representam um fator causal de doenças humanas, incluindo o câncer (Cork, Lennard et al. 2008; Tazi, Bakkour et al. 2009).

Apesar do enorme conhecimento acumulado nos últimos anos, muitos dos eventos genéticos responsáveis pelo desenvolvimento e pela progressão neoplásica permanecem desconhecidos e poucos marcadores moleculares são comumente utilizados na prática médica (Goerner, Seiwert et al. 2010). A busca de novos marcadores moleculares para esse grupo de doenças é, portanto, muito importante, especialmente aqueles relacionados com detecção precoce que podem elevar consideravelmente a taxa de sobrevivência dos pacientes.

OBJETIVOS GERAIS

O objetivo geral do presente trabalho foi investigar biomarcadores de diagnóstico e prognóstico em carcinomas epidermóides de cabeça e pescoço. Os objetivos específicos compreenderam:

1. Identificar diferenças no padrão de metilação do DNA entre linhagens celulares tratadas e não tratadas com droga desmetilantes, bem como em tumores primários de carcinomas epidermóides de cabeça e pescoço.
2. Analisar, pelos métodos de SAGE (*Serial Analysis of Gene Expression*) e PCR quantitativo em tempo real (qRT-PCR), o perfil de expressão gênica global em amostras de carcinomas epidermóides de cabeça e pescoço.
3. Analisar o perfil de expressão de variantes de *splicing* alternativo em linhagens celulares submetidas a condições de estresse de temperatura, bem como validar a expressão diferencial de transcritos alternativos em amostras primárias de carcinomas epidermóides de cabeça e pescoço.

CAPÍTULO 2

INTRODUÇÃO

O carcinoma epidermóide de cabeça e pescoço (CECP) é uma doença heterogênea de origem epitelial que atinge a cavidade oral, a faringe e a laringe, sendo seu principal fator etiológico a exposição crônica ao álcool e ao tabaco. Embora tenham ocorrido avanços significativos no tratamento dessa doença, levando à diminuição da morbidade, a taxa de sobrevida em cinco anos permaneceu praticamente inalterada ao longo das últimas décadas. A busca de novos marcadores moleculares para esse grupo de doenças é, portanto, muito importante, especialmente aqueles relacionados com detecção precoce que podem elevar consideravelmente a taxa de sobrevida dos pacientes.

O fenótipo maligno é caracterizado pelo acúmulo de mutações e eventos epigenéticos que modificam a função de proteínas e/ou causam alterações nos padrões transcricionais. A metilação do DNA é uns dos mais importantes mecanismos de silenciamento gênico em tumores e geralmente confere vantagens às células neoplásicas. Sua análise compreende uma ferramenta importante na avaliação de alguns dos mecanismos envolvidos no processo neoplásico.

MATERIAIS E MÉTODOS

Neste estudo, os métodos de hibridização subtrativa rápida (*Rapid Subtractive Hybridization - RaSH*), microarranjos de cDNA (*cDNA microarrays*) e PCR quantitativa em tempo real foram combinados de maneira hierárquica para selecionar genes que são supostamente reativados pelo agente desmetilante 5-aza-2'-deoxicitidina (5-Aza-dC) em linhagens celulares de carcinomas epidermóides de cabeça e pescoço (*FaDu*, *UM-SCC-14A*, *UM-SCC-17A*, *UM-SCC-38A*). O perfil de metilação do DNA das ilhas CpGs associadas aos promotores dos genes reativados pela droga 5-Aza-dC foram investigados pela técnica de sequenciamento por bissulfito. A análise de metilação diferencial do DNA foi analisada, pelo método de MSP (*methyl-specific PCR*), em um painel de 140 amostras de carcinomas primários de cabeça e pescoço. Além disso, a expressão de proteínas de interesse foi avaliada por imunohistoquímica em 75 amostras de CECP, com o objetivo de correlacionar a expressão protéica com o perfil de metilação do DNA e com variáveis clínico-patológicas.

RESULTADOS E DISCUSSÃO

No presente trabalho, as técnicas de *RaSH* e microarranjos de cDNA foram combinadas com o objetivo de identificar genes com expressão elevada em linhagens celulares de cabeça e pescoço após a utilização de droga desmetilante. Utilizando essa abordagem, foram identificados 78 genes reativados após o tratamento com 5-Aza-dC. Dentre esses, 35 genes que apresentaram expressão elevada em pelo menos duas linhagens celulares e que possuíam ilha CpG associada aos seus promotores foram selecionados para experimentos de validação por PCR em tempo real. Três deles (*CRABP2*, *MXI* e *SLC15A3*) exibiram expressão aumentada nas linhagens tratadas com 5-Aza-dC em relação às linhagens não tratadas, com valor superior ao *cut off* estabelecido, ou seja, um valor de expressão pelo menos três vezes maior que o controle.

O perfil de metilação dos promotores gênicos desses genes foi investigado pela técnica de sequenciamento por bissulfito. As análises de metilação global das ilhas CpG mostraram que os genes *CRABP2*, *MXI* e *SLC15A3* são metilados em pelo menos uma das linhagens celulares não tratadas com o agente desmetilante. O próximo passo foi determinar se os genes seu padrão de metilação em tumores primários de cabeça e pescoço. Utilizando a metodologia de MSP-PCR, os resultados mostraram uma alta frequência de metilação dos genes *CRABP2* (58,1%) e *MXI* (46.3%) em amostras de CECP. As análises imunohistoquímicas revelaram que a ausência da proteína CRABP2 está associada com sobrevida livre de doença. Estes dados sugerem que a expressão de CRABP2 pode ser utilizada como um fator prognóstico para pacientes com CECP.

Epigenetic Silencing of *CRABP2* and *MX1* in Head and Neck Tumors^{1,2}

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease affecting the epithelium of the oral cavity, pharynx and larynx. Conditions of most patients are diagnosed at late stages of the disease, and no sensitive and specific predictors of aggressive behavior have been identified yet. Therefore, early detection and prognostic biomarkers are highly desirable for a more rational management of the disease. Hypermethylation of CpG islands is one of the most important epigenetic mechanisms that leads to gene silencing in tumors and has been extensively used for the identification of biomarkers. In this study, we combined rapid subtractive hybridization and microarray analysis in a hierarchical manner to select genes that are putatively reactivated by the demethylating agent 5-aza-2'-deoxycytidine (5Aza-dC) in HNSCC cell lines (FaDu, UM-SCC-14A, UM-SCC-17A, UM-SCC-38A). This combined analysis identified 78 genes, 35 of which were reactivated in at least 2 cell lines and harbored a CpG island at their 5' region. Reactivation of 3 of these 35 genes (*CRABP2*, *MX1*, and *SLC15A3*) was confirmed by quantitative real-time polymerase chain reaction (PCR; fold change, ≥ 3). Bisulfite sequencing of their CpG islands

Abbreviations: 5Aza-dC, 5-aza-2'-deoxycytidine; HNSCC, head and neck squamous cell carcinoma; MSP, methylation-specific polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; RaSH, rapid subtractive hybridization; TSS, transcription start site; TMA, tissue microarray
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revealed that they are indeed differentially methylated in the HNSCC cell lines. Using methylation-specific PCR, we detected a higher frequency of *CRABP2* (58.1% for region 1) and *MX1* (46.3%) hypermethylation in primary HNSCC when compared with lymphocytes from healthy individuals. Finally, absence of the *CRABP2* protein was associated with decreased disease-free survival rates, supporting a potential use of *CRABP2* expression as a prognostic biomarker for HNSCC patients.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises a heterogeneous disease, which arises from the epithelium of the oral cavity, pharynx, and larynx [1], and is associated with tobacco and alcohol abuse [2]. According to worldwide cancer statistics, approximately 450,000 new oral and laryngeal carcinomas are diagnosed annually, and the incidence varies between countries, probably as a result of environmental risk factors [3]. For example, the incidence rates for oral cancer in males are high in France and comparatively low in the United States and Brazil [4–6].

Although detection of HNSCC in early stages improves the survival rate, most patients present advanced stages of the disease at the time of diagnosis, and no sensitive and specific predictors of aggressive behavior have been identified. Lymph node status is still the most powerful prognostic factor, but the routine histopathologic examination of neck dissection specimens is unable to detect all micrometastases [7]. Therefore, the identification of early detection and prognostic biomarkers is highly desirable for planning an efficient and appropriate treatment procedure.

Evidence for a fundamental role for epigenetic modifications in head and neck cancer cells has been widely reported in the literature, including DNA methylation and histone deacetylation [8,9]. Both promoter hypermethylation of specific genes [10–12] and global hypomethylation are implicated in head and neck tumorigenesis [13,14].

Aberrant DNA methylation, such as regional gains or global loss, is an early event that occurs as a nonrandom signature in almost all tumors [15] and may be used for the identification of biomarkers. Strategies for assessing genome-wide methylation changes include genomic scanning after methylation-specific cleavage of the DNA and two-dimensional electrophoresis, amplification of intermethylated sites by arbitrarily primed polymerase chain reaction (PCR), and microarray gene expression analysis after treatment with DNA demethylating agents such as the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5Aza-dC) [16,17]. 5Aza-dC is incorporated into genomic DNA during replication, where it acts as an irreversible inhibitor of methyltransferase by forming a covalent complex with methyltransferase active sites. This suicide inhibition depletes methyltransferase activity, resulting in generalized DNA demethylation and release of specific genes from methylation-mediated transcriptional silencing [18].

In the present study, we carried out a genome-wide screening of 5Aza-dC-reactivated genes in four human squamous cell carcinoma cell lines derived from different topographical sites, using a combination of rapid subtractive hybridization (RaSH) and complementary DNA (cDNA) microarray analysis. This analysis revealed two genes reactivated by 5Aza-dC (*CRABP2* and *MX1*), and they were frequently hypermethylated in primary HNSCCs. Furthermore, the ab-

sence of *CRABP2* protein was associated with decreased disease-free survival rates, supporting a potential use of *CRABP2* expression as a prognostic biomarker for HNSCC.

Materials and Methods

Tumor Cell Lines and 5Aza-dC Treatment

Four HNSCC cell lines derived from distinct topographical sites, pharynx (FaDu), floor of the mouth (UM-SCC-14A), supraglottis (UM-SCC-17A), and tonsil (UM-SCC-38A), were used in this study. UM-SCC-14A, UM-SCC-17A, and UM-SCC-38A cell lines were kindly provided by Dr. Thomas E. Carey of the University of Michigan, USA, and FaDu (HTB-43) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were routinely cultured as monolayers in minimum essential medium (Eagle) supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin, at 37°C and 5% carbon dioxide. Cells were seeded at a density of 10^6 cells/10-cm dish, cultured for 48 hours, and treated for 4 days with freshly prepared 2.5 μ M 5Aza-dC (Sigma, St Louis, MO) dissolved in 50% acetic acid.

Tumor Samples

For methylation-specific PCR (MSP) analysis, 140 HNSCC samples and 10 lymphocyte samples from normal individuals were used. The samples were obtained from the Tumor Tissue Biobank of the Medical and Research Center – A.C. Camargo Hospital, São Paulo, and from the Head and Neck Genome Project/Gencapo – Brazil after Institutional Ethics Committee approval. Tumor samples were microdissected to enrich for tumor cells. Five-micrometer-thick sections from the frozen tumors were cut onto glass slides, fixed, and stained with hematoxylin and eosin. The hematoxylin and eosin-stained section was used as a guide for manual dissection, and only samples with more than 70% of tumor area were used in this study. All samples were reviewed by two independent pathologists. The main clinicopathological characteristics corresponding to these samples are shown in Table W3. None of the patients received preoperative treatment, and 41.3%, 42.0%, and 16.7% of patients were treated by surgery alone, surgery + radiotherapy, or surgery + radiotherapy + chemotherapy, respectively. The mean follow-up for these patients was approximately 31 months.

For the tissue microarray (TMA) analysis, an independent set of 75 HNSCC samples was used. These samples were obtained from the archives of the Department of Anatomic Pathology, A.C. Camargo Hospital, São Paulo, Brazil, and were reviewed by two independent pathologists. The main clinicopathological characteristics corresponding to these samples are also shown in Table W3. None of the patients received preoperative treatment, and 41.3%, 42.0%, and

6.7% of patients were treated by surgery alone, surgery + radiotherapy, or surgery + radiotherapy + chemotherapy, respectively. The average follow-up period was approximately 56 months.

DNA and RNA Extraction

Genomic DNA from tumor cell lines was purified using a Super Quick Gene DNA Isolation kit (Analytical Genetic Testing Center) following the protocol instructions. Total RNA was isolated using TRIzol Reagent for Molecular Biology (Invitrogen/Life Technologies, Carlsbad, CA). Genomic DNA from tumor samples was purified by standard phenol/chloroform purification. DNA quality was verified by electrophoresis through agarose gel on visualization with ethidium bromide. For microarray experiment, total RNA was further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). For quantitative real-time PCR (qRT-PCR), 5 µg of total RNA was previously treated with the RQ1 RNase-free DNase (Promega, Madison, WI). The RNA integrity after the purification procedure was evaluated using the Agilent 2100-Bioanalyser revealing a minimal RIN value of 7.9.

Rapid Subtractive Hybridization

RaSH cDNA libraries were prepared by a modified protocol taken from Jiang et al. [19]. The cDNA were initially digested with *Mbol* (Gibco, Gaithersburg, MD) at 37°C for 1 hour. The fragments were inserted into *Xba*I-digested pZERO plasmid (1 µg/µl) at 16°C for 3 hours. The constructs were introduced into the DH10-B competent cells. Two RaSH cDNA libraries were prepared: one using cDNA from the FaDu cell line treated with 5Aza-dC as tester and the mock-treated FaDu cell line as driver and the other using cDNA from the mock-treated FaDu cell line as tester and cDNA from the FaDu cell line treated with 5Aza-dC as driver.

Bacterial colonies were selected randomly and PCR amplified, using M13 forward and reverse primers. Inserts were sequenced with forward and reverse M13 primers using a DYEnamic ET Dye Terminator Sequencing kit (Amersham Biosciences, Piscataway, NJ) and a MegaBACE 1000 sequencer (Amersham Biosciences). The sequences were analyzed, using an annotation pipeline that consists of four steps: 1) quality checking, phred base-calling, cutoff 0.05 [20,21]; 2) vector trimming and removal of undesirable sequences such as bacterial, mitochondrial, and rRNA sequences; 3) masking of repetitive elements and screening of low-complexity regions by Repeat Masker, using the default settings [22]; and 4) annotation against existing databases, using BLASTN with default parameters. Significant hits were determined by using an *E*-value threshold of 10^{-15} for searches against nucleotide sequence databases [23].

cDNA Microarray

A total of 151 RaSH cDNA clones were amplified by PCR, purified, and spotted onto glass slides (Corning, Corning, NY) with a Flexys Robot (Genomic Solutions, Ann Arbor, MI). A total of 2352 spots, including 151 RaSH cDNA clones, 496 negative controls (pure H₂O or DMSO), 48 positive controls (*Q gene* fragment from phage lambda), and 1657 cDNA fragments derived from other projects were arranged on this customized cDNA platform. Positive hybridization signals from all spots were considered for evaluation of hybridization quality, normalization, and statistical analysis. However, for the purpose of this study, only differences in the expression levels of RaSH cDNA clones were used.

Total RNA extracted from HNSCC cell lines was further purified using the RNeasy Mini Kit (Qiagen) before the RNA amplification procedure. A two-round RNA amplification procedure was carried out as previously described [24]. Amplified RNA was used in a reverse transcription reaction in the presence of random hexamer primer (Invitrogen/Life Technologies), Cy3- or Cy5-labeled dCTP (Amersham Biosciences), and SuperScript II (Invitrogen/Life Technologies). Equal amounts of Cy3- or Cy5-labeled cDNA derived from cell lines treated or not with 5Aza-dC were mixed and cohybridized to the customized platform. Dye swap was performed, and hybridizations were carried out in duplicates, resulting in four independent hybridizations for each cell line. Self-self hybridization experiments were performed by pooling the cDNA derived from the four untreated cell lines, labeling them with Cy3 and Cy5 independently. Labeled cDNA were then cohybridized to the customized platform. Dye swap was also performed, and hybridizations were carried out in duplicates, resulting in four independent hybridizations for self-self experiments. Arrays were scanned and extracted as previously described [25]. Self-self experiments-based statistical test for low-replication microarray studies was performed to select genes reactivated by 5Aza-dC treatment. This strategy has been used to derive intensity-dependent cutoffs to classify a gene as differentially expressed in microarray studies [26]. The cutoff for all comparisons between treated and untreated samples was 99%.

Selection of Genes for Validation by qRT-PCR

Genes that were reactivated by 5Aza-dC in at least two of the four cell lines and that possessed a CpG island in their 5' region were selected for qRT-PCR validation. Genomic sequences corresponding to 5' regions of reactivated genes were analyzed for the presence of a CpG island using the UCSC Genome Browser (<http://genome.ucsc.edu/>). Criterion for a CpG island was based on those of Gardiner-Garden and Frommer [27], as a GC content of 50% or greater, length greater than 200 bp, and a ratio greater than 0.6 of the observed number of CG dinucleotides to the expected number for the total number of Gs and Cs in the segment. Repeat Masker (<ftp.genome.washington.edu/cgi-bin/Repeat-Masker>) was used to determine whether selected CpG islands contained repetitive elements.

Quantitative Real-time Polymerase Chain Reaction

qRT-PCR amplification was performed with Power SYBR Green and an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). The PCR total volume was 20.0 µl containing 10.0 µl of PCR Power SYBR Green Master Mix, 2.0 µl of diluted cDNA, and optimized primer concentrations for each primer pair (Table W4). Conditions were set as an initial polymerase activation step for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C for template denaturation, 1 minute at 60°C for extension and fluorescence measurement. Afterward, a dissociation protocol was used for each primer pair to verify the specificity of the qRT-PCR reaction and the absence of primer dimer. All samples were amplified in triplicates and the mean was used for qRT-PCR analysis, and a no-template control was also included. Primers were located in different exons and designed for optimal hybridization kinetics with Primer Express 2.0 (Applied Biosystems). Relative quantification of gene expression was carried out with the mathematical model developed by Pfaffl [28]. All PCR efficiencies were greater than 95%. Non-treated cell lines were used as reference samples, and *TUBA1C* (*TUBA6*) was selected as endogenous control gene after GeNorm [29].

Bisulfite Sequencing

Genomic DNA was subjected to sodium bisulfite treatment to modify unmethylated cytosine to uracil, as previously described [30]. Hypermethylation in HNSCC cell lines was determined by the bisulfite sequencing. Bisulfite-treated DNA was amplified by a nested-PCR protocol, using primers designed to amplify CpG-rich regions located at the 5' regions of *CRABP2* (-450 to -117 relative to transcription start site [TSS] encompassing 22 CpG dinucleotides), *MX1* (-71 to +845 relative to TSS encompassing 78 CpG dinucleotides), and *SLC15A3* (+351 to +845 relative to TSS encompassing 58 CpG dinucleotides; Figure 2). Primer sequences and PCR amplification conditions are available on request. Amplified products were cloned using the InsTAclone PCR Cloning Kit (Fermentas, Hanover, MD). Five positive clones were sequenced for each cell line using the Big Dye Terminator v3.1 Cycle Sequencing kit and an ABI3130 sequencer, in accordance with manufacturer's instructions (Applied Biosystems). The methylation percentage for each sample was calculated as the proportion of unconverted CpG dinucleotides among all the CpGs analyzed in all five positive clones.

Methylation-Specific PCR

Hypermethylation in head and neck tumors was determined by the MSP method as reported by Herman et al. [30], but amplified fragments were analyzed on silver-stained 8% polyacrylamide gels. Bisulfite-modified DNA was PCR amplified with primers specific for methylated versus unmethylated DNA. Two primer pairs were designed for MSP analysis of the *CRABP2* gene, and a single primer pair was used for the *MX1* gene, as indicated in Figure 2. Primer sequences and PCR amplification conditions are available on request.

Tissue Microarray

To construct the TMA, core biopsies were taken from previously defined areas, with a Tissue Microarrayer (Beecher Instruments, Silver Springs, MD). Tissue cores with a dimension of 1.0 mm were punched from each specimen and arrayed in duplicate on a recipient paraffin block. Each core was spaced 0.2 mm apart. After cutting (3 μ m) on the recipient block and transferring with an adhesive tape to coated slides for subsequent UV cross-linkage (Instrumedics Inc, Hackensack, NJ), the slides were dipped in a layer of paraffin to prevent oxidation and kept in a -20°C freezer.

For immunostaining, the sections were deparaffinized and rehydrated in graded ethanol solutions, treated with peroxide to quench endogenous peroxidase (0.3% H₂O₂ for 15 min), and blocked for avidin/biotin (Biotin Blocking System; DAKO, Carpinteria, CA) and for protein (Protein Block Serum-Free; DAKO). Antigenic recovery was performed by wet heating in a pressure cooker. Slides were incubated with anti-CRABP2 (MAB5488, 1:2000; Chemicon, Inc, Temecula, CA). The immunohistochemical reaction was carried out in duplicate at different TMA levels, representing four-fold redundancy for each case. Positive and negative controls were included in all reactions. Positive controls were obtained using normal breast slides incubated with the CRABP2 antibody. CRABP2 binding was assessed by two kinds of negative controls: 1) omitting the primary antibody and incubating slides with phosphate-buffered saline; 2) replacing the primary antibody with normal mouse serum.

After scanning each tumor specimen in low power field to choose the most stained area, at least five fields were evaluated under high power. The presence of a clearly visible dark brown precipitation was

considered an immunoreaction. Evaluation of CRABP2 included the proportion of reactive cells within the tumors and the staining intensity. The proportion score described the fraction of positively stained tumor cells (<10% of positive cells; $\geq$ 10% of tumors cells stained).

The immunostaining intensities were assessed visually by two pathologists using blind analysis by determining the color intensity of stained cells. The samples were classified as negative (no visible reaction or positivity in <10% of positive cells) or positive reaction (weak and strongly positive present in $\geq$ 10% of tumors cells stained). For statistical analysis, the samples were categorized into two groups: negative and weakly/strongly positive cases.

Statistical Analysis

For frequency analysis in contingency tables, statistical analyses of associations between variables were performed by the χ^2 test or Fisher's exact test (with significance set at $P < .05$). The overall survival was defined as the interval between the beginning of treatment and the date of death or the last information for censored observations. The disease-free interval was measured from the date of the treatment to the date when locoregional recurrence or distant metastasis was diagnosed. Overall survival and disease-free survival probabilities were estimated by the Kaplan-Meier method, and the log-rank test was applied to assess the significance of differences among actuarial survival curves. Multivariate analysis was carried out using Cox proportional hazards model (stepwise forward selection). All variables presenting $P < .20$ on the univariate analysis were selected for building a multiple model. For all tests, type I error (α) was established as 0.05, and results were considered statistically significant when $P < .05$. All statistical analyses were performed with the STATA software (Intercooled Stata release 7.0; Stata Corporation, College Station, TX).

Results

Identification of Genes Reactivated by 5Aza-dC

To identify novel methylation-silenced genes in HNSCC cell lines, we treated the FaDu cell line with the demethylating agent 5Aza-dC and used a combination of RaSH and cDNA microarray analysis to identify genes reactivated on treatment. Initially, two RaSH cDNA libraries were constructed: one using cDNA prepared from the FaDu cell line treated with 5Aza-dC as tester and the mock-treated FaDu cell line as driver (5Aza-dC tester library) and the other using cDNA prepared from mock-treated FaDu cell line as tester and cDNA from the FaDu cell line treated with 5Aza-dC as driver (mock tester library; Figure 1). A total of 1773 cDNA clones were sequenced, corresponding to 876 and 897 clones derived from the 5Aza-dC tester and mock tester libraries, respectively. After similarity searches in public databases, we found that these sequences correspond to 415 known genes of which 65 were present in both RaSH libraries. A total of 181 and 169 genes were found exclusively in the 5Aza-dC tester and mock tester libraries, respectively. Of the 181 cDNA clones reactivated by the 5Aza-dC, 151 were successfully amplified and spotted onto glass slides (Figure 1; Table W1).

Microarray analysis was then carried out with RNA extracted from FaDu and three additional HNSCC cell lines (UM-SCC-14A, UM-SCC-17A, and UM-SCC-38A) derived from distinct topographical sites and treated with 5Aza-dC. Self-self experiments based on a statistical test for low-replication microarray studies were performed to

Figure 1. Flowchart for the identification of differentially methylated genes in HNSCC cell lines. FaDu cell line was treated with 5Aza-dC, and purified mRNA was used to construct RaSH cDNA libraries. A set of 151 nonredundant genes was used to prepare an enriched cDNA platform for microarray analysis. A total of 48 genes were reactivated in at least two cell lines. From them, 35 genes harboring a CpG island located at their 5' region were submitted to qRT-PCR. Up-regulation of gene expression by 5Aza-dC was confirmed for three genes. Bisulfite sequencing revealed three differentially methylated genes.

identify genes reactivated on treatment. A total of 78 reactivated genes were identified using this combined approach, of which 31, 18, 47, and 46 were identified in the FaDu, UM-SCC-14A, UM-SCC-17A, and UM-SCC-38A cell lines, respectively. A total of 48 genes were reactivated in at least two cell lines and used for further investigation. We reasoned that commonly reactivated genes, inactivated in at least two HNSCC, were more likely to represent genes frequently inactivated in tumors.

Validation Analysis by qRT-PCR of Genes Reactivated by 5Aza-dC in HNSCC Cell Lines

Of the 48 genes selected by the microarray analysis, 35 harbored a bona fide CpG island in the 5' region and were further selected for qRT-PCR quantification of gene expression in the same cell lines used in the microarray analysis (Table W2). Three genes (*CRABP2*, *MXI*, and *SLC15A3*) were confirmed to be reactivated at least three-fold in at least one of the cell lines after 5Aza-dC treatment. All these genes were upregulated in the FaDu cell line. In addition, the *CRABP2* and *MXI* genes were also upregulated in the UM-SCC-14A and UM-SCC-38A cell lines, respectively (Table 1).

DNA Methylation Analysis in HNSCC Cell Lines

The methylation status of the CpG island at the 5' region site of the *CRABP2*, *MXI*, and *SLC15A3* genes was then investigated by bisulfite sequencing in HNSCC cell lines that showed induction of gene expression after 5Aza-dC treatment.

A significant reduction in the global methylation level (from 40.00% to 5.50%) of the 5' region of *CRABP2* was observed in the FaDu cell line after treatment with the demethylating agent, which is in agreement with the 4.6-fold increase in the *CRABP2* messenger RNA (mRNA) level observed in this cell line treated with 5Aza-dC. Corroborating this result (Figure 2), reduction in the methylation levels was observed for almost all CpG dinucleotides analyzed.

A less pronounced reduction in the DNA methylation level of the *MXI* 5' region was observed in the FaDu (from 27.70% to 12.70%) and UM-SCC-14A (from 7.80% to 1.02%) cell lines (Figure 2). The reduction was limited to dinucleotides 39 to 56 located within the first exon of the *MXI* gene. Reduction in the methylation levels of these specific dinucleotides was directly correlated with induction of gene expression on treatment observed in these cell lines, as measured by qRT-PCR (22.4-fold for FaDu and 4.5-fold for UM-SCC-14A), suggesting that these dinucleotides play a critical role in transcription regulation.

In the case of *SLC15A3* gene, an increase of 7.3- and 3.0-fold in its expression level was observed in the FaDu and UM-SCC-38A cell lines, respectively. However, a significant reduction (from 96.6% to 5.5%) in the DNA methylation level of the *SLC15A3* 5' region was exclusively observed in the UM-SCC-38A cell line. These results suggest that other epigenetic mechanisms, such as histone modification, may also play a critical role in regulating the expression of the *SLC15A3* gene.

DNA Methylation Analysis in Primary HNSCC

We next sought to determine whether *CRABP2* and *MXI* hypermethylation, identified in the preceding experiments using cell lines, was also present in primary HNSCC. The methylation status of *SLC15A3* was not further investigated in primary tumors because

Table 1. Validation of Gene Expression Reactivation by Microarray Analysis and qRT-PCR in 5Aza-dC-Treated HNSCC Cell Lines.

Official Symbol	Microarray				qRT-PCR (Fold Change)			
	FaDu	UM-SCC-14A	UM-SCC-17A	UM-SCC-38A	FaDu	UM-SCC-14A	UM-SCC-17A	UM-SCC-38A
<i>CRABP2</i>	Up	Up	Up	—	4.6	1.9	1.7	ND
<i>MXI</i>	Up	Up	—	Up	22.4	4.5	ND	0.2
<i>SLC15A3</i>	Up	Up	Up	Up	7.3	2.2	1.0	3.0

ND indicates not determined; Up, upregulated.

Figure 2. A representative result of bisulfite sequencing. Each panel represents a schematic representation of the genome structure of each gene including their 5' CpG islands. Exons and untranslated regions are represented by filled or open boxes, respectively. The transcription initiation site is represented by +1. Expanded view shows the position of CpG islands and the region analyzed by bisulfite sequencing. Vertical marks represent individual CpG dinucleotides and their spacing accurately reflects the CpG density of the region. MSP primers are represented by horizontal arrows in the panels. Primer sets M and U were designed for the same CpG dinucleotide (indicated by an asterisk). Methylation profiles of the treated (5Aza-dC) and untreated (mock) cell lines are indicated in the lower part of the panels. Each row represents one sequenced clone, and open and filled circles represent unmethylated and methylated CpG dinucleotides, respectively. (A) Bisulfite sequencing of *CRABP2* in the FaDu cell line. (B) Bisulfite sequencing of *MX1* in the FaDu and UM-SCC-14A cell lines. (C) Bisulfite sequencing of *SLC15A3* in the FaDu and UM-SCC-38A cell lines.

Figure 3. MSP analysis of *CRABP2* and *MX1* genes. Representative results of MSP analysis of *CRABP2* and *MX1* genes in the FaDu cell line and five different HNSCC samples. Methylated tumors are indicated with asterisks. The lanes indicated by M and U correspond to the products amplified by MSP primer sets specific for methylated and unmethylated DNA, respectively. *In vitro* methylated DNA (IVD) and normal human peripheral lymphocytes (NL) were used as positive and negative methylation controls, respectively. (A) MSP of *CRABP2* R1. (B) MSP of *CRABP2* R2. (C) MSP of *MX1*.

we were unable to detect a direct correlation between DNA methylation level and gene expression. MSP analysis of *CRABP2* and *MX1* was carried out in 140 HNSCC samples and 10 lymphocyte samples from normal individuals. The main clinicopathological characteristics of the HNSCC patients are shown in supplementary data (Table W3), and representative MSP results are shown in Figure 3. MSP primers were designed to target the most frequently methylated CpG dinucleotides and those with a direct correlation with mRNA expression as revealed by bisulfite sequencing of the tumor cell lines. Two primer pairs were designed for MSP analysis of the *CRABP2* (one

pair for region 1 – R1 and one pair for region 2 – R2) and a single primer pair was used for the *MX1* gene, as indicated in Figure 2.

CRABP2 hypermethylation was detected in 56.4% (79/140) of the samples when primers for R1 were used and in 10.0% (14/140) of the samples when reactions were carried out with R2 primers. For 13 patients (9.3%), hypermethylation was detected with both primer pairs. No *CRABP2* hypermethylation was detected in normal lymphocytes for both primer pairs. We then investigated the association between *CRABP2* hypermethylation and well-established clinicopathological parameters used for HNSCC. As shown in Table 2,

Table 2. Relationship between Methylation Analyses of *CRABP2* and *MX1* and Clinicopathological Variables in HNSCC Patients.

Variables	Category	<i>CRABP2</i> R1,* n (%)			<i>CRABP2</i> R2,* n (%)			<i>MX1</i> ,* n (%)		
		Unmethylated	Methylated	P	Unmethylated	Methylated	P	Unmethylated	Methylated	P
Age	≤53	26 (44.07)	23 (29.49)	.078	47 (38.52)	2 (14.29)	.074	29 (40.85)	20 (30.30)	.198
	>53	33 (55.93)	55 (70.51)		75 (61.48)	12 (85.71)		42 (59.15)	46 (69.70)	
Tumor site	Oral cavity	29 (49.16)	34 (43.04)	.010 [†]	58 (47.15)	4 (28.57)	.097	39 (54.17)	24 (36.36)	.095
	Larynx	15 (25.42)	8 (10.13)		22 (17.89)	1 (7.14)		9 (12.50)	14 (21.21)	
	Hypopharynx	15 (25.42)	37 (46.84)		43 (34.96)	9 (64.29)		24 (33.33)	28 (42.43)	
Tumor size	T1 + T2	14 (25.00)	20 (26.32)	.864	30 (25.42)	4 (30.77)	.676	13 (18.31)	21 (34.43)	.035 [‡]
	T3 + T4	42 (75.00)	56 (73.68)		88 (74.58)	9 (69.23)		58 (81.69)	40 (65.57)	
Lymph nodes	N0	10 (17.54)	16 (21.33)	.588	21 (17.80)	5 (38.46)	.076	15 (21.13)	11 (18.03)	.656
	N+	47 (82.46)	59 (78.67)		97 (82.20)	8 (61.54)		56 (78.87)	50 (81.97)	
Grade	1	11 (19.64)	25 (34.25)	.184	34 (29.57)	2 (15.38)	.595	21 (31.34)	15 (24.19)	.563
	2	36 (64.29)	39 (53.42)		65 (56.52)	9 (69.23)		36 (53.73)	39 (62.90)	
	3	9 (16.07)	9 (12.33)		16 (13.91)	2 (15.38)		10 (14.93)	8 (12.90)	
Vascular invasion	No	35 (60.34)	51 (68.92)	.305	76 (64.41)	10 (76.92)	.367	40 (57.14)	45 (72.58)	.055
	Yes	23 (39.66)	23 (31.08)		42 (35.59)	3 (23.08)		30 (42.86)	17 (27.42)	
Lymphatic permeation	No	49 (84.48)	64 (87.67)	.599	100 (84.75)	12 (100)	.145	57 (82.61)	56 (90.32)	.200
	Yes	9 (15.52)	9 (12.33)		18 (15.25)	0		12 (17.39)	6 (9.68)	
Perineural infiltration	No	27 (46.55)	42 (57.53)	.211	60 (51.28)	9 (69.23)	.219	38 (55.07)	31 (50.00)	.562
	Yes	31 (53.45)	31 (42.47)		57 (48.72)	4 (30.77)		31 (44.93)	31 (50.00)	

R1 indicates region 1; R2, region 2.

*Percentages considering cases with complete information.

[†]Statistically significant.

a statistically significant association was found between tumor site and *CRABP2* hypermethylation in region 1 ($P = .010$). It was observed that hypopharynx tumors showed a higher frequency of methylation (37/52 or 71.2%) when compared with the other tumor sites. Kaplan-Meier analysis was then used to estimate the relationship between the methylation status of the *CRABP2* gene with overall and disease-free survival. No difference in terms of overall or disease-free survival was observed between patients with or without *CRABP2* hypermethylation in their primary tumors.

Hypermethylation in the 5' region of the *MX1* gene was detected in 45.0% (63/140) of the HNSCC patients. Similar to what was observed for *CRABP2*, no *MX1* hypermethylation was detected in normal lymphocytes. A statistically significant association was observed between *MX1* hypermethylation and tumor size ($P = .035$) and a marginal association with vascular invasion ($P = .055$; Table 2). However, as for *CRABP2*, no difference in terms of overall or disease-free survival was observed between patients that did or did not show *MX1* hypermethylation in primary tumors.

CRABP2 Protein Expression in HNSCC Primary Tumors

CRABP2 protein expression was then analyzed in HNSCC tumors by immunohistochemistry using a TMA containing 75 HNSCC samples. As shown in Figure 4A, *CRABP2* protein immunostaining was detected in the morphologically normal epithelium samples used as controls, more intense staining being detected in the suprabasal (post-mitotic) epithelial cells. Among the 75 HNSCC samples, 8 cases (11%) were negative, 37 cases (51%) were weakly positive, and 28 cases (38%) were strongly positive (Figure 4, B–D). Two samples did not con-

tain representative sections of the tumor tissue and were not considered in the analysis. For all further analysis, weakly and strongly positive tumors were treated as a single group with positive *CRABP2* protein expression.

We then analyzed the possible association between the expression of *CRABP2* protein and clinicopathological variables. As shown in Table 3, a statistically significant association was observed between *CRABP2* staining and increased tumor size (T3 + T4; $P = .029$) and absence of lymphatic permeation ($P = .014$). Univariate analysis was used to estimate the association between *CRABP2* protein expression and overall or disease-free survival. No difference in terms of overall survival was observed with *CRABP2* protein expression. Interestingly, our data show that an absence of *CRABP2* expression was associated with a worse disease outcome because patients whose tumors were negative for *CRABP2* expression had a higher risk of locoregional recurrence or distant metastasis than patients with *CRABP2*-positive tumors (log-rank test, $P = .0531$; Figure 5). In the Cox regression univariate model, *CRABP2* protein expression showed a protective hazard ratio of 0.40 (95% confidence interval, 0.1–1.0). However, in the multivariate analysis, *CRABP2* protein expression was not shown to be an independent prognostic factor for disease-free survival owing to the small number of *CRABP2*-negative tumors or to the presence of confounding variables such as tumor size and site and the occurrence of lymphatic permeation.

Discussion

Treatment with 5Aza-dC in combination with histone deacetylase inhibitors has been widely used to reactivate epigenetically silenced

Figure 4. *CRABP2* protein immunostaining patterns. Representative immunostainings of (A) morphologically normal epithelium and (B–D) HNSCC samples for *CRABP2*. Chromogenic detection (brown precipitate) counterstained with hematoxylin. Original magnifications: A, $\times 200$ (A); B–D, $\times 400$. Staining was scored as negative (B), weakly positive (C), or strongly positive (D).

Table 3. Relationship between CRABP2 Protein Expression and Clinicopathological Variables in HNSCC Patients.

Variables	Category	CRABP2,* n (%)		P
		Negative	Positive	
Age	≤53	1 (50.00)	13 (55.38)	>.999
	>53	7 (50.00)	52 (44.62)	
Tumor site	Oral cavity	3 (37.50)	20 (30.77)	NA
	Larynx	4 (50.00)	26 (40.00)	
	Hypopharynx	1 (12.50)	19 (29.23)	
Tumor size	T1 + T2	5 (71.43)	17 (29.82)	.029 [†]
	T3 + T4	2 (28.57)	40 (70.18)	
Lymph nodes	N0	5 (62.50)	56 (91.80)	.075
	N+	3 (37.50)	5 (8.20)	
Histologic grade	1	3 (37.5)	22 (33.85)	NA
	2	5 (62.5)	36 (55.38)	
	3	0	7 (10.77)	
Vascular invasion	No	6 (75.00)	50 (80.65)	.707
	Yes	2 (25.00)	12 (19.35)	
Lymphatic permeation	No	2 (25.00)	43 (69.35)	.014 [‡]
	Yes	6 (75.00)	19 (30.65)	
Perineural infiltration	No	6 (75.00)	34 (54.84)	.278
	Yes	2 (25.00)	28 (45.16)	

NA indicates not applied.

*Percentages considering cases with complete information.

[†]Statistically significant.

genes in cell lines from several types of tumor [31–35]. Although this approach can lead to secondary effects on gene expression, it seems to be very efficient compared with alternative strategies in which CpG island arrays are hybridized with genomic DNA digested with methylation-sensitive restriction enzyme [15,36] because it relies directly on the reactivation of gene expression rather than on the presence of a CpG island.

In the present study, we have used 5Aza-dC to identify genes putatively silenced by DNA hypermethylation. Histone deacetylase inhibitors were not used because previous studies using similar strategies have demonstrated that most genes are reactivated by high-dose 5Aza-dC treatment, and only a small subset of genes is induced by the synergistic treatment with demethylating agents and deacetylase inhibitors [32,33].

To evaluate changes in gene expression induced by 5Aza-dC treatment, we used a combination of RaSH and cDNA microarray analysis. The RaSH methodology has been extensively used in the identification of differentially expressed genes, and in our study, it was used in an attempt to enrich the cDNA microarray for 5Aza-dC reactivated genes and not limit our analysis to a set of predefined genes represented in commercial arrays. Two RaSH cDNA libraries were constructed: 5Aza-dC tester and mock tester. The mock tester library was constructed in an attempt to identify false-positive genes that were found in both cDNA libraries and as a control of the subtraction efficiency. We identified 415 known genes of which only 65 were present in both RaSH libraries, indicating a high subtraction efficiency. In addition, we were able to identify 181 genes putatively induced by 5Aza-dC in the FaDu cell line, and 151 of these genes were spotted on the microarray platform and analyzed for gene reactivation in three additional HNSCC cell lines. Using this combined approach, we identified 78 genes induced on treatment and selected 35 genes, induced in at least two of the cell lines and which contained a CpG island at the 5' region for technical validation by qRT-PCR. Gene expression increased at least three-fold on 5Aza-dC treatment in 3 of the 35 genes analyzed (*CRABP2*, *MX1*, and *SLC15A3*) and 2 of them (*CRABP2* and *MX1*) were indeed hypermethylated in the cell lines used in this study. MSP

was then used to examine the DNA methylation status of *CRABP2* and *MX1* in a set of 140 primary HNSCCs. MSP primers were designed to target the most frequently methylated CpG dinucleotides and those with a direct correlation with mRNA expression as revealed by bisulfite sequencing of the tumor cell lines. Because the CpG-rich region located at the 5' region of *CRABP2* contains 22 CpG dinucleotides, two primer pairs (R1 and R2) were designed for MSP analysis of *CRABP2* in primary tumors. Unfortunately, owing to the higher GC content of the 5' region of the *MX1* gene, we were able to design a single primer pair for MSP analysis of *MX1*. *CRABP2* hypermethylation was specifically detected in 56.4% and in 10.0% of the tumors when reactions were carried out with R1 and R2 primers, respectively. The difference in the methylation frequencies observed for different primer pairs is expected because MSP primers were placed at different CpG dinucleotides. *MX1* hypermethylation was specifically detected in 45.0% of primary HNSCCs. Taken together, these results suggest that *CRABP2* and *MX1* mRNA expression is regulated by DNA methylation and that *CRABP2* and *MX1* hypermethylation is frequent among HNSCCs. Interestingly, *CRABP2* (R1) or *MX1* hypermethylation showed statistically significant association with tumor site or tumor size, respectively. In the case of *CRABP2*, hypopharynx tumors showed a higher frequency of promoter methylation when compared with the other tumor sites.

The human *MX1* (myxovirus resistance 1) gene, also named IFI78 (interferon-inducible protein p78), encodes a member of the dynamin superfamily of large GTPases, which mediates vesicle trafficking and organelle homeostasis [37]. Similarly to other Mx proteins, human *MX1* has antiviral activities against several RNA viruses and is transcriptionally induced by interferon through the JAK/STAT pathway [38]. *MX1* is upregulated in cells of some Fanconi anemia complementation groups, which may be related to phenotypic features of this disease, particularly bone marrow failure [23]. *MX1* is downregulated in prostate carcinomas [39] and methylated in acute myeloid leukemia cells [40], which provide a potential link between *MX1* silencing and tumorigenesis. Supporting this link are the observations by Mibayashi et al. [41] that *MX1* promotes cell death induced by apoptotic stimuli. Therefore, low levels of *MX1* protein might contribute to apoptosis resistance during cancer development.

Figure 5. Kaplan-Meier disease-free survival estimates from 73 patients for CRABP2 expression. Continuous and dashed lines depict patients with positive (weak and strong) or negative CRABP2 expression ($P = .0531$), respectively.

CRABP2 (cellular retinoic acid binding protein 2) encodes a small protein (15 kDa) harboring a lipocalin domain involved in retinoic acid (RA) binding [42,43]. CRABP2 binds to all-*trans* RA in the cytoplasm, which triggers its nuclear targeting to associate with RA receptors (RARs). Owing to the poor water solubility of RA, its binding by CRABP2 allows the intracellular RA levels and availability to increase [44]. The association between CRABP2 and RAR in the nucleus enables direct channeling of RA and increases the RAR-RXR heterodimer transcriptional activity at RA-responsive sites [44].

RA and its derivatives (retinoids) are responsible for the regulation of multiple biologic processes, such as embryogenesis, apoptosis, cell proliferation, and differentiation [45]. Several authors have described retinoids as useful pharmaceuticals for the prevention and treatment of various types of human cancer. These studies demonstrated their efficiency in the treatment of tumors of head and neck [46,47], lung [48], skin [49], breast [50], and also of acute promyelocytic leukemia [51]. However, development of RA resistance frequently occurs [52]. Importantly, two major RA pathways were described as responsible for the antiproliferative and proliferative effects observed, respectively: the classic CRABP2/RAR and, more recently described, the FABP5/PPAR β / δ [53]. Therefore, both endogenous and exogenous retinoids may only inhibit tumor growth if the RAR pathway is predominant in the tumor cells, and this idea is supported by data showing that diverting RA from PPAR β / δ to RAR is sufficient to overcome RA resistance of mammary carcinomas [54]. In a related manner, abnormalities in the expression or in the function of retinoid receptors, particularly the suppression of RARB expression, have been found in several types of cancer, including premalignant oral lesions [55] and HNSCC [56,57]. Interestingly, CRABP2 mRNA and protein levels were shown to be downregulated in carcinoma cells, relative to normal glandular cells, in the prostate [58,59]. Also, CRABP2 was identified as downregulated in an oligomicroarray analysis of genes related to lymph node metastasis in esophageal squamous cell carcinoma [60]. More recently, CRABP2 expression was demonstrated to be suppressed as a result of promoter DNA methylation in non-small cell lung tumor [61].

Finally, in confirmation that CRABP2 is an important component of the antiproliferative effects of the RAR pathway in response to RA, it was shown that CRABP2 induces apoptosis in MCF-7 mammary carcinoma cells because of the induction of transcription of cell cycle-regulating genes [62,63] and that overexpression of CRABP2 in the HaCaT keratinocyte cell line significantly increased tumor necrosis factor α -induced apoptosis [53]. The correlation of CRABP2 protein with the antiproliferative effect of RA and the induction of apoptosis in various cells are in agreement with the results of our study showing that CRABP2 expression loss leads to survival disadvantage.

In the present study, we have demonstrated for the first time that CRABP2 and MX1 mRNA expression is regulated by DNA methylation and that hypermethylation of both genes is frequent among HNSCC. Although a direct correlation between CRABP2 hypermethylation and absence of protein expression was not directly evaluated in the present work, we observed similar frequencies for both CRABP2 hypermethylation (region 2 – 10.2%) and absence of CRABP2 protein (11.0%) in different sets of HNSCC samples, suggesting that DNA hypermethylation might also affect CRABP2 protein levels. Moreover, a statistically significant association between absence of CRABP2 protein and lower survival rates was observed in our study, suggesting that CRABP2 could be used as a prognostic biomarker for patients with HNSCC.

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Table W1. List of the 181 Reactivated Genes after 5Aza-dC Treatment Identified by RaSH cDNA Library.

Sequence	Official Symbol	CpG Island	Spotted in Array
CV341138	—	Yes	Yes
NM_000700	<i>ANXA1</i>	No	Yes
NM_001008897	<i>TCP1</i>	Yes	Yes
NM_002462	<i>MX1</i>	Yes	Yes
NM_005562	<i>LAMC2</i>	No	Yes
NM_005347	<i>HSPA5</i>	Yes	Yes
NM_004509	<i>SP110</i>	Yes	No
NM_005610	<i>RBBP4</i>	Yes	Yes
NM_001001977	<i>ATP5E</i>	Yes	Yes
NM_022073	<i>EGLN3</i>	Yes	Yes
NM_006476	<i>ATP5L</i>	Yes	Yes
NM_002893	<i>RBBP7</i>	Yes	No
NM_001799	<i>CDK7</i>	Yes	Yes
NM_016237	<i>ANAPC5</i>	Yes	Yes
NM_002463	<i>MX2</i>	Yes	Yes
NM_001852	<i>COL9A2</i>	Yes	No
NM_022121	<i>PERP</i>	Yes	Yes
NM_001012456	<i>SEC61G</i>	Yes	Yes
NM_002535	<i>OAS2</i>	Yes	No
NM_015013	<i>AOF2</i>	Yes	Yes
NM_006135	<i>CAPZA1</i>	Yes	Yes
NM_018835	<i>RC3H2</i>	Yes	No
NM_144596	<i>TTC8</i>	Yes	No
NM_002227	<i>JAK1</i>	No	Yes
NM_014352	<i>POU2F3</i>	Yes	Yes
NM_004923	<i>MTL5</i>	Yes	Yes
NM_003406	<i>YWHAZ</i>	Yes	Yes
NM_001007074	<i>RPL32</i>	Yes	Yes
NM_033407	<i>DOCK7</i>	Yes	No
NM_001686	<i>ATP5B</i>	Yes	Yes
NM_017688	<i>BSPRY</i>	Yes	Yes
NM_000979	<i>RPL18</i>	Yes	No
NM_005056	<i>JARID1A</i>	No	Yes
NM_020675	<i>SPC25</i>	Yes	Yes
NM_004371	<i>COPA</i>	Yes	No
NM_012420	<i>IFT15</i>	Yes	Yes
NM_019606	<i>MEPCE</i>	Yes	Yes
NM_015091	<i>FAM179B</i>	Yes	Yes
NM_014612	<i>FAM120A</i>	Yes	No
NM_139266	<i>STAT1</i>	Yes	Yes
NM_014969	<i>WDR47</i>	Yes	Yes
NM_006004	<i>UQCRH</i>	Yes	Yes
NM_006819	<i>STIP1</i>	Yes	Yes
NM_004487	<i>GOLGB1</i>	Yes	Yes
NM_002421	<i>MMP1</i>	No	No
NM_006603	<i>STAG2</i>	Yes	Yes
NM_015340	<i>LARS2</i>	Yes	Yes
NM_000602	<i>SERPINE1</i>	No	Yes
NM_198076	<i>FAM36A</i>	Yes	Yes
NM_003746	<i>DYNLL1</i>	Yes	Yes
NM_005782	<i>THOC4</i>	Yes	Yes
NM_018660	<i>ZNF395</i>	Yes	Yes
NM_003792	<i>EDF1</i>	Yes	Yes
NM_005381	<i>NCL</i>	Yes	Yes
NM_005762	<i>TRIM28</i>	Yes	Yes
NM_002231	<i>CD82</i>	Yes	Yes
NM_002797	<i>PSMB5</i>	No	Yes
NM_001748	<i>CAPN2</i>	Yes	No
NM_001009925	<i>C20orf30</i>	Yes	Yes
NM_000646	<i>AGL</i>	Yes	Yes
NM_183356	<i>ASNS</i>	Yes	Yes
NM_005100	<i>AKAP12</i>	Yes	Yes
NM_006421	<i>ARFGEF1</i>	Yes	No
NM_198829	<i>RAC1</i>	Yes	Yes
NM_006931	<i>SLC2A3</i>	No	Yes
NM_000366	<i>TPM1</i>	Yes	Yes
NM_005365	<i>MAGEA9</i>	No	Yes
NM_006815	<i>TMED2</i>	Yes	Yes
NM_022754	<i>SPXN1</i>	Yes	Yes
NM_004755	<i>RPS6KA5</i>	Yes	Yes
NM_015382	<i>HECTD1</i>	Yes	No
NM_005243	<i>EWSR1</i>	Yes	Yes

Table W1. (continued)

Sequence	Official Symbol	CpG Island	Spotted in Array
NM_004130	<i>GYGI</i>	Yes	Yes
NM_153649	<i>TPM3</i>	Yes	Yes
NM_021101	<i>CLDN</i>	Yes	Yes
NM_006549	<i>CAMKK2</i>	Yes	Yes
NM_001175	<i>ARHGD1B</i>	No	Yes
NM_002673	<i>PLXNB1</i>	Yes	Yes
NM_198147	<i>LOC116236</i>	Yes	Yes
NM_014758	<i>SNX19</i>	Yes	Yes
NM_016565	<i>CHCHD8</i>	Yes	Yes
NM_020914	<i>RNF213</i>	Yes	No
NM_000576	<i>IL1B</i>	No	Yes
NM_005388	<i>PDCL</i>	Yes	No
NM_001080	<i>ALDH5A1</i>	Yes	Yes
NM_014611	<i>MDN1</i>	Yes	No
NM_015509	<i>NECAP1</i>	Yes	Yes
NM_003932	<i>ST13</i>	Yes	Yes
NM_002274	<i>KRT13</i>	No	Yes
NM_005789	<i>PSME3</i>	Yes	Yes
NM_182972	<i>IRF2BP2</i>	Yes	No
NM_004859	<i>CLTC</i>	Yes	Yes
NM_133337	<i>FER1L3</i>	Yes	Yes
NM_178868	<i>CMTM8</i>	Yes	Yes
NM_018043	<i>ANO1</i>	Yes	Yes
NM_003405	<i>YWHAH</i>	Yes	Yes
NM_016816	<i>OAS1</i>	Yes	Yes
NM_001457	<i>FLNB</i>	Yes	Yes
NM_003670	<i>BHLHB2</i>	Yes	Yes
NM_014014	<i>ASCC3L1</i>	Yes	No
NM_002094	<i>GSPT1</i>	Yes	No
NM_002080	<i>GOT2</i>	Yes	Yes
NM_005113	<i>GOLGA5</i>	Yes	Yes
NM_032547	<i>SCOC</i>	Yes	Yes
NM_000526	<i>KRT14</i>	No	No
NM_203459	<i>CAMSAP1L</i>	Yes	Yes
NM_030920	<i>ANP32E</i>	Yes	Yes
NM_015575	<i>GIGYF2</i>	Yes	Yes
NM_005744	<i>ARIH1</i>	Yes	Yes
NM_032565	<i>EBPL</i>	Yes	Yes
NM_177423	<i>PPFIA1</i>	Yes	Yes
NM_000989	<i>RPL30</i>	Yes	Yes
NM_014752	<i>SPCS2</i>	Yes	Yes
NM_201517	<i>H2AFV</i>	Yes	Yes
NM_031689	—	Yes	Yes
NM_031430	<i>RILP</i>	Yes	No
NM_213646	<i>WARS</i>	Yes	No
NM_001975	<i>ENO2</i>	Yes	No
NM_032940	<i>POLR2C</i>	Yes	Yes
NM_032366	<i>C16orf13</i>	Yes	Yes
NM_024881	<i>SLC35E1</i>	Yes	Yes
NM_005389	<i>PCMT1</i>	Yes	Yes
NM_020899	<i>ZBTB4</i>	Yes	Yes
NM_002822	<i>TWFI</i>	Yes	Yes
NM_016582	<i>SLC15A3</i>	Yes	Yes
NM_016286	<i>DCXR</i>	Yes	Yes
NM_001614	<i>ACTG1</i>	Yes	Yes
NM_001878	<i>CRABP2</i>	Yes	Yes
NM_144570	<i>HN1L</i>	Yes	Yes
NM_002272	<i>KRT4</i>	No	Yes
NM_001539	<i>DNAJA1</i>	Yes	Yes
NM_018156	<i>VPS13D</i>	Yes	Yes
NM_001005340	<i>GPNNMB</i>	Yes	Yes
NM_003039	<i>SLC2A5</i>	Yes	Yes
NM_003467	<i>CXCR4</i>	Yes	Yes
NM_000661	<i>RPL9</i>	Yes	Yes
NM_014831	<i>LBA1</i>	Yes	No
NM_017830	<i>OCIAD1</i>	Yes	Yes
NM_007146	<i>VEZF1</i>	Yes	Yes
NM_016091	<i>EIF3EIP</i>	Yes	Yes
NM_001331	<i>CTNND</i>	Yes	No
NM_013230	<i>CD24</i>	Yes	Yes
NM_005556	<i>KRT7</i>	Yes	Yes
NM_002211	<i>ITGB1</i>	Yes	No
NM_031899	<i>GORASP1</i>	Yes	Yes

Table W1. (*continued*)

Sequence	Official Symbol	CpG Island	Spotted in Array
NM_003972	<i>BTAF1</i>	Yes	Yes
NM_016284	<i>CNOT1</i>	Yes	Yes
NM_006362	<i>NXF1</i>	Yes	Yes
NM_006018	<i>GPR109B</i>	No	Yes
NM_181777	<i>UBE2A</i>	Yes	Yes
NM_006306	<i>SMC1A</i>	Yes	Yes
NM_013236	<i>ATXN10</i>	Yes	Yes
NM_015384	<i>NIPBL</i>	Yes	Yes
NM_002828	<i>PTPN2</i>	Yes	No
NM_018127	<i>ELAC2</i>	Yes	Yes
NM_002880	<i>RAF1</i>	Yes	Yes
NM_001008493	<i>ENAH</i>	Yes	Yes
NM_025137	<i>SPG11</i>	Yes	Yes
BC038574	—	Yes	Yes
NM_003376	<i>VEGFA</i>	Yes	No
NM_001025	<i>RPS23</i>	Yes	No
CV571660	—	Yes	Yes
CV411881	—	Yes	Yes
AL523333	—	Yes	Yes
NM_005121	<i>MED13</i>	Yes	Yes
NM_016072	<i>GOLT1B</i>	Yes	Yes
NM_004966	<i>HNRPF</i>	Yes	Yes
NM_015630	<i>EPC2</i>	Yes	Yes
NM_001002857	<i>ANXA2</i>	Yes	Yes
NM_016520	<i>C9orf78</i>	Yes	Yes
NM_006472	<i>TXNIP</i>	No	Yes
NM_003324	<i>TULP3</i>	Yes	Yes
NM_201281	<i>MTMR2</i>	Yes	Yes
NM_006796	<i>AFG3L2</i>	Yes	Yes
NM_005358	<i>LMO7</i>	Yes	Yes
BC027471	—	Yes	Yes
BM685726	—	Yes	Yes
NM_182926	<i>KTNI</i>	Yes	Yes
NM_001087	<i>AAMP</i>	Yes	Yes
NM_001008844	<i>DSP</i>	Yes	Yes
AW105461	—	Yes	Yes

Table W2. Validation of Gene Expression Reactivation by qRT-PCR in 5Aza-dC–Treated HNSCC Cell Lines.

Official Symbol	Cell Lines			
	FaDu	UM-SCC-14A	UM-SCC-17A	UM-SCC-38A
<i>AAMP</i>	1.7	1.7	ND	ND
<i>ACTG1</i>	ND	ND	0.8	0.6
<i>AFG3L2</i>	ND	ND	1.0	0.5
<i>ASNS</i>	ND	ND	1.1	0.3
<i>ATP5E</i>	0.8	1.7	1.6	ND
<i>ATXN10</i>	ND	ND	0.9	0.4
<i>CAPZA1</i>	ND	2.5	1.7	ND
<i>CLDN1</i>	1.9	2.4	ND	ND
<i>CRABP2</i>	4.6	1.9	1.7	0.4
<i>DCXR</i>	ND	ND	1.3	1.1
<i>EIF3EIP</i>	ND	ND	1.0	0.8
<i>EPC2</i>	1.3	ND	ND	0.7
<i>MEPCE</i>	0.9	ND	1.5	1.7
<i>GOT2</i>	0.6	1.1	ND	ND
<i>HNRPF</i>	ND	ND	0.8	0.5
<i>KTNI</i>	ND	ND	1.2	2.7
<i>MED13</i>	1.3	1.8	ND	ND
<i>MXI</i>	22.4	4.5	ND	0.2
<i>OCIAD1</i>	1.1	ND	1.4	0.7
<i>PCMT1</i>	0.9	ND	1.2	ND
<i>PERP</i>	2.1	1.7	ND	ND
<i>PLXNB1</i>	1.8	ND	ND	0.3
<i>PSME3</i>	ND	ND	1.0	0.6
<i>RAC1</i>	1.3	ND	1.1	ND
<i>RAF1</i>	ND	ND	1.2	1.1
<i>RPL30</i>	ND	1.3	1.1	0.6
<i>SFXN1</i>	0.7	ND	1.3	0.6
<i>SLC15A3</i>	7.3	2.2	1.0	3.0
<i>SPG11</i>	1.0	2.0	1.0	ND
<i>STAG2</i>	1.4	ND	1.9	1.8
<i>THOC4</i>	ND	ND	0.8	0.8
<i>TNRC15</i>	0.8	ND	ND	0.7
<i>UQCRH</i>	ND	1.6	1.3	ND
<i>WDR47</i>	1.5	ND	1.6	0.4
<i>YWHAH</i>	ND	ND	0.9	1.0

ND indicates not determined.

Table W3. Distribution of the HNSCC Cases According to Demographic, Lifestyle, and Clinicopathological Variables.

Variables	Category	TMA, <i>n</i> (%)	MSP, <i>n</i> (%)
Age	≤53	14 (19.18)	51 (36.43)
	>53	59 (80.82)	89 (63.57)
Tumor site	Oral cavity	23 (30.67)	64 (45.71)
	Larynx	31 (41.33)	24 (17.14)
	Hypopharynx	21 (28.00)	52 (37.14)
Tumor size	T1 + T2	22 (33.85)	34 (25.37)
	T3 + T4	43 (66.15)	100 (74.63)
Lymph nodes	N0	8 (11.43)	26 (19.40)
	N+	62 (88.57)	108 (80.60)
Grade	1	25 (34.24)	36 (27.48)
	2	41 (56.16)	77 (58.78)
	3	7 (9.60)	18 (13.74)
Vascular invasion	No	57 (79.17)	115 (86.47)
	Yes	15 (20.83)	18 (13.53)
Lymphatic permeation	No	46 (63.89)	87 (64.93)
	Yes	26 (36.11)	47 (35.07)
Perineural infiltration	No	40 (55.56)	69 (51.88)
	Yes	32 (44.44)	64 (48.12)

Table W4. Sequence and Concentration of the Primer Pairs Used in the qRT-PCR Validation of Gene Induction.

Official Symbol	Sense	Sequence (5' → 3')	μM
<i>AAMP</i>	Forward	CACCTTTGCATTGCACTCAG	0.4
	Reverse	TATGGCCTGCACACTCAAAG	
<i>ACTG1</i>	Forward	AGCCTTCCTTCTGGGTATG	0.5
	Reverse	TGTTGGCGTACAGGTCTTTG	
<i>AFG3L2</i>	Forward	ACGAGGTGGCAAGAAAGATG	0.4
	Reverse	ATGACTCCACCCAGAACAG	
<i>ASNS</i>	Forward	AAGACAGCCCGATTCTACTG	0.5
	Reverse	AGAGCCTGAATGCCCTTCCTC	
<i>ATP5E</i>	Forward	TGGCAGCAACGTAAAAATTG	0.4
	Reverse	ACATGTGCCACACATCTTC	
<i>ATXN10</i>	Forward	GCGAGTGGAAAGGAATCTC	0.5
	Reverse	AGTTCTGGGAAAGCATGCAC	
<i>CAPZA1</i>	Forward	TCCGATGAGGAGAAGGTACG	0.5
	Reverse	CCCTTCCTTGAGGAGATTG	
<i>CLDN1</i>	Forward	GGTGCAGAAAGATGAGGATGG	0.4
	Reverse	CATTGACTGGGGTCTATAGGG	
<i>CRABP2</i>	Forward	GACCTCGTGGACGAGAACTG	0.4
	Reverse	CCTGGTGACACAACGTCAT	
<i>DCXR</i>	Forward	GGCCTTTGACAGATCCTTTG	0.5
	Reverse	AGCACTGGCTGGAGACAATC	
<i>EIF3EIP</i>	Forward	ACTACCAGGCCATCAAGGTG	0.5
	Reverse	GATGGCATCCTGGTAACGAC	
<i>EPC2</i>	Forward	GCAAGACATGCCTGATCTC	0.4
	Reverse	TTGCTGTGCTGAAATTGCTC	
<i>GOT2</i>	Forward	AATGTTTGCTCTGCGCAATC	0.4
	Reverse	CATCCGCATCTTTGCAGAC	
<i>HNRPF</i>	Forward	GCCTGGTAGCAACAGAAACC	0.5
	Reverse	CAGGTGATCTTGGGTGTGG	
<i>KTN1</i>	Forward	GTTTCCCAAGAACGGAGTC	0.5
	Reverse	TGTGAGCTGTTGGTTTACCG	
<i>MED13</i>	Forward	TGAAGAGCATATCACCCTTGC	0.4
	Reverse	TTGAATGCTGTCTGTGAG	
<i>MEPCE</i>	Forward	CAACCCCTGGTCGTGATG	0.4
	Reverse	CTGGCTTCAATTGGATTCC	
<i>MX1</i>	Forward	CAATCAGCTGCTGACATTG	0.4
	Reverse	TGTCTCTGCTCTGGATG	
<i>OCIAD1</i>	Forward	GAATGGGAGGGCTGATTTTC	0.4
	Reverse	TGCATTCTGCGAAGACTCTC	
<i>PCMT1</i>	Forward	CTACAGACCGCTCCACTATG	0.6
	Reverse	GGAGCACTGATTGTTGCTTG	
<i>PERP</i>	Forward	TACGAGGAGGGCTGTCAGAG	0.4
	Reverse	GGCGAAGAAGGAGAGGATG	
<i>PLXNB1</i>	Forward	TGGTTGCAAGCCATCAGAG	0.4
	Reverse	CTCTTGAGGAGGGTCTGG	
<i>PSME3</i>	Forward	CCAAGGAACCAAGGTGTTTG	0.5
	Reverse	TGGGAATCAGGAGCTGTACC	
<i>RAC1</i>	Forward	TGCTTTTCCCTTGTGAGTCC	0.4
	Reverse	ATGGGAGTGTGGGACAGTG	
<i>RAF1</i>	Forward	GCCGAACAAGCAAGAACAG	0.5
	Reverse	AACACTGCACAGCACTCTGG	
<i>RPL30</i>	Forward	CCTGGGTACAAGCAGACTC	0.4
	Reverse	TGATGGACACCAGTTTATGCC	
<i>SFXN1</i>	Forward	GCAAGTTGTGCTGTCCAGGATT	0.6
	Reverse	TTCCAAAGTGTTCAATGAATGG	
<i>SLC15A3</i>	Forward	AAGCTCGCTCTCCAAACTG	0.4
	Reverse	GCACATTGACGGTCTCTGG	
<i>SPG11</i>	Forward	TCTCCCAGGATAAGTCCAG	0.4
	Reverse	GAGGGCTTCAGGGGAATATG	
<i>STAG2</i>	Forward	AAACCAAAAGCAAGGCAAG	0.4
	Reverse	GGTTTTCCTCTCCATTCC	
<i>THOC4</i>	Forward	CTCAGACGCCGATATTCAGG	0.5
	Reverse	CTCAAAGTGCACGTCTGCTG	
<i>TNRC15</i>	Forward	CTCCAGGAGAACCCCTTC	0.6
	Reverse	TCCTCTCGTCTGTCAATC	
<i>UQCRH</i>	Forward	CGGAGGAGCTCTTTGACTTC	0.5
	Reverse	TGCCAGATGATGAAGACTG	
<i>WDR47</i>	Forward	CCAGGGGTACAAAACCTCAG	0.4
	Reverse	GATAGGCCTCTGTGCATCAAC	
<i>YWHAH</i>	Forward	GCGGTGACAGAGCTGAATG	0.5
	Reverse	TGGTTTCTGCTCAATGCTG	

CAPÍTULO 3

INTRODUÇÃO

A programação epigenética do genoma é muito importante para o desenvolvimento e o crescimento normal dos mamíferos. Esse mecanismo é essencial para os processos de gametogênese, embriogênese, expressão de genes tecido-específicos e silenciamento gênico (Li 2002) e, quando alterado está associado com diversas doenças humanas (Robertson 2005; Park, Claus et al. 2011). Os dois principais mecanismos de regulação epigenética relacionada com expressão gênica são a metilação da região promotora dos genes e a modificação das histonas.

METILAÇÃO DO DNA

A metilação do DNA é um processo complexo pelo qual as metiltransferases de DNA (DNMTs) catalisam a adição de um grupo metil ($-CH_3$) do doador universal S-adenosil-L-metionina para a posição carbono-5 das citosinas presentes em posições 5' de guaninas, formando os dinucleotídeos CpGs. Atualmente, são conhecidas três metiltransferases de DNA em mamíferos, DNMT1, DNMT3A e DNMT3B.

A metilação de citosinas em dinucleotídeos CpG é a mais prevalente alteração epigenética no genoma mamífero (Hendrich and Bird 2000) e influencia profundamente muitos processos, incluindo regulação transcricional, modulação da estrutura da cromatina, inativação do cromossomo X e silenciamento de elementos parasitas do DNA (Jones and Laird 1999; Baylin, Esteller et al. 2001; Robertson 2001). Ao contrário, a metilação de citosinas que não estão em dinucleotídeos CpG é um fenômeno incomum em mamíferos e ocorre somente em células tronco embrionárias.

No genoma humano, estima-se que as citosinas metiladas correspondam a 3-6% do total de bases do DNA. Essa proporção vem diminuindo ao longo da evolução, pois muitos dinucleotídeos CpGs foram convertidos em TpGs, por desaminação hidrolítica espontânea (Yoder, Walsh et al. 1997; Egger, Liang et al. 2004). Os sítios CpGs remanescentes apresentam uma alta frequência de metilação e participam de mudanças na estrutura da cromatina e do bloqueio da transcrição de determinados genes.

A metilação do DNA não está distribuída aleatoriamente no genoma. Ao contrário, regiões discretas, incluindo a maioria dos elementos repetitivos e de

elementos parasitas, são hipermetiladas, enquanto outras regiões são hipometiladas (Bird 2002; Suzuki and Bird 2008). Também, existem no genoma seqüências curtas de DNA ricas em CpGs, conhecidas como ilhas CpG, que não exibem metilação e estão geralmente localizadas nos promotores de genes essenciais para a função celular.

As ilhas CpGs são usualmente definidas como uma região com pelo menos 200 pares de bases, cujo conteúdo de guaninas e citosinas é igual ou superior a 50%, e que apresentam uma razão entre a freqüência observada e a freqüência esperada de dinucleotídeos CpGs igual ou superior a 0,6 (Gardiner-Garden and Frommer 1987). Essas regiões são encontradas em locais próximos aos promotores dos genes de expressão constitutiva e em 40% das regiões promotoras dos genes de expressão tecido-específica (Wang and Leung 2004). A ausência de metilação na região promotora pode ser um pré-requisito para a atividade transcricional desses últimos (Jaenisch and Bird 2003).

O padrão de metilação do DNA muda radicalmente durante o desenvolvimento embrionário. Uma onda de desmetilação generalizada ocorre na fertilização e é seguida por ondas de metilação *de novo* durante a implantação do embrião. Entretanto, nem todas as seqüências do genoma são desmetiladas no momento da fertilização e também nem todas são metiladas *de novo* após a implantação. Tais exceções enfatizam a especificidade regional do padrão de metilação do DNA (Reik, Dean et al. 2001). Como evidências da importância de tal padrão podem ser citadas as mutações em genes envolvidos no controle da metilação do DNA que resultam nas síndromes de Rett, do X frágil, da imunodeficiência do tipo ICF (*immunodeficiency, centromere instability and facial anomalies*) e da alfa talassemia ATRX (*alpha-thalassemia/mental retardation*) (Robertson and Wolffe 2000; Robertson 2005).

O silenciamento gênico, por meio da metilação do DNA, parece ser um processo regulado por diversos mecanismos. Um desses mecanismos consiste no bloqueio da interação de fatores de transcrição com seus sítios de ligação no DNA. Membros da família das proteínas de ligação a CpGs metilados (do inglês *Methyl-CpG binding proteins* - MBP), como MeCP2, MBD1-MBD4, induzem o silenciamento gênico por se associarem a vários complexos de remodelamento da cromatina e atraírem histonas desacetilases (HDACs), que promovem a compactação da cromatina (Boyes and Bird 1992; Wade 2001). Os dados da literatura sugerem que a ligação do fator CTCF (*binding factor-CCCTC*) a ilhas CpG resulta em um efeito protetor contra a metilação e que o fator BORIS (também chamado de *CCCTC-binding factor like*)

compete pelos mesmos sítios, deslocando o CTCF e permitindo a atuação de DNMTs e subsequente metilação (Robertson 2005) (Figura 1A).

Recentemente, um grande número de estudos tem mostrado o envolvimento de proteínas do complexo *polycomb* (PcG; do inglês *Polycomb complex group*) no estabelecimento de padrões de metilação. As proteínas desse complexo, principalmente PRC1 e PRC2 (*Polycomb repressive complex 1 e 2*), interagem com DNA metiltransferases e promovem o silenciamento gênico (Vire, Brenner et al. 2006; Jin, Yao et al. 2009; Margueron and Reinberg 2011) (Figura 1A).

MODIFICAÇÃO DE HISTONAS

Além da metilação do DNA, outro mecanismo epigenético envolve modificações de histonas, principalmente nos resíduos de lisina. Tais modificações incluem acetilação/desacetilação e metilação/desmetilação. As três primeiras são catalisadas enzimaticamente, mas a desmetilação compreende um processo mais complexo. Em geral, a acetilação de histonas está associada com cromatina ativa e a desacetilação com uma estrutura condensada da cromatina (Tyler and Kadonaga 1999; Wade 2001; Jaenisch and Bird 2003) (Figura 2).

A acetilação de resíduos específicos nas histonas H3 e H4 é tipicamente associada com a ativação da transcrição. Por outro lado, a metilação de resíduos específicos nestas histonas promove a ativação ou a repressão da transcrição, dependendo de qual resíduo é modificado e do grau de metilação. Por exemplo, a tri-metilação da lisina 4 em histona H3 (H3K4me3) está associada a regiões de promotores de genes transcricionalmente ativos enquanto a tri-metilação das lisinas 9 e 27 em histona H3 (H3K9me3 e H3K27me3) está presente em promotores de genes inativos (Bannister and Kouzarides 2011; Wong, Wong et al. 2011) (Figura 2).

As modificações de histonas mais associadas ao câncer são: a desacetilação de histonas H3 e H4, a perda de trimetilação da lisina 4 da histona H3 (H3K4me3) (Ellinger, Kahl et al. 2010; Benedikt, Baltruschat et al. 2011), a metilação de H3K9 (Chen, Hua et al. 2010) e a trimetilação de H3K27 (Sneeringer, Scott et al. 2010; Testoni, Schinzari et al. 2011). Um fato importante é que estas modificações são acompanhadas de metilação do DNA e, juntas, estas marcas epigenéticas levam a repressão transcricional (Figura 2).

METILAÇÃO DO DNA E CÂNCER

A ausência do padrão normal de metilação é um dos achados mais comuns em células transformadas e vários estudos têm revelado que tal alteração é um evento precoce no processo tumorigênico e contribui diretamente para a transformação maligna. Nas células tumorais, a regulação normal da maquinaria de metilação do DNA está severamente comprometida, de tal forma que a especificidade regional dos padrões de metilação é revertido, resultando em metilação *de novo* das ilhas CpG e hipometilação do DNA repetitivo (Jones and Laird 1999; Baylin, Esteller et al. 2001; Robertson 2001; Gaudet, Hodgson et al. 2003; Kaneda, Tsukamoto et al. 2004).

As ilhas CpG em regiões não promotoras são geralmente mantidas desmetiladas em células normais e são mais sensíveis à metilação que as ilhas em regiões promotoras (Nguyen, Liang et al. 2001). Sua metilação, que não bloqueia a transcrição, é observada tanto em câncer como em tecidos não neoplásicos, embora em alguns tumores sejam marcadores úteis de diagnóstico (Ushijima 2005). Na verdade, esse padrão varia ao longo da ilha CpG.

Figura 1. Mecanismos de regulação epigenética na célula normal e no câncer. O diagrama mostra uma região representativa do genoma de uma célula normal (A) e (B) a mesma região em uma célula tumoral. Essa região genômica contém sequências de elementos repetitivos (ER) e de um gene supressor de tumor (GST) com promotor associado a uma ilha CpG. (A) Em células normais, nas regiões de genes supressores de tumor, o DNA é mantido desmetilado e a cromatina contém alta densidade de histonas desacetiladas. Nestas regiões, atuam diversos elementos reguladores da maquinaria epigenética (CTCF, HAT) que permitem à cromatina ser permissiva aos fatores de transcrição (FT), o que contrasta com as regiões de elementos repetitivos. (B) Em células tumorais, ocorre a alteração desses mecanismos epigenéticos, o que resulta na hipermetilação do DNA e na desacetilação de histonas em regiões de GST, com subsequente ligação de complexos repressivos (BORIS, MBP) a esse DNA metilado impedindo, dessa forma, a transcrição gênica. PcG – complexo repressivo *polycomb*; MBP – proteína de ligação à metil-CpG; DNMT – DNA metiltransferases; HDAC – histonas desacetilases; HAT – histonas acetilases; SWI/SNF – remodelador da cromatina dependente de ATP; FT – fatores de transcrição; GST – gene supressor de tumor; CTCF/BORIS – reguladores de metilação do DNA. Modificado de Esteller, 2007 (Esteller 2007).

Figura 2. Principais modificações no código de histonas em célula normal e no câncer. Os nucleossomos consistem de um octâmero de histonas (H2A, H2B, H3 e H4), representadas como semi-esferas. Em células normais, as regiões genômicas que incluem genes supressores de tumor são ricas em histonas associadas a cromatina transcricionalmente ativa, como a acetilação de resíduos de lisina (K) em histona H3 (K9) e H4 (K5, K8, K12, K16) e a trimetilação de H3K4. Nessas células, regiões de DNA repetitivo ou outras regiões de heterocromatina são caracterizadas pela trimetilação de H3K27, dimetilação de H3K9 e trimetilação de H4K20, que funcionam como marcas repressivas. Em células tumorais, essas marcas são perdidas e frequentemente invertidas. Por exemplo, em regiões de genes supressores de tumor, as histonas perdem as marcas de cromatina ativa e ganham marcas repressoras, como a trimetilação de H3K27 e a dimetilação de H3K9. O contrário ocorre em regiões de DNA repetitivo e de heterocromatina, nas quais a perda de metilação da H3K20 e de trimetilação de H3K27 leva à formação de uma cromatina mais “relaxada” e, conseqüentemente, ativa. Modificado de Esteller, 2007 (Esteller 2007).

A metilação consistentemente associada com silenciamento gênico ocorre em um pequeno segmento da região promotora que cobre o sítio de início de transcrição (Hagihara, Miyamoto et al. 2004). No entanto, também pode ocorrer fora deste segmento principal mas ainda dentro da ilha, sem bloquear a transcrição, sendo frequentemente observada em câncer e mesmo em células normais (Issa, Ahuja et al. 2001).

A metilação isolada do segmento principal, não associada àquela de regiões flanqueadoras, é rara (Hagihara, Miyamoto et al. 2004). Alguns pesquisadores sugerem que a metilação dessas seqüências flanqueadoras ou de seqüências repetitivas próximas invadem o segmento principal e eventualmente levam à perda de expressão gênica (Jones and Baylin 2002). Outros pesquisadores acreditam que a perda de expressão e a metilação esparsa na ilha CpG são as responsáveis pela indução da metilação densa no segmento principal da ilha (Ushijima 2005). As duas hipóteses não são exclusivas e o padrão de metilação de segmentos flanquadores é potencialmente útil como marcador de risco.

Em diferentes tipos de câncer, já foram identificados muitos genes mostrando alterações epigenéticas. Entre eles estão genes envolvidos na regulação da divisão, da diferenciação e da proliferação celular. Muitos são supressores de tumor cujos promotores apresentam-se metilados nas células tumorais, contudo estão desmetilados nas células normais. Alguns exemplos de genes com padrões epigenéticos aberrantes podem ser citados, como *RASSF1*, *RARB*, *DAPK1*, *CDKN2A*, *CDKN2B*, *MGMT* e *GSTP1* em câncer de pulmão, *CDKN2A*, *CALCA*, *MGMT* e *TIMP3* em câncer de esôfago, *GSTP1* em câncer de próstata e *HIC1* e *TP53* em câncer de mama (Esteller and Herman 2002). O gene *CDX1*, cujo produto está envolvido na regulação do desenvolvimento normal do epitélio intestinal *in útero*, também exhibe correlação positiva entre o estado de metilação de sua região promotora e o padrão de expressão em células de carcinoma colorretal (Wong, Britton et al. 2004). A reativação de muitos desses genes em células tumorais pode levar à supressão do crescimento celular ou à alteração da sensibilidade das células neoplásicas a terapias anticâncer.

Ao contrário do que ocorre com os genes supressores de tumor, os proto-oncogenes exibem um nível baixo de metilação em células tumorais. Por exemplo, a hipometilação dos genes *RAF1*, *MYC*, *FOS*, *HRAS* e *KRAS* foi descrita em câncer de fígado, em animais modelo (Verma and Srivastava 2002). Outros exemplos incluem hipometilação do gene *RAS* em cânceres humanos (Fang, Zhu et al. 1996) e a correlação

inversa entre metilação e grau de expressão do gene *BCL-2* em leucemia linfocítica aguda de células B (Hanada, Delia et al. 1993).

A metilação anormal do DNA é um evento epigenético frequente em tumores de cabeça e pescoço (Smiraglia and Plass 2003; Ha and Califano 2006; Worsham, Chen et al. 2006) e representa uma fonte importante de marcadores moleculares. A recorrência de CECp parece ser mais frequente quando há inativação do gene *p16* (*CDKN2A*) (Kresty, Mallery et al. 2002), enquanto a invasão óssea está associada com metilação do gene *DCC* (Ogi, Toyota et al. 2002). Entre os genes comumente metilados estão o *CDKN2A*, o *FIHT*, o *DAPK1*, o *CDH1*, o *RASSF1A* e o *RARB* (Rosas, Koch et al. 2001; Hasegawa, Nelson et al. 2002; Kwong, Lo et al. 2002; Ha and Califano 2006; Perez-Ordóñez, Beauchemin et al. 2006; Paluszczak, Misiak et al. 2011). Tais resultados abrem a possibilidade de utilização de painéis de genes metilados para o prognóstico desses pacientes.

Realmente, as alterações epigenéticas podem ser empregadas para monitoramento da gênese e da progressão do câncer e constituem alvos terapêuticos potenciais em função de sua reversibilidade. Tratamentos com inibidores de metilação do DNA podem restaurar a atividade de genes silenciados e diminuir a taxa de crescimento de células tumorais em um modo herdável. Por exemplo, a capacidade das células em reparar o DNA danificado pode ser restaurada pela ativação do gene *MLH1*. A reversão parcial do fenótipo tumoral pelo uso de inibidores de metilação é, portanto, possível {Costa, 2010 #163; Datta, 2009 #164}.

Recentemente, algumas drogas que reverterem a inativação epigenética estão sendo utilizadas em protocolos clínicos para tratamento de pacientes com câncer, como a 5-aza-2'-deoxicitidina (5-Aza-dC). Entretanto, sua alta citotoxicidade, possivelmente decorrente de efeitos não diretamente relacionados com suas atividades desmetilantes, restringe sua aplicação clínica (Silverman, Demakos et al. 2002; Villar-Garea, Fraga et al. 2003). Observações recentes referem que o uso de doses baixas de 5-Aza-dC parece facilitar a reativação de genes metilados. Também têm sido vistas como promissoras sua administração associada a inibidores de desacetilases de histonas (Bryan, Jabbour et al. 2010) e a utilização de oligonucleotídeos antissense para DNA metiltransferases (Winqvist, Knox et al. 2006).

Embora os resultados sejam muito interessantes, a relação definitiva entre eficiência clínica e desmetilação de genes-alvo ainda não foi estabelecida. Os dados disponíveis indicam que a cooperação entre diferentes mecanismos de regulação

epigenética (metilação do DNA, metilação e acetilação de histonas, remodelamento da cromatina) é essencial para o controle apropriado da transcrição gênica. Os diferentes níveis epigenéticos envolvidos nessa complexa cooperação significam que as estratégias de desenvolvimento de drogas devem considerar ambos os eventos, metilação do DNA e modificação das histonas. Realmente, até o presente, inibidores de DNMT, tal como a 5-Aza-dC, são aparentemente os compostos mais ativos para induzir a expressão de genes silenciados epigeneticamente em células tumorais modelos. Entretanto, inibidores das acetilases de histonas podem aumentar os níveis de expressão gênica e existem evidências de que trabalham junto com inibidores de DNMT nesse processo (Bryan, Jabbour et al. 2010).

Os dados acima referidos indicam que o estudo da metilação do DNA e dos elementos da cromatina associados ao silenciamento gênico e à caracterização cuidadosa dos padrões de metilação no câncer humano é muito importante. A precisa quantificação do status de metilação de ilhas CpG pode, sem dúvida, resultar no desenvolvimento de um marcador molecular poderoso para diagnóstico e prognóstico de neoplasias e levar à identificação de novos alvos terapêuticos.

OBJETIVOS

Em função do (a) papel relevante da metilação do DNA na carcinogênese, (b) da importância e da necessidade de identificação de marcadores moleculares precoces em tumores de cabeça e pescoço e (c) dos resultados já obtidos anteriormente pelo nosso grupo, o presente trabalho teve como objetivo geral investigar o perfil de metilação de ilhas CpG em carcinomas epidermóides de cabeça e pescoço (CECP), bem como identificar e iniciar estudos funcionais de biomarcadores candidatos para diagnóstico e prognóstico desses tumores. Os objetivos específicos compreenderam:

1. Analisar, em amostras de carcinomas epidermóides de cabeça e pescoço, o perfil de metilação do DNA na região dos promotores dos genes *MXI* e *SLC15A3* e correlacionar os resultados obtidos com os dados clínico-patológicos dos pacientes e com a evolução da doença.
2. Analisar, em amostras de carcinomas epidermóides de cabeça e pescoço, o perfil de metilação do DNA em sequência próxima ao sítio de início de transcrição de genes conhecidamente metilados em diversas neoplasias humanas e correlacionar os resultados obtidos com os dados clínico-patológicos dos pacientes e com a evolução da doença.
3. Analisar, em linhagens celulares, o perfil de expressão de dois transcritos variantes do gene *MXI* e correlacioná-lo com o padrão de metilação das ilhas CpGs associadas aos seus promotores.
4. Analisar, em linhagens celulares, o perfil de expressão do gene *SLC15A3* e correlacioná-lo com o padrão de metilação das ilhas CpGs associadas aos seus promotores.
5. Investigar o efeito do silenciamento da expressão gênica por RNA de interferência contra o *MXI* pela análise do índice de proliferação e por estudos de migração e do ciclo celular.

MATERIAIS E MÉTODOS

Amostras

Linhagens celulares de cabeça e pescoço

No presente trabalho, foram utilizadas linhagens celulares de carcinomas epidermóides de cabeça e pescoço e de outros tipos tumorais, sendo a maioria proveniente da “*American Type Culture Collection*” (ATCC, Manassas - VA E.U.A.). As características gerais dessas células são mostradas na Tabela 1.

Tabela 1. Características das linhagens utilizadas no estudo.

Linhagem	Tecido de origem	ATCC	Dados dos pacientes	
			Idade	Sexo
<i>SCC-4</i>	Carcinoma epidermóide de língua	CRL-1624	55	homem
<i>SCC-9</i>	Carcinoma epidermóide de língua	CRL-1629	25	homem
<i>SCC-15</i>	Carcinoma epidermóide de língua	CRL-1623	55	homem
<i>SCC-25</i>	Carcinoma epidermóide de língua	CRL-1628	70	homem
<i>SCC-38</i>	Carcinoma epidermóide de laringe	ND	66	homem
<i>FaDu</i>	Carcinoma epidermóide de hipofaringe	HTB-43	56	homem
<i>TALL</i>	Leucemia linfoblástica aguda	CRL-11386	2	homem
<i>LOUCY</i>	Leucemia linfoblástica aguda de células T	CRL-2629	38	mulher
<i>WDT4</i>	Leucemia linfocítica aguda (<i>BCR/ABL</i> +)	ND	ND	ND
<i>WDT5</i>	Leucemia linfocítica aguda (<i>BCR/ABL</i> +)	ND	ND	ND
<i>HL60</i>	Leucemia promielocítica aguda	CCL-240	36	mulher
<i>CEM</i>	Leucemia linfoblástica aguda	CRL-2264	4	mulher
<i>BV-173</i>	Leucemia aguda (Ph +)	ND	46	homem
<i>RS4</i>	Leucemia linfoblástica aguda	CRL-1873	32	mulher
<i>MCF12</i>	Epitélio de mama	CRL-10782	60	mulher
<i>CAMA1</i>	Adenocarcinoma de mama	HTB-21	51	mulher
<i>MCF7</i>	Adenocarcinoma de mama	HTB-22	69	mulher
<i>MB-468</i>	Adenocarcinoma de mama	HTB-132	51	mulher
<i>SKBR3</i>	Adenocarcinoma de mama	HTB-30	43	mulher
<i>HCT116</i>	Carcinoma colorretal	CCL-247	adulto	homem
<i>SW620</i>	Adenocarcinoma colorretal	CCL-227	51	homem
<i>SW480</i>	Adenocarcinoma colorretal	CCL-228	50	homem
<i>KM12</i>	Adenocarcinoma colorretal	ND	ND	ND
<i>H460</i>	Carcinoma de pulmão	HTB-177	ND	homem

ND – não determinado.

Amostras de doadores normais (sangue)

Como controle, foram utilizadas sete amostras de sangue periférico de doadores normais, gentilmente cedidos pela prof.a. Fernanda Carregaro.

Amostras de carcinomas epidermóides de cabeça e pescoço

No presente estudo, também foram utilizadas amostras de tumores e margens cirúrgicas provenientes de 54 pacientes com carcinoma epidermóide das vias aerodigestivas superiores. Na Tabela 2, estão apresentados os dados clínico-patológicos de cada paciente. Tais amostras foram colhidas após ressecção cirúrgica pelas equipes de médicos do Projeto Temático (Processo FAPESP 04/12054-9) “Marcadores de agressividade em tumores de cabeça e pescoço” (Instituto Arnaldo Vieira de Carvalho, SP; Hospital Heliópolis; Hospital das Clínicas da Faculdade de Medicina, USP, SP; UNIVAP, São José dos Campos) e imediatamente armazenadas em nitrogênio líquido ou freezer -80°C , para posterior microdissecção, extração de RNA, DNA e proteínas. De todos os pacientes foram obtidas informações clínicas e laboratoriais. O projeto temático foi aprovado pelo CONEP (Parecer 1763/05 - Proc. 25000.152864/2005-50, Registro no. 12380/CONEP, Data 18/095/2005).

Tabela 2 – Característica clínico patológicas das amostras de carcinomas epidermóides de cabeça e pescoço

Caso	Sexo	Idade	Fuma	Etilista	Sítio	TNM	Estadio	Diferenciação Histológica	Infiltração Vascular Sanguínea	Infiltração Linfática	Invasão Perineural	Infiltrado Inflamatório peri-tumoral	Óbito
CP1/0012	M	55	Sim	Sim	Faringe	T4N2BM0	IV	Moderadamente	Ausente	Presente	Ausente	Escasso	Pela Neoplasia
CP1/0019	F	59	No passado	No passado	Língua	T4N2CM0	IV	Bem diferenciado	Ausente	Presente	Presente	Moderado	Pela Neoplasia
CP1/0174	M	68	Sim	No passado	Laringe	T2N0M0	II	Bem diferenciado	Ausente	Ausente	Ausente	Intenso	Não
CP1/0271	F	50	Sim	Nunca	Laringe	T2N1M0	III	Pouco diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP3/0018	M	61	Sim	No passado	Laringe	T4N2BM0	IV	Moderadamente	Ausente	Ausente	Ausente	Moderado	Pela Neoplasia
CP3/0019	M	54	Sim	Sim	Laringe	T4N0M0	IV	Moderadamente	Ausente	Ausente	Ausente	Escasso	Não
CP3/0020	M	53	No passado	No passado	Laringe	T1N1M0	III	Moderadamente	Ausente	Ausente	Presente	Moderado	Não
CP3/0031	M	54	Sim	Sim	Laringe	T4N2CM0	IV	Moderadamente	Ausente	Ausente	Presente	Moderado	Pela Neoplasia
CP3/0069	M	58	Sim	Sim	Língua	T1N0M0	I	Pouco diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP3/0076	M	64	No passado	No passado	Laringe	T4N2BM0	IV	Pouco diferenciado	Ausente	Ausente	Presente	Escasso	Não
CP3/0084	M	61	Sim	Nunca	Laringe	T3N0M0	III	Bem diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP3/0085	M	59	Sim	Sim	Laringe	T4N3M0	IV	Pouco diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP3/0096	M	54	Sim	Nunca	Laringe	T4N0M0	IV	ND	Ausente	Ausente	Ausente	Escasso	Não
CP3/0102	M	49	Sim	Sim	Laringe	T2N1M0	III	Bem diferenciado	Presente	Ausente	Ausente	Escasso	Não
CP3/0105	M	67	No passado	Sim	Laringe	T4N2CM0	IV	Moderadamente	Ausente	Ausente	Presente	Moderado	Pela Neoplasia
CP3/0113	M	67	Nunca	Sim	Língua	T4N1M0	IV	Bem diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP3/0119	M	67	Sim	Sim	Laringe	T4N2CM0	IV	Bem diferenciado	Ausente	Presente	Presente	ND	Pela Neoplasia
CP3/0124	M	68	Sim	Sim	Laringe	T4N2CM0	IV	Moderadamente	Ausente	Ausente	ND	Escasso	Pela Neoplasia
CP3/0125	M	63	No passado	No passado	Laringe	T4N3M0	IV	Pouco diferenciado	Presente	Presente	Presente	Moderado	Não
CP3/0136	M	65	Sim	Sim	Laringe	T4N2BM0	IV	Bem diferenciado	Ausente	Ausente	Ausente	Escasso	Pela Neoplasia
CP3/0147	M	44	No passado	Nunca	Laringe	T2N0M0	II	Moderadamente	Ausente	Ausente	Ausente	ND	Não
CP3/0153	M	49	Sim	No passado	Laringe	T3N3M0	IV	Moderadamente	Presente	Presente	Presente	Moderado	Pela Neoplasia
CP3/0193	M	49	No passado	No passado	Língua	T2N1M0	III	Bem diferenciado	Ausente	Ausente	Ausente	ND	Não
CP3/0199	M	74	Sim	Nunca	Laringe	T4N0M0	IV	Bem diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP3/0209	M	67	No passado	No passado	Laringe	T4N0M0	IV	Bem diferenciado	Ausente	Ausente	Presente	Escasso	Pela Neoplasia
CP3/0246	M	69	No passado	Nunca	Laringe	T4N0M-ND	ND	Moderadamente	Ausente	Ausente	Presente	Escasso	Não
CP3/0377	M	78	No passado	No passado	Laringe	T4N0M-ND	ND	Moderadamente	Ausente	Ausente	Presente	ND	Outras Causas
CP3/0390	M	55	Sim	Sim	Laringe	T4N0M-ND	ND	Moderadamente	Ausente	Ausente	Ausente	Intenso	Não
CP3/0411	M	49	Sim	Sim	Laringe	T4N2BM-ND	ND	Moderadamente	Presente	Ausente	Presente	Intenso	Outras Causas
CP3/0433	M	56	Sim	Sim	Laringe	T4N0M-ND	ND	Bem Diferenciado	Ausente	ND	Ausente	Escasso	Não
CP3/0464	M	79	Sim	No passado	Laringe	T4N2BM-ND	ND	Moderadamente	Ausente	Ausente	Ausente	Intenso	Pela Neoplasia
CP3/0485	M	47	Sim	No passado	Laringe	T3N0M-ND	ND	Moderadamente	Ausente	Ausente	Ausente	ND	ND
CP4/0002	M	56	Sim	Sim	Laringe	T3N0M-ND	ND	Bem Diferenciado	Ausente	Ausente	Ausente	Moderado	Não
CP4/0008	M	46	Sim	Sim	Laringe	T3N0M-ND	ND	Moderadamente	Ausente	Ausente	Ausente	Intenso	Não

CP4/0016	F	52	Sim	Sim	Laringe	T1N0M-ND	ND	Bem diferenciado	Ausente	Ausente	Ausente	Escasso	Não
CP4/0024	M	45	Sim	No passado	Laringe	T4N0M-ND	ND	Bem diferenciado	Ausente	Ausente	Ausente	Intenso	ND
271901	M	56	Sim	Sim	Faringe	ND	ND	ND	ND	ND	ND	ND	ND
275296	M	50	No passado	Nunca	Laringe	ND	ND	ND	ND	ND	ND	ND	ND
279422	F	58	Sim	Sim	Laringe	ND	ND	ND	ND	ND	ND	ND	ND
281415	M	50	Sim	Sim	Laringe	T4N1M0	ND	ND	ND	ND	ND	ND	ND
CP1/0001	M	53	Sim	Sim	Laringe	T2N2M0	IV	Moderadamente	Presente	Presente	Presente	Moderado	Pela neoplasia
CP1/0006	M	57	Sim	Sim	Laringe	T4N1M0	IV	Moderadamente	Ausente	Presente	Presente	Escasso	Pela neoplasia
CP1/0017	M	55	Sim	No passado	Língua	T2N0M0	II	Moderadamente	Ausente	Ausente	Ausente	Moderado	Não
CP1/0027	M	48	Sim	Sim	Laringe	T4N2CM0	IV	Bem diferenciado	Ausente	Presente	Ausente	Escasso	Pela neoplasia
CP1/0023	M	56	Sim	Sim	Faringe	T4N0M0	IV	Moderadamente	Ausente	Ausente	Ausente	Moderado	Pela neoplasia
CP1/0042	M	70	Sim	Sim	Laringe	T4N2CM0	IV	Moderadamente	Ausente	Presente	Ausente	Escasso	Pela neoplasia
CP1/0036	M	66	No passado	No passado	Laringe	T4N0M0	IV	Moderadamente	Ausente	Ausente	Presente	Escasso	Pela neoplasia
CP1/0037	M	55	Sim	No passado	Faringe	T4N2BM0	IV	Moderadamente	Presente	Presente	Presente	Moderado	Pela neoplasia
CP1/0041	M	44	Sim	No passado	Faringe	T4N2BM0	IV	Moderadamente	Ausente	Presente	Ausente	Moderado	Não
CP1/0046	M	60	No passado	Sim	Laringe	T4N2CM0	IV	Moderadamente	Ausente	Presente	Presente	Escasso	Outra causa
CP1/0058	M	50	Sim	No passado	Laringe	T4N2CM0	IV	Moderadamente	Presente	Presente	Ausente	Moderado	Não
CP1/0049	M	44	Sim	No passado	Língua	T4N2CM0	IV	Moderadamente	Presente	Presente	Presente	Moderado	Não
CP1/0066	M	72	Sim	Sim	Laringe	T4N2BM0	IV	Bem diferenciado	Ausente	Presente	Ausente	Moderado	Pela neoplasia
CP1/0065	M	46	Sim	No passado	Faringe	T2N0M0	II	Bem diferenciado	Ausente	Ausente	Ausente	Moderado	Não

ND – Não determinado

Extração de DNA

O DNA das amostras de carcinomas epidermóides de cabeça e pescoço foi obtido pelo método de extração por *TRIzol*[®] de RNA, DNA e proteínas (*Invitrogen*, Carlsbad - CA, E.U.A) seguindo protocolo fornecido pelo fabricante. Os DNAs das linhagens celulares utilizadas no presente trabalho foram gentilmente cedidos pelo Dr. Jean-Pierre Issa, MD Anderson Cancer Center, Houston, Texas.

Conversão do DNA por Bisssulfito de Sódio

Após a determinação da concentração por espectrofotometria, 2 µg de DNA genômico foi ressuspendido em 20 µl de DDW (*Double Distilled Water*). Em seguida, os DNAs foram tratados com bissulfito de sódio, que promove a desaminação de citosinas não metiladas em uracilas, posteriormente substituídas por timinas durante a síntese de uma nova fita de DNA. As citosinas metiladas (5-metilcitosinas), ao contrário, são mantidas intactas e permanecem como citosinas após a reação de amplificação. A reação foi realizada com a utilização do *EZ DNA Methylation Gold Kit*[™] (*Zymo*, Irvine – CA, E.U.A) de acordo com as instruções fornecidas pelo fabricante.

Desenho de iniciadores para Nested PCR e Pirosequenciamento

As regiões promotoras dos genes *SLC15A3*, *MX1*, *CDH1*, *CDH13*, *DAPK*, *CDKN2A*, *RASSF1A*, *SOCS3*, *TIMP3* foram amplificadas por PCR Nested e posteriormente sequenciadas. Foram desenhados, com o auxílio do programa *PyroMark Assay Design Software* (*Qiagen*, Valencia – CA, E.U.A.), iniciadores externos e internos aos CpGs presentes nas regiões promotoras de cada gene, além de uma sonda de sequenciamento. Uma sequência universal de nucleotídeos (5' – GGGACACCGCTGATCGTTTA – 3'), necessária para a ligação da biotina, foi adicionada a um dos iniciadores utilizados nas reações de nested PCR. Após amplificação dos fragmentos de interesse, a fita do DNA biotinilada foi purificada e, posteriormente, a porcentagem de metilação dessas regiões foi avaliada por pirosequenciamento. A Tabela 3 mostra a sequência dos iniciadores externos, dos iniciadores internos e da sonda de sequenciamento de cada gene, bem como os tamanhos dos produtos de amplificação.

PCR e Nested PCR

As reações da primeira PCR e da Nested PCR foram processadas em 25µL, contendo 2,5 µl de tampão 10X, 4,0 µl de dNTP mix a 1,25 µM), 1µl de $MgCl^{2+}$ a 50mM, 0,4 µl de oligonucleotídeos a 10 pmol e 0,2 µl de polimerase de DNA a 5U/µl. Em todos os experimentos, um dos tubos não recebeu DNA, como controle de contaminação. Para a primeira PCR, foram utilizados cerca de 50 ng de DNA tratado e 1µl desse produto foi utilizado para a Nested PCR.

A reação da primeira PCR compreendeu um passo inicial de 10 minutos a 95°C para ativação da enzima *Taq Platinum (Invitrogen)*, seguido de 30 ciclos de: 50 segundos a 95°C, 50 segundos à temperatura de anelamento de cada seqüência, 1 minuto a 72°C e um passo final de cinco minutos a 72°C para extensão dos fragmentos.

Tabela 3 - Iniciadores externos, iniciadores internos e sonda de sequenciamento de cada gene de interesse, bem como os tamanhos dos respectivos produtos de amplificação.

Gene	Localização	Oligo	5' → 3'
MXI (Variante longa)	+ 289/+317	F1	TAGGGAGGATGGGTAGGTTGT
		R1	CACCACCAAAAAACAATATTACTC
		F2	GATGGGTAGGTTGTGGGATGT
		RU	GGGACACCGCTGATCGTTTACACCACCAAAAAACAATATTACTC
		S	GTAGGTTGTGGGATGTT
MXI (Variante curta)	+53/+97 (região 1)	F1	GAAGGGGGGAAGGATATGTTTA
		R1	CCCCAACCCAAACCTACTAAATC
		F2	GGGGGAAGGATATGTTTAGGTT
		RU	GGGACACCGCTGATCGTTTACCCCAACCCAAACCTACTAAATC
		S	GGATATGTTTAGGTTTAAGG
	+668/+709 (região 2)	F1	TTAGAGGTTTTTTTGTAGGAGAGG
		R1	AAAAATCCACCAAAAAACAATAATC
		F2	TTTTTTGTAGGAGAGGTTGGGAAG
		RU	GGGACACCGCTGATCGTTTAAAAATCCACCAAAAAACAATAATC
		S	TGGGAAGGGGTGGG
SLC15A3	+369/+403	F1	TTGTTGGTGGAGATGTTGGA
		R1	CTACTCCCCCATCCAATTAAAA
		FU	GGGACACCGCTGATCGTTTATTGTTGGTGGAGATGTTGGA
		R2	CCCATCCAATTAAAAATTAATAC
		S	AAAATTAATACTATTAAAAATACAA
CDHI	-33/+2	F1	TTAGGAGAGAGGAAGGGATTGG
		R1	CCCCATTTCTCATTCCTACCA
		F2	TTTATTTGGGGTTTTTGAGGGT
		RU	GGGACACCGCTGATCGTTTACCCCATTTCTCATTCCTACCA
		S	TTTTAGTTTAGGTTTTATTATAAG
CDHI3	+74/+102	F1	TTTGGGAAGTTGGTTGGTTG
		R1	ACAACCCCTCTTCCCTACct
		F2	AGTTTGGTTTTTAAGGAAAATATGTTTAGT
		RU	GGGACACCGCTGATCGTTTAAACCAATTCTCCACTACATTTTATCC
		S	TAAGGAAAATATGTTTAGTGTA
RASSF1A	+213/+244	F1	ATGTAGGGGAGTTTGAGTTTATTGA
		R1	CACCACCCCCCAAATAAAATC
		F2	GTTGGGAGAGTTGGGAAGGGT
		RU	GGGACACCGCTGATCGTTTACACCACCCCCCAAATAAAATC
		S	AGGGTYGTATTYGGTTGGA
DAPK1	-24/+10	F1	AGAGAGGAGTTGGGAGGAGTAG
		R1	CCCACAAACCACCAATAAAAAAC
		F2	GTAGAGTAGGTTTGGTAGGGTAGTT
		RU	GGGACACCGCTGATCGTTTACCCACAAACCACCAATAAAAAAC
		S	GTAGGGTAGTTYGGAGGTG
CDKN2A	-16/+25	F1	GGGGTAGGTGGGAGGAGTT
		R1	CCCCTCCTCTTCTTCTCTCC
		F2	GGTTGTTTTAGGTTGGTGTTTT
		*R	*-ACCCTATCCCTCAAATCCTCTAAAA
		S	GATTTTAGGGGTGTTATATT
SOCS3	-3/+39	F1	GGAGGGGATTAGGAGAGGGAT
		R1	ACCCCCCAATTCCAAAAATC
		F2	GAAGAGGTAGTAGAGGAGTAGGGAGTTTA
		RU	GGGACACCGCTGATCGTTTACCCCCCAATTCCAAAAATC
		S	AGAGGAGTAGGGAGTTTAAG
TIMP3	+538/+619	F1	GGTGGGTGGGTGTTAGTTGG
		R1	CAAACCTCCTACCCCTTCTC
		F2	GGTTTTGGTTTGGGTTAGAGATA
		RU	GGGACACCGCTGATCGTTTCAAACCTCCTACCCCTTCTC
		S	GGTTAGAGATATTTAGTGTTTA

TSS (*Transcript Start Site*) – posição da região analisada em relação ao sítio de início da transcrição. * - primer biotinilado diretamente. Sequência em azul – sequência universal de ligação à biotina.

A reação de Nested PCR compreendeu um passo inicial de 10 minutos a 95 °C para ativação da enzima *Taq Platinum*, seguido de 5 ciclos de: 50 segundos a 95°C, 50 segundos à temperatura de anelamento de cada gene, 1 minuto a 72°C e um passo final de cinco minutos a 72°C para extensão dos fragmentos. Os produtos das amplificações foram submetidos à corrida eletroforética em gel de poliacrilamida 8% para visualização das bandas de interesse.

Pirosequenciamento

A metodologia de pirosequenciamento foi realizada neste trabalho por possibilitar a análise quantitativa de metilação do DNA em regiões promotoras dos genes de interesse. Essa técnica necessita que o produto de PCR esteja na forma de fita simples de DNA, o que permite à sonda de sequenciamento anelar-se à sequência alvo. Diversos métodos têm sido descritos e mostraram-se eficientes na obtenção da fita molde de DNA para as reações de pirosequenciamento (Nordstrom, Nourizad et al. 2000; Nordstrom, Alderborn et al. 2002; Diggle and Clarke 2003). No presente trabalho, foi utilizado o método descrito por Ronaghy e colaboradores, 1996 (Ronaghi, Karamohamed et al. 1996), que produz um DNA de alta qualidade sem a necessidade de purificação prévia do produto de PCR. Nessa técnica, uma das fitas do produto de PCR é marcada com biotina. Essa fita é capturada em microesferas revestidas por estreptoavidina e o DNA não biotinilado é removido por tratamento com solução alcalina (NaOH 0,2M). O DNA biotinilado é então lavado com uma solução de Tris (10mM, pH 7,6) para neutralizar o pH básico. Finalmente, a fita simples de DNA purificada é transferida para uma placa de pirosequenciamento contendo um tampão de anelamento (20mM Tris acetato, 2mM Mg acetato, pH7,6) e a sonda de sequenciamento.

Estes últimos experimentos foram desenvolvidos no laboratório do Dr. Jean-Pierre Issa do departamento de Leucemia, MD Anderson Cancer Center, Universidade do Texas, Houston-TX.

Cultivo Celular

Os experimentos seguintes foram realizados no laboratório do Roger Chammas do departamento de Oncologia, Faculdade de Medicina, Universidade de São Paulo - FMUSP.

As linhagens celulares de carcinoma epidermóide de cabeça e pescoço (*SCC-4*, *SCC-9*, *SCC-15* e *SCC-25*) foram cultivadas em frascos plásticos de 25 ou 75 cm² em meio de cultura DMEM/F-12 (*Invitrogen*), suplementado com 10% de soro fetal bovino (SFB), penicilina (100U/mL), estreptomicina (100µg/mL), glutamina (4mM), a 37°C em atmosfera contendo 5% de CO₂ e 95% de umidade. As células utilizadas nos experimentos foram subcultivadas sempre que atingiram uma confluência de aproximadamente 60-70%. Para isto, o meio de cultura foi removido, as células lavadas com 10 ml de solução salina tamponada com fosfato pH 7,4 (PBS) e então incubadas a 37°C com 0,5 ml ou 2 ml de tripsina a 2%, para frascos de 25 cm² ou 75 cm², respectivamente. Após um período que variou de 5 a 25 minutos, dependendo da linhagem, as células destacaram-se do soallo do frasco de cultura (o que foi determinado por observação em microscópio de contraste de fase), sendo então a ação da tripsina interrompida pela adição de meio de cultura DMEM (*Invitrogen*) com 10% de SFB. A suspensão de células foi transferida para tubos *falcon* de 15 ml estéreis e centrifugada a 1500 rpm por 5 minutos. O sobrenadante foi removido e descartado e o *pellet* de células ressuspendido em 5 ml de meio de cultura DMEM/F-12 com 10% de SFB, as células contadas e replaqueadas em novos frascos de 25 cm² ou 75 cm² (1x 10³ células em 5 ml de meio para frascos de 25 cm² e 1x10⁵ células em 10 ml de meio para frascos de 75cm²). O meio de cultura foi trocado a cada 48 horas. Com o objetivo de manter o estoque congelado em nitrogênio líquido e trabalhar sempre com as células em passagens semelhantes, várias amostras de cada linhagem celular foram congeladas antes da realização dos experimentos. Para isto, as células foram suspensas em solução 1/1 de DMEM/F-12 contendo 20% de di-metilsulfóxido (DMSO - *Sigma*, Munich, Alemanha) e DMEM/F-12 com 50% de SFB e congeladas em nitrogênio líquido.

Curvas de proliferação celular

O potencial proliferativo das linhagens SCC foi avaliado por curvas de proliferação. Um total de 5x10³ células foi plaqueado em 1ml de meio DMEM/F12 contendo 10% de SFB em cada poço de uma placa para cultura celular de 24 poços (*Nunc Brand Products*, Roskilde, Dinamarca). Após 24 horas, o meio foi trocado por DMEM/F-12 livre de SFB e as células incubadas por mais 48 horas. Para estimular o crescimento celular, o meio foi substituído por DMEM/F-12 contendo 10% de FBS. Após 24, 48, 72, 96, 120, 144, 168 e 192 horas, as células de 2 poços de cada placa foram lavadas duas vezes com PBS a 37 °C e incubadas com 0,3 ml de tripsina a 2%,

também a 37°C por 20-25 minutos, até que estivessem completamente separadas do soalho da placa. A tripsina foi inativada com 2 ml de meio DMEM com 10% de FBS. O crescimento e a morfologia celular foram avaliados diariamente em microscópio invertido.

Silenciamento de expressão gênica por RNA de interferência

O silenciamento da expressão do gene *MX1* foi realizados nas linhagens *SCC-4*, *SCC-9*, *SCC-15* e *SCC-25*. Para tanto, foi utilizado o kit *TriFECTa (Integrated DNA Technologies - Coralville, IA, E.U.A.)* que contém três oligos duplex de 27mer, específicos para o gene *MX1*, e um controle negativo (duplex *Scrambled* universal).

Para cada experimento, foi feito o plaqueamento de 2×10^4 células em placa de 24 poços. As células foram cultivadas até atingirem entre 40 e 60% de confluência e, 24 horas antes da realização dos experimentos de transfecção, o meio foi substituído por outro sem antibiótico. No momento da transfecção, o meio foi removido e as células lavadas com PBS e a transfecção foi realizada com meio *Opti-MEM (Invitrogen)*. Durante a etapa de padronização, as células foram transfectadas com 1nM, 10nM e 20nM de duplex para *MX1* e 10nM para os controles, e os lipossomos foram utilizados como agentes de transfecção. Foram testados dois tipos de lipossomos; a *Lipofectamine 2000 (Invitrogen)* e a *Lipofectamine RNAiMax (Invitrogen)*. As células foram mantidas em incubadora úmida a 37°C em atmosfera de 5% de CO₂. Após um período de 24 horas, os RNAs das linhagens celulares foram extraídos, os cDNAs foram confeccionados e foram realizadas reações de PCR em tempo real para avaliação do nível de expressão do gene *MX1*.

Extração de RNA e síntese de cDNA

O RNA total das amostras foi isolado com *TRIzol[®] (Invitrogen)* e sua qualidade e integridade foram avaliadas por espectrometria e eletroforese em gel de agarose. Depois de isolados, os RNAs foram utilizados para síntese de DNA complementar (cDNA) com a enzima transcriptase reversa e posterior amplificação e quantificação por PCR em tempo real.

O RNA total foi previamente tratado com *RQ1 Rnase-Free Dnase (Promega Corporation, Madison – WI, E.U.A.)*, seguindo especificações do fornecedor, a fim de eliminar qualquer vestígio de DNA genômico. A síntese de cDNA foi realizada com o *High Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad - CA, E.U.A.)*,

segundo protocolo do fornecedor. A qualidade da síntese foi avaliada por reação de RT-PCR para o gene *GAPDH*.

PCR em tempo real

A metodologia de PCR em tempo real foi realizada segundo protocolo estabelecido no laboratório. Todas as reações de PCR em tempo real foram realizadas no aparelho *7500 Real-Time PCR System (Applied Biosystems)*. Foram desenhados iniciadores específicos para cada transcrito por meio da utilização da ferramenta *Primer3* (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>) e as reações de padronização para cada par de oligonucleotídeo foram realizadas com o objetivo de selecionar a concentração que garantisse sua saturação. As concentrações de oligonucleotídeos testadas foram 0,2µM; 0,4µM; 0,6µM e 0,8µM.

As reações de padronização foram processadas em volumes de 20µL, contendo 10ng de cDNA proveniente de pool de amostras tumorais de língua, 10µL de *Power Master Mix SYBR GreenTM* (*Applied Biosystems*) e os oligonucleotídeos iniciadores nas concentrações referidas na Tabela 2. O *Power Master Mix SYBR GreenTM* contém todos os componentes para a reação de PCR em tempo real, exceto o *template*, os oligonucleotídeos iniciadores e a água. Para uma melhor análise dos resultados, os ensaios de otimização de concentração de oligonucleotídeos foram realizados em duplicata para cada gene analisado. Além disso, todos os experimentos incluíram controles negativos sem cDNA.

As reações compreenderam uma etapa inicial de 2 minutos a 50°C para a ativação da enzima UNG, seguida de um passo de 10 minutos a 95 °C para inativação da UNG, evitando, dessa forma, que a enzima degradasse os produtos da termociclagem a ser iniciada em seguida, e também para a ativação da enzima *AmpliTaq Gold®* (*Applied Biosystems*). Posteriormente, seguiram-se 40 ciclos de 15 segundos a 95°C e um minuto a 60°C para extensão. Foi acrescentado um passo de dissociação que compreende duas etapas de 15 segundos a 95°C, intercaladas por uma etapa de 30 segundos a 60°C. A curva de dissociação tem como objetivo a verificação de ocorrência de *primer dimers*, produtos inespecíficos e de contaminações.

As eficiências de amplificação dos iniciadores específicos para os transcritos alvos e para o gene normalizador foram calculadas de acordo com a equação:

$$E = 10^{(-1/slope)}$$

Nessa equação, o *slope* corresponde à inclinação da reta obtida quando se analisa a variação do Ct dos transcritos alvo e do normalizador em função do log de diferentes quantidades de cDNA. Para verificar a eficiência de amplificação, foram realizadas diluições seriadas de cDNA da linhagem celular *FaDu*, sendo as concentrações finais iguais a 20ng, 10ng, 5ng, 2,5ng e 1,25ng. As amostras de cDNA nas concentrações citadas foram submetidas à amplificação por PCR em tempo real. O teste foi realizado para cada gene, sendo a concentração de oligonucleotídeo otimizada previamente.

Para cálculo da expressão diferencial, foi utilizado o modelo matemático proposto por PFAFFL, 2001 (Pfaffl 2001) segundo a fórmula,

$$R = (E \text{ alvo})^{\Delta Ct \text{ alvo}} / (E \text{ normalizador})^{\Delta Ct \text{ normalizador}}$$

Nessa fórmula, o “E alvo” corresponde à eficiência de amplificação dos iniciadores específicos para o transcrito alvo, o “E normalizador” corresponde à eficiência de amplificação dos iniciadores específicos para o gene normalizador, o “ ΔCt alvo” corresponde à diferença entre o Ct do transcrito alvo obtido para a amostra em análise e o Ct do transcrito alvo obtido para a amostra referência, o “ ΔCt normalizador” corresponde à diferença entre o Ct do gene endógeno obtido para a amostra em análise e o Ct do gene endógeno obtido para a amostra referência.

PCR em tempo real

Após a etapa de padronização, foi realizada a análise da expressão diferencial do gene *MXI*. As amostras foram testadas em duplicata com controles negativos. Foram considerados como diferencialmente expressos os transcritos que exibiram expressão diminuída nas linhagens transfectadas com os duplex para o *MXI* em relação às linhagens transfectadas com o controle negativo (duplex *Scrambled*), com valor superior ao *cut off* (≥ 3.0) com base nos dois controles endógenos (*GAPDH* e *TUBA1C*).

Avaliação de ciclo e morte celular

As células *SCC-15* e *SCC-25* foram mantidas em cultura por um período de 36 horas após ensaio de silenciamento gênico. As células foram tripsinizadas e centrifugadas juntamente com o meio de cultura, e o *pellet* foi fixado em etanol 70% e mantido congelado até o momento da análise por citometria de fluxo (citômetro *FACScalibur Becton & Dickson*[®], Franklin Lakes – NJ, E.U.A). A avaliação de ciclo e morte celular foi realizada por meio da marcação com iodeto de propídeo (PI, do inglês *propidium iodide*). O PI se incorpora estequiometricamente ao DNA, permitindo uma quantificação relativa do conteúdo de DNA.

A análise da citometria de fluxo foi realizada utilizando o programa *Cell Quest Pro* (*Becton & Dickson*[®]). Cada condição foi avaliada em triplicata, a porcentagem das células nas diferentes fases do ciclo celular foi quantificada e os dados foram apresentados em histogramas. Para avaliar o papel do gene *MX1* sobre a proliferação celular em linhagens celulares de CECP foi realizado o cálculo da fração proliferativa das células. Para tanto, foi utilizada a seguinte razão:

$$SG_2M/G_0G_1,$$

“*SG₂M*” corresponde à população de células contidas em M3 e “*G₀G₁*” corresponde à população de células contidas em M2 dos histogramas obtidos por citometria de fluxo. Uma razão maior ou menor que 1 indica aumento ou redução de proliferação, respectivamente.

Ensaio de migração celular

O efeito do silenciamento do gene *MX1* sobre o processo de migração celular foi investigado por meio do ensaio de “cicatrização de ferida” em cultura de monocamada. As células foram semeadas sobre lamínulas de vidro de 13mm em placas de 24 poços e em câmaras de cultura de 2 poços. O experimento de silenciamento gênico por RNA de interferência foi realizado quando as células atingiram aproximadamente 90% de confluência. Transcorridas 24h após a transfecção, foram realizadas, com o auxílio de uma ponteira de micropipeta, descontinuidades na monocamada confluenta, criando dessa forma uma área livre de células (ferida). Em seguida, o meio foi retirado e as células foram lavadas três vezes com PBS para

remoção dos restos celulares. Após um novo período de incubação de 12h, as células foram lavadas com PBS e fixadas com paraformaldeído 4%. Foi realizada então a marcação dos núcleos com *DAPI* (4',6-diamidino-2-phenylindole), segundo protocolo estabelecido no laboratório. Para determinação da taxa de migração celular, as imagens das feridas foram capturadas com câmera digital *Nikon DXM 1200F* (*Nikon Corporation*, Tokyo, Japão) acoplada ao microscópio de fluorescência *Nikon Eclipse E600* (*Nikon Corporation*) utilizando objetiva de 10 vezes. Duas barras paralelas às feridas foram inseridas sempre na mesma posição em todas as imagens obtidas e os núcleos localizados no interior destas barras foram contados.

Análise estatística

O programa *GraphPad prism* (*GraphPad Software Inc.*, San Diego – CA, E.U.A.) foi utilizado para a confecção dos gráficos e análises estatísticas. A comparação das porcentagens médias de metilação entre os grupos controle (sangue normal), margens cirúrgicas e tumores foi realizada pela análise de variância (*one-way ANOVA*). Utilizou-se o Teste exato de *Fisher* para comparar a proporção de metilação do gene *SLC15A3* e variáveis clínicas dos pacientes. Para tanto, foram considerados metilados os casos que apresentaram uma porcentagem de metilação maior que a média de metilação do grupo controle (sangue normal). A análise estatística dos resultados dos ensaios funcionais (migração, morte e ciclo celular) foi realizada pelo Teste *t Student* (não paramétrico).

A significância estatística foi definida para os valores de $p \leq 0,05$ em todas as etapas da análise.

RESULTADOS E DISCUSSÃO

Casuística

Foram analisadas 54 amostras de carcinomas epidermóides de cabeça e pescoço, que incluíram 42 amostras procedentes de carcinoma de laringe, 6 de carcinoma de língua e 6 de faringe. Cinquenta pacientes eram do sexo masculino e 4 do sexo feminino, com idades variando de 44 a 79 anos (média = 57,4 anos); 53 referiram exposição ao tabaco, sendo 13 ex-fumantes; 47 pacientes faziam uso de álcool, mas 20 haviam abandonado o hábito etílico; 24 relataram exposição ao fumo e ao álcool. A classe TNM mais frequente foi a T4N2CM0 (Tabela 4).

Avaliação de metilação do DNA por pirosequenciamento

O perfil de metilação dos genes *MXI* e *SLC15A3* e de outros genes conhecidamente metilados em cabeça e pescoço (*CDH1*, *CDH13*, *RASSF1A*, *DAPK1*, *CDKN2A*, *SOC3* e *TIMP3*) foi avaliado por pirosequenciamento. O perfil de metilação da ilha CpG associada ao promotor da variante curta do gene *MXI* já havia sido avaliado anteriormente por MSP-PCR (*methyl-specific PCR*) e foi comparado com dados de pirosequenciamento em um painel de amostras independentes. Uma pequena parcela das amostras analisadas pelo método de pirosequenciamento também já havia sido avaliada previamente por MSP-PCR.

Diversas metodologias têm sido utilizadas para avaliar perfis anormais de metilação do DNA em tumores humanos (Herman, Graff et al. 1996; Colella, Shen et al. 2003; Ogino, Odze et al. 2006), sendo a mais frequentemente utilizada a técnica de MSP-PCR (Herman, Graff et al. 1996). A MSP-PCR é um método de baixo custo, altamente sensível e reprodutível. Apesar disto, a técnica de MSP-PCR apresenta algumas limitações, como a possibilidade de falsos positivos devido à conversão inadequada de citosinas não metiladas para uracila. Outras variáveis, como número de ciclos de PCR, iniciadores inadequados, bem como qualidade do DNA utilizado, podem gerar dados dúbios. Por esse motivo, foi utilizada a metodologia de pirosequenciamento para avaliação do perfil de metilação de possíveis biomarcadores.

Tabela 4. Frequência dos parâmetros sexo, idade, exposição a fumo e álcool, sítio primário, classificação estadio e diferenciação histológica das lesões em 54 pacientes portadores de carcinomas epidermóides de cabeça e pescoço segundo tamanho (T), comprometimento de linfonodos (N1-3) ou não (N0).

Parâmetros	Grupos	Total de pacientes (%)
Sexo	Masculino	50 (92,6)
	Feminino	4 (7,4)
Idade*	≥ 60	37 (68,5)
	< 60	17 (31,5)
Fumante	Sim	40 (74,2)
	No passado	13 (24,0)
	Não	1 (1,8)
Etilista	Sim	27 (50,0)
	No Passado	20 (37,0)
	Não	7 (13,0)
Sítio Primário	Laringe	42 (78,0)
	Faringe	6 (11,0)
	Língua	6 (11,0)
Classificação	T1	3 (5,5)
	T2	8 (14,8)
	T3	5 (9,2)
	T4	35 (65)
	T-ND	3 (5,5)
	N0	21 (39,0)
	N1	7 (13,0)
	N2	20 (37,0)
	N3	3 (5,5)
	N-ND	3 (5,5)
	I	1 (1,8)
Estadio	II	4 (7,4)
	III	5 (9,3)
	IV	29 (53,7)
	ND	15 (27,8)
Diferenciação histológica	Pouco diferenciado	5 (9,3)
	Moderadamente diferenciado	27 (50)
	Bem diferenciado	17 (31,4)
	ND	5 (9,3)

ND – Não determinado

Em estudo prévio, o perfil de metilação do gene *MXI* foi encontrado como diferencialmente metilado em linhagens de CECP, bem como em amostras primárias de CECP (Calmon, Rodrigues et al. 2009). São conhecidos três transcritos do gene *MXI* derivados de *splicing* alternativo, duas variantes curtas e uma terceira recentemente descrita. Esta última apresenta uma ilha CpG próxima à sua região promotora e as duas primeiras compartilham uma outra ilha CpG que tem início no final do primeiro exon e engloba grande parte do intron adjacente. (Figura 3).

Figura 3 – Ferramenta BLAT mostrando localização e transcritos variantes do gene *MXI*. Caixas verdes – ilhas CpG analisadas no presente trabalho

Levando-se em conta as limitações técnicas do método de MSP-PCR, foi realizada a reavaliação de metilação do gene *MXI* por pirosequenciamento de DNA após tratamento com bissulfito de sódio. Para tanto, um ensaio de pirosequenciamento (sonda de pirosequenciamento da região 2, isoformas curtas – Tabela 3) foi desenhado para a mesma região analisada pela técnica de MSP-PCR (Figura 4). Também foram desenhados dois outros ensaios próximos aos promotores associados a cada uma destes transcritos (Tabela 3).

Figura 4 – Ensaios de pirosequenciamento e de MSP-PCR para detecção de metilação do DNA em dinucleotídeos CpG no promotor do gene *MXI*. Setas vermelhas – localização dos iniciadores utilizados nas reações de MSP-PCR. Seta azul – sonda de sequenciamento utilizada no ensaio de pirosequenciamento. Verde – dinucleotídeos CpG. Sublinhado – dinucleotídeos CpG analisados nos ensaios de pirosequenciamento.

Como pode ser observado, a ilha CpG associada à variante longa não está metilada em amostras de indivíduos saudáveis, em tumores e suas respectivas margens cirúrgicas (Figura 5A). Os dinucleotídeos CpGs próximos ao sítio de início de transcrição da variante curta (região 1) também não estão metilados em amostras normais, margens cirúrgicas e tumores (Figura 5B). No entanto, a análise de dinucleotídeos CpGs distantes do promotor (região 2) mostrou que algumas amostras de CECp encontram-se metiladas (Figura 5C).

Como discutido acima, os resultados por pirosequenciamento mostraram muitas diferenças em relação aos dados obtidos pelo método de PCR-MSP. Um grupo de quinze amostras foi utilizado na comparação do perfil de metilação do gene *MXI* por estas duas metodologias. Dentre as amostras que apresentaram resultado positivo pelo método de PCR-MSP, apenas 10% foram confirmadas pelo pirosequenciamento. Essa enorme diferença deve-se, provavelmente, à característica qualitativa e à alta especificidade e sensibilidade do método de PCR-MSP. Por outro lado, o pirosequenciamento não possui a mesma especificidade e sensibilidade do MSP-PCR, pois seus iniciadores são desenhados fora de dinucleotídeos CpG, o que favorece a amplificação de ambos os DNAs, metilado e não metilado. Dessa forma, caso a amostra tumoral apresente contaminação por DNA normal, a metilação presente no DNA tumoral pode ser mascarada pela presença de DNA não metilado proveniente da amostra normal.

O gene *SLC15A3* foi encontrado como diferencialmente expresso e metilado em linhagens celulares tratadas com 5-AzadC, porém o seu perfil de metilação não foi avaliado anteriormente pela metodologia de MSP devido a dificuldades encontradas na padronização da reação de PCR. Por esse motivo, avaliamos o perfil de metilação deste gene apenas por pirosequenciamento. Como pode ser observado, o gene encontra-se desmetilado em amostras de indivíduos sadios, porém o nível de metilação aumenta em margens cirúrgicas aparentemente livres de doença e está elevado em vários tumores (Figura 5D).

O perfil de metilação do DNA de alguns genes com papel importante na tumorigênese de cabeça e pescoço foi analisado com o objetivo de identificar uma assinatura molecular para esses tumores (Figura 6 A-G). Com exceção de uma pequena proporção de casos, a maioria desses genes encontra-se desmetilados nas amostras analisadas (Figura 6 A-G).

Figura 5. Metilação da ilha CpG associada às variantes do gene *MXI* e de dinucleotídeos CpG localizados na região promotora do gene *SLC15A3* em amostras de indivíduos saudáveis, em tumores e suas respectivas margens cirúrgicas.

Figura 6. Metilação de ilhas CpGs associadas aos promotores dos genes *CDH1*, *CDH13*, *RASSF1A*, *CDKN2A*, *DAPK1* e *TIMP3* em amostras de indivíduos saudáveis, em tumores e suas respectivas margens cirúrgicas, bem como em linhagens.

As análises estatísticas não mostraram diferenças significativas para a maioria dos genes analisados, com exceção do gene *SLC15A3*, que apresentou diferença significativa ($p < 0,05$) entre tumores e amostras de sangue de doadores saudáveis. No entanto, não houve diferença entre tumores e margens cirúrgicas aparentemente livres de doença. A Tabela 5 apresenta os dados da comparação de variáveis clínicas dos pacientes com o perfil de metilação do gene *SLC15A3*, que mostrou associação com o grau de diferenciação dos tumores (Teste exato de Fisher; $p < 0,05$).

O gene *SLC15A3* faz parte de uma superfamília de transportadores de oligopeptídeos dependentes de prótons (POT), também denominados transportadores de peptídeos (PTR) (Steiner, Naider et al. 1995), que utilizam um mecanismo dependente de prótons para o transporte transmembrânico de pequenas cadeias peptídicas (Herrera-Ruiz and Knipp 2003; Daniel and Kottra 2004). As proteínas POT possuem entre 450 e 700 aminoácidos, com as variantes de procariotos sendo menores que as de eucariotos. As predições protéicas indicam que essas proteínas possuem 12 domínios transmembrânicos (TMD) com as porções carboxi- e amino-terminais voltadas para o citosol (Steiner, Naider et al. 1995).

Em humanos, foram descritos primeiramente dois membros dessa família, *PEPT1* (*SLC15A1*) e *PEPT2* (*SLC15A2*), ambos com a capacidade de transportar uma ampla variedade de di e tripeptídeos. Entretanto, essa capacidade diminui drasticamente com o aumento do tamanho dos peptídeos (Liu, Liang et al. 1995). Posteriormente, foram descritos dois outros genes dessa família, *PHT1* (*SLC15A4*) e *PHT2* (*SLC15A3*), e suas isoformas derivadas de *splicing* alternativo (Botka, Wittig et al. 2000; Herrera-Ruiz, Wang et al. 2001).

Os genes da família POT possuem um padrão de expressão tecido-específico e, interessante, também estão relacionados com o transporte de diversas drogas peptídeo-miméticas (Terada, Sawada et al. 2000; Shu, Shen et al. 2001; Watanabe, Sawano et al. 2002; Wenzel, Kuntz et al. 2002). Isto faz com que sejam de particular interesse para o desenvolvimento de drogas tecido-específicas.

YAMASHITA *et. al.* (1997) (Yamashita, Shimada et al. 1997), analisando o perfil de expressão de *PHT1* em tecidos de rato por *Northern blot*, obtiveram confirmação de expressão em cérebro, olho, pulmão e baço. As análises em tecidos humanos mostraram expressão de *PHT1* em intestino delgado (Bhardwaj, Herrera-Ruiz et al. 2006) e expressão elevada de seu produto (proteína hPept1) (Qual gene? O *PHT1* ou *SLC15A4*) em pancreatite (Gonzalez, Covitz et al. 1998) e na linhagem celular Caco-

2 derivada de adenocarcinomas de cólon (Walker, Thwaites et al. 1998). Uma revisão recente sobre o gene *SLC15A3* (*peptide/histidine transporter 2*) mostrou escassez de dados sobre sua função.

Tabela 5. Relação entre perfil de metilação do gene *SLC15A3* e variáveis clínico-patológicas de pacientes com CECp.

Variáveis clínico - patológicas	Categoria	<i>SLC15A3</i>		
		Não metilado	Metilado	P
Idade	≤60	11	26	0,4073
	>60	7	10	
Tamanho do tumor	T1+T2	4	7	0,8112
	T3+T4	14	29	
Grau	1	4	1	0.0326* (1-2)
	2	8	19	0.6945 (2-3)
	3	6	11	0.0776 (1-3)
Sítio anatômico	Laringe	13	27	0.6761 (1-2)
	Faringe	2	6	0.334 (2-3)
	Língua	3	3	0.4013 (1-3)
Estadio	I+II	3	2	0,1755
	III+IV	10	24	
Comprometimento linfonodal	N0	9	18	1,000
	N+	9	18	

*p<0,05 (teste exato de Fisher)

Comparação do perfil de expressão gênica com o padrão de metilação do promotor

Nesta etapa do trabalho, foi investigado se a expressão dos genes *MXI* e *SLC15A3* está associada com o padrão de metilação do DNA em seus promotores. Para tanto, os perfis de expressão gênica e de metilação do DNA foram analisados em diversas linhagens celulares. Os transcritos dos genes *MXI* e *SLC15A3* apresentam expressão diminuída em linhagens celulares em relação aos linfócitos normais (Figura 7 A, C, E). Entretanto, a ilha CpG associada ao promotor da variante longa do gene *MXI* apresenta baixos níveis de metilação (Figura 7B) na maioria das linhagens celulares analisadas. Ao contrário, a região do DNA próxima ao promotor da ilha CpG (+53/+97) associada aos transcritos curtos do gene *MXI* está altamente metilada em praticamente todas as linhagens celulares (Figura 7D), o que concorda com as análises de transcritos. O gene *SLC15A3* também apresentou altos níveis de metilação do DNA próxima ao início sítio de transcrição (Figura 7F).

Ensaio funcionais do gene *MXI* em linhagens celulares de cabeça e pescoço

Para investigar o efeito do silenciamento da expressão do gene *MXI* em linhagens celulares de cabeça e pescoço, foram realizadas análises do índice de proliferação, migração e ciclo celular. Esses parâmetros foram avaliados após o silenciamento da expressão gênica por RNA de interferência contra o *MXI*.

Curvas de proliferação celular

A morfologia e o potencial proliferativo das linhagens celulares de carcinomas epidermóides de cavidade oral (*SCC-4*, *SCC-9*, *SCC-15*, *SCC-25*) foram comparados. Essas linhagens, apesar de derivarem de CECs de língua, apresentam características biológicas bastante diferentes. As linhagens *SCC-4* e *SCC-15* mostraram-se similares no aspecto morfológico, ambas com células grandes e arredondadas com nucléolos evidentes, multinucleadas e crescimento formando redes (Figura 8A e 8C).

Figura 7. Perfis de expressão gênica e de metilação de ilhas CpGs associadas aos promotores dos genes *MXI* e *SLC15A3* em linhagens celulares provenientes de tumores de cabeça e pescoço, mama, cólon, leucemias e pulmão. **A** – qRT-PCR do gene *MXI* (transcrito variante longo), **B** - % metilação da ilha CpG associada ao promotor da variante longa do gene *MXI*. **C** – qRT-PCR do gene *MXI* (transcritos curtos), **D** - % metilação promotor ilha CpG associada ao promotor das variantes curtas do gene *MXI*. **E** – qRT-PCR do gene *SLC15A3*, **F** - % metilação promotor ilha CpG associada ao promotor do gene *SLC15A3*.

A linhagem *SCC-9* apresentou um padrão de crescimento em rede e suas células mostraram núcleos grandes com nucléolos bastante evidentes, também arredondadas, porém raramente multinucleadas, e menores que as observadas em *SCC-4* e *SCC-15* (Figura 8B). A linhagem *SCC-25* apresentou células pequenas, com aspecto variando de arredondado a fusiforme, citoplasma escasso, núcleo grande e nucléolos evidentes (Figura 8D). O padrão de crescimento desta linhagem foi diferente, sendo observada a formação de pequenos grumos.

Figura 8. Aspecto morfológico das linhagens celulares *SCC-4* (A), *SCC-9* (B), *SCC-15* (C) e *SCC-25* (D) em culturas subconfluentes, observadas em aumento de 100x. Em A observa-se a presença de nucléolos evidentes, em B a formação de redes de células característica da linhagem *SCC-9*, em C células multinucleadas (seta) e em D células fusiformes presentes na linhagem *SCC-25* com seu típico crescimento em grumos (*).

A linhagem que apresentou maior potencial proliferativo foi a *SCC-25*, com maior número de células em todos os períodos. As linhagens *SCC-4*, *SCC-9* e *SCC-15* comportaram-se de maneira semelhante, não havendo grandes diferenças entre elas nos intervalos de tempo analisados. De maneira geral, o pico de proliferação variou entre 144 e 168 horas para todas as linhagens. No período de 192 horas após o plaqueamento, observou-se com frequência um declínio no número de células, provavelmente por causas da proximidade da fase de confluência, o que provoca morte celular (Figura 9).

Curva de crescimento das linhagens de carcinomas epidermóides de cavidade oral

Figura 9. Curvas de proliferação das linhagens de carcinomas epidermóides de cabeça e pescoço *SCC-4*, *SCC-9*, *SCC-15*, *SCC-25*.

Silenciamento da expressão do gene *MX1* por RNA de interferência

A padronização dos experimentos de silenciamento da expressão do gene *MX1* foi realizada, primeiramente, com a linhagem *SCC-9*, utilizando três oligos duplex de 27mer, específicos para o gene *MX1*, em diferentes concentrações (1nM, 10nM e 20nM). O RNA foi isolado e serviu de molde para a síntese de cDNA. Os níveis dos transcritos do *MX1* foram avaliados por PCR em tempo real. Nesses experimentos, foi verificado que a expressão do gene *MX1* está reduzida após tratamento com oligos

específicos para esse transcrito. A *lipofectamine RNAiMax* apresentou menor toxicidade que a *lipofectamine 2000* e mostrou-se mais eficiente no silenciamento do transcrito (Figura 10). Foram considerados como diferencialmente expressos os valores superiores ao *cut off* (≥ 3.0) com base nos dois controles endógenos (*GAPDH* e *TUBA1C*). Os dados de eficiência de amplificação dos oligonucleotídeos utilizados nas reações de PCR em tempo real, bem como sua sequencias e concentrações, estão apresentados na Tabela 6.

Figura 10 –Expressão relativa do gene *MXI* após ensaio de silenciamento com RNAi. **A** – agente de transfecção *Lipofectamine RNAi Max*. **B** – agente de transfecção *Lipofectamine 2000*.

Tabela 6. Sequência, concentração otimizada, *slope* e *R2* dos *oligonucleotídeos* utilizados para as amplificações por PCR em tempo real.

Genes		5' → 3'	Concentração	Slope	R2
<i>MXI</i>	senso	CAATCAGCCTGCTGACATTG	0,4	-3,450	0,99
	antisenso	TGTCTCCTGCCTCTGGATG			
<i>TUBA6</i>	senso	TCAACACCTTCTTCAGTGAAACG	0,6	-3,341	0,99
	antisenso	AGTGCCAGTGCGAACTTCATC			
<i>GAPDH</i>	senso	ACCCACTCCTCCACCTTTGA	0,4	-3,392	0,99
	antisenso	CTGTTGCTGTAGCCAAATTCGT			

Avaliação de ciclo e morte celular

Para avaliar o efeito silenciamento do gene *MXI* sobre o processo de morte celular em linhagens celulares de cabeça e pescoço, foi realizada análise por citometria de fluxo, após incorporação de iodeto de propídeo, das células transfectadas com o oligo para o transcrito de *MXI* e com o controle negativo (*Scrambled*) após 36 horas da transfecção. Aparentemente, o silenciamento do gene *MXI* não interfere nos eventos de morte celular nas linhagens avaliadas, sendo a porcentagem de morte (M1) baixa em ambas as linhagens avaliadas e sem diferenças consideráveis entre os grupos controle e tratado (Tabelas 7-8) (Figuras 11-12).

Tabela 7. Porcentagem de eventos em cada fase na linhagem *SCC-15*

	<i>SCC-15</i>			
	M1(%)	M2(%)	M3(%)	M4(%)
<i>Scrambled</i>	3,79	27,51	37,45	6,45
<i>MXI</i>	3,19	24,93	44,57	9,01

Figura 11. Exemplos de histogramas obtidos com as análises de morte e ciclo celular por citometria de fluxo na linhagem *SCC-15*.

Tabela 8. Porcentagem de eventos em cada fase na linhagem *SCC-25*

	<i>SCC-25</i>			
	M1(%)	M2(%)	M3(%)	M4(%)
<i>Scrambled</i>	2,31	43,01	46,76	2,96
<i>MXI</i>	3,57	39,71	50,70	2,76

Figura 12. Exemplos de histogramas obtidos com as análises de morte e ciclo celular por citometria de fluxo na linhagem *SCC-25*.

A avaliação da fração de células em estado proliferativo mostrou um aumento aparente do número de células em fase proliferativa quando o gene *MX1* está silenciado em ambas as linhagens (Figuras 13 A-B). A linhagem *SCC-15* proliferou aproximadamente 1,31 vezes mais na amostra transfectada com o oligo para o *MX1* do que o controle negativo (Figura 13A), enquanto a linhagem *SCC-25* apresentou um aumento de 1,18 vezes (Figura 13B).

Figura 13. Gráficos da fração proliferativa, construídos a partir da análise dos resultados obtidos por citometria de fluxo, de linhagens celulares de carcinoma epidermóide de cabeça e pescoço após ensaios de *RNAi*. **A** - linhagem *SCC-15*. **B** - linhagem *SCC-25*

Ensaio de migração celular (Ensaio de “cicatrização de ferida” em monocamada)

O ensaio de migração celular foi realizado com as linhagens *SCC-4* e *SCC-25* transfectadas com oligo contra o transcrito do gene *MX1* (Figuras 14B-D) e com o controle negativo (Figura 14A-C). Foram capturadas imagens das “feridas” em cada uma das condições experimentais doze horas após a realização do ensaio de RNA de interferência.

A linhagem *SCC-25* mostrou um aumento significativo da migração nas células tratadas com o oligo contra o gene *MX1* quando comparada com as células transfectadas com o controle negativo ($p < 0,05$) (Figura 15B). A linhagem *SCC-4* também apresentou um aumento número de células que migraram após transfecção com o oligo contra o *MX1* quando comparado com o controle. No entanto, esse aumento de migração não foi significativo ($p = 0,06$) (Figura 15A).

Figura 14. Fotomicrografias das regiões submetidas ao ensaio de ferida mostrando núcleos corados com *DAPI*. As barras paralelas vermelhas foram usadas como referencial para a contagem dos núcleos. Objetiva de 10X.

Figura 15. Gráficos comparativos da contagem de núcleos em experimentos de migração com linhagens celulares de carcinoma epidermóide de cabeça e pescoço após ensaios de transfecção de oligos para o gene *MX1* e controle negativo. **A** - linhagem *SCC-4* (n=8 campos). **B** - linhagem *SCC-25* (n=5 campos) Teste *t Student* (não paramétrico) - *p<0,05

O gene *MX1* (*myxovirus resistance 1*) está mapeado no braço longo do cromossomo 21 na posição 22.3 e codifica um membro da superfamília de dinaminas de grandes GTPases, que modulam o tráfico de vesículas e homeostase de organelas (Haller, Staeheli et al. 2007). Similarmente a outras proteínas Mx, a proteína humana MX1 contém um domínio de GTPase na região amino-terminal, um domínio interativo no meio da molécula e um zíper de leucina na região C-terminal (Horisberger, McMaster et al. 1990).

A MX1 possui atividade antiviral contra diversos vírus de RNA e é transcricionalmente induzida por interferon pela via JAK/STAT (Holzinger, Jorns et al. 2007). Aparentemente, essa proteína citoplasmática reconhece e liga-se ao nucleocapsídeo viral e previne a transcrição e a replicação viral (Schwemmle, Richter et al. 1995; Kochs and Haller 1999). O *MX1* mostra expressão elevada em células de alguns grupos de complementação da anemia de Fanconi e pode estar relacionado com algumas características fenotípicas dessa doença (Altschul, Madden et al. 1997). Por outro lado, exibe expressão reduzida em câncer de próstata (Schulz, Alexa et al. 2007) e encontra-se metilado em células de leucemia mielóide aguda (Desmond, Raynaud et al. 2007). Os dados do presente estudo mostraram que a região promotora do gene *MX1* também encontra-se metilada em linhagens celulares e tumores primários de carcinoma epidermóide de cabeça e pescoço (Calmon, Rodrigues et al. 2009), sugerindo uma provável ligação entre o silenciamento de *MX1* e a tumorigênese. Essa hipótese é reforçada pelos achados de MIBAYASHI e colaboradores (Mibayashi, Nakad et al. 2002), de que o *MX1* promove morte celular induzida por estímulos apoptóticos. Portanto, baixos níveis de MX1 devem contribuir para a resistência à apoptose durante o desenvolvimento do câncer.

NOSER e colaboradores (Noser, Mael et al. 2007) também apontam o silenciamento de *MX1* como um importante processo na tumorigênese. Esses autores observaram que células humanas com a via RAS/Raf1/MEK/ERK ativada e sensíveis à morte celular induzida pelo vírus da estomatite vesicular mostram baixa eficiência na indução da expressão de *MX1*. No entanto, nos ensaios iniciais de citometria de fluxo, não foram identificadas modificações significativas nos padrões de morte celular em linhagens celulares de cabeça e pescoço com o gene *MX1* silenciado.

Recentemente, MUSHINSKI e colaboradores (Mushinski, Nguyen et al. 2009), demonstraram que a proteína MX1 inibe a motilidade e a capacidade invasiva de linhagens celulares de próstata e seus dados sugerem que a indução da expressão dessa

proteína pode ser usada na prevenção e no tratamento de metástases. Os resultados de experimentos de migração celular (“cicatrização de ferida”), obtidos no presente trabalho, apóiam essa hipótese e indicam que o *MXI* atua nos processos de migração celular e que sua ausência pode favorecer a progressão tumoral e a formação de metástases.

CONCLUSÕES

O presente trabalho teve as seguintes conclusões:

1. A ilha CpG associada ao promotor da variante longa do gene *MX1* não está metilada em carcinomas epidermóides de cabeça e pescoço. Uma pequena parcela das amostras analisadas no presente trabalho está metilada na ilha CpG próxima ao sítio de início de transcrição dos transcritos curtos do gene *MX1*. O promotor do gene *SLC15A3* é frequentemente metilado em carcinomas epidermóides de cabeça e pescoço e está relacionado com o grau de diferenciação do tumor.
2. Os genes *CDH1*, *CDH13*, *DAPK*, *CDKN2A*, *RASSF1A*, *SOCS3* e *TIMP3* são metilados em uma pequena parcela dos tumores analisados no presente trabalho. Não existe um padrão de metilação destes genes entre as amostras analisadas.
3. A comparação dos níveis de expressão com o perfil de metilação do gene *MX1*, em linhagens celulares tumorais, indicou que a metilação do promotor deste gene não regula sua expressão.
4. A comparação dos níveis de expressão com o perfil de metilação do gene *SLC15A3*, em linhagens celulares tumorais, indicou que a metilação do promotor deste gene não regula sua expressão.
5. A redução dos níveis dos transcritos do gene *MX1* modula a proliferação e ciclo celular, bem como aumenta a motilidade de linhagens celulares de carcinomas epidermóides de cabeça e pescoço.

RESUMO

A programação epigenética do genoma por metilação do DNA, modificação das histonas e remodelamento da cromatina é crucial para o desenvolvimento e o crescimento normal dos mamíferos e alterações nesses mecanismos contribuem diretamente para a transformação maligna. Em função do papel relevante da metilação do DNA na carcinogênese, da importância e da necessidade de identificação de marcadores moleculares em tumores de cabeça e pescoço e dos resultados já obtidos pelo grupo, o presente trabalho teve por objetivo geral investigar o perfil de metilação de ilhas CpG em carcinomas primários de cabeça e pescoço (CECP), bem como identificar e iniciar estudos funcionais de biomarcadores candidatos para diagnóstico e prognóstico desses tumores. Alguns genes previamente descritos com padrões anormais de metilação em neoplasias humanas (*CDH1*, *CDH13*, *DAPK*, *CDKN2A*, *RASSF1A*, *SOCS3* e *TIMP3*), além de outros dois genes (*MX1* e *SLC15A3*) identificados em nosso estudo anterior, foram analisados em amostras normais e margens cirúrgicas de CECP por meio da técnica de pirosequenciamento de DNA após tratamento com bissulfato de sódio. As análises estatísticas não mostraram diferenças significativas para a maioria dos genes analisados, com exceção do gene *SLC15A3*, que apresentou diferença significativa ($p < 0,05$) entre tumores e amostras de sangue de doadores saudáveis. Diferentemente, os resultados obtidos com a metodologia de PCR-MSP em nosso trabalho anterior mostraram que o gene *MX1* está metilado em CECP. Os estudos funcionais do *MX1*, por RNA de interferência, ensaios de migração e citometria de fluxo mostraram que seu produto contribui para migração e proliferação celular, e talvez para resistência à apoptose. Os resultados sugeriram que o nível de expressão do *MX1* pode ser um preditor do potencial metastático em carcinoma epidermóide.

ABSTRACT

The genome epigenetic programming by DNA methylation, histone modification and chromatin remodeling is crucial for normal growth and development in mammals and changes in these mechanisms contributes directly to malignant transformation. Regarding the role of DNA methylation in carcinogenesis, the importance and necessity for the identification of molecular markers in head and neck tumors, and the results already obtained by the group, this study was aimed at investigating the methylation profile of CpG islands in primary head and neck squamous cell carcinomas (HNSCC), and to identify and initiate functional studies of candidate biomarkers for diagnosis and prognosis of these tumors. Therefore, some genes previously described with abnormal methylation pattern in humans tumors (*CDH1*, *CDH13*, *DAPK*, *CDKN2A*, *RASSF1A*, and *TIMP3 SOCS3*), and two other genes (*MXI* and *SLC15A3*) identified in our previous study were analyzed in normal samples, surgical margins, and in HNSCC by pyrosequencing after sodium bisulfite treatment. The statistical analysis showed no significant differences for most of analyzed genes, except for the *SLC15A3*, which showed significant difference ($p < 0.05$) between tumors and blood samples from healthy donors. However, our earlier results showed a higher frequency of *MXI* hypermethylation in primary HNSCC using the PCR-MSP methodology. To gain a better understanding of the role *MXI* in cancer biology we investigated whether a downregulation of *MXI* by interference RNA contribute to apoptosis resistance and cell migration during cancer development. Wound healing and flow cytometry assays were performed to determine changes in cell motility, death and cell cycle in SCC cells. The results indicated that low levels of *MXI* could regulate the cell cycle, increase proliferation, and enhance tumor cell migration in HNSCC cell lines, but it might not contribute to apoptosis resistance. It also suggests that the level of *MXI* expression may be a predictor of metastatic potential in HNSCC.

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CAPÍTULO 4

INTRODUÇÃO

O carcinoma epidermóide de cabeça e pescoço (CECP) está entre as mais frequentes neoplasias em humanos. Esta doença afeta principalmente homens e tem como principal fator etiológico o consumo crônico de álcool e tabaco. A taxa de sobrevivência em cinco anos é uma das mais baixas quando comparada à de outras neoplasias, permanecendo praticamente inalterada ao longo das últimas décadas.

Esses tumores possuem prognóstico favorável quando detectados precocemente mas, nas fases iniciais da doença, os pacientes apresentam poucos sintomas. A maioria destes tumores é diagnosticado tardiamente, quando muitos possuem metástases. Não há dúvidas, portanto, de que marcadores de diagnóstico e prognóstico são particularmente importantes para o carcinoma epidermóide de cabeça e pescoço.

Várias técnicas de análise de expressão gênica global têm fornecido uma lista crescente de biomarcadores potenciais. Entre essas técnicas, estão a de microarranjos de cDNA e de análise serial de expressão gênica (SAGE - do inglês, *Serial Analysis of Gene Expression*), ambas capazes de avaliar a expressão de milhares de genes simultaneamente. No entanto, poucos destes genes têm sido utilizados efetivamente na prática clínica ou como alvos terapêuticos.

MATERIAIS E MÉTODOS

Com o objetivo de identificar genes envolvidos no desenvolvimento e na progressão de carcinomas epidermóides de laringe, foi realizada a análise de expressão gênica diferencial por meio do método de SAGE. Para tanto, foram construídas duas bibliotecas, uma a partir de tumor com metástase em linfonodos regionais (N+) e outra de tumor sem evidência de metástases (N0), além de uma biblioteca gerada de um *pool* de duas amostras de mucosa normal de laringe. Dentre os genes que apresentaram expressão diferencial nos experimentos de SAGE, foram selecionados quatro para validação por PCR em tempo real. Tais validações foram realizadas em um conjunto de 26 amostras de tumores primários de laringe (15 N0 and 11 N+) e em 36 amostras de tumores primários de língua (18 N0 and 18 N+), além de dois pools de margens cirúrgicas normais de laringe e língua.

RESULTADOS E DISCUSSÃO

O sequenciamento do material obtido a partir das três bibliotecas SAGE gerou aproximadamente 54 mil *tags* únicas. As análises estatísticas identificaram um conjunto de 1.216 *tags* com expressão diferencial entre as bibliotecas de tumores e amostras normais e 894 *tags* entre carcinomas metastáticos e não metastáticos. Três genes que apresentaram expressão diferencial entre tumores e amostras normais foram selecionados para validações por PCR em amostras de CECP: um deles com expressão reduzida (*KRT31*) e dois outros com expressão aumentada (*BST2*, *MFAP2*). Além desses, um quarto gene (*GNAI5*) sem diferenças significativas de expressão em relação ao tecido normal foi escolhido como controle negativo.

Os resultados dos experimentos por PCR em tempo real foram consistentes com as análises estatísticas dos dados de SAGE. Estes resultados confirmaram a expressão elevada dos transcritos *BST2* e *MFAP2* e diminuída no caso de *KRT31* nas amostras de CECP em relação às margens cirúrgicas normais. Como esperado, o gene *GNAI5* não mostrou diferenças significativas de expressão em tumores, quando comparados com amostras normais.

Este estudo foi o primeiro a utilizar a metodologia de SAGE em amostras de CECP que, após o tratamento estatístico empregado, mostrou-se efetiva na identificação de genes diferencialmente expressos e potencialmente envolvidos no desenvolvimento do câncer. Os dados gerados por este trabalho sugerem a existência de um conjunto de biomarcadores potenciais para prognóstico e de possíveis alvos para o desenvolvimento de terapias específicas para esse tipo de tumor.

Research article

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Searching for molecular markers in head and neck squamous cell carcinomas (HNSCC) by statistical and bioinformatic analysis of larynx-derived SAGE libraries

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Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies in humans. The average 5-year survival rate is one of the lowest among aggressive cancers, showing no significant improvement in recent years. When detected early, HNSCC has a good prognosis, but most patients present metastatic disease at the time of diagnosis, which significantly reduces survival rate. Despite extensive research, no molecular markers are currently available for diagnostic or prognostic purposes.

Methods: Aiming to identify differentially-expressed genes involved in laryngeal squamous cell carcinoma (LSCC) development and progression, we generated individual Serial Analysis of Gene Expression (SAGE) libraries from a metastatic and non-metastatic larynx carcinoma, as well as from a normal larynx mucosa sample. Approximately 54,000 unique tags were sequenced in three libraries.

Results: Statistical data analysis identified a subset of 1,216 differentially expressed tags between tumor and normal libraries, and 894 differentially expressed tags between metastatic and non-metastatic carcinomas. Three genes displaying differential regulation, one down-regulated (*KRT31*) and two up-regulated (*BST2*, *MFAP2*), as well as one with a non-significant differential expression

pattern (*GNAI5*) in our SAGE data were selected for real-time polymerase chain reaction (PCR) in a set of HNSCC samples. Consistent with our statistical analysis, quantitative PCR confirmed the upregulation of *BST2* and *MFAP2* and the downregulation of *KRT31* when samples of HNSCC were compared to tumor-free surgical margins. As expected, *GNAI5* presented a non-significant differential expression pattern when tumor samples were compared to normal tissues.

Conclusion: To the best of our knowledge, this is the first study reporting SAGE data in head and neck squamous cell tumors. Statistical analysis was effective in identifying differentially expressed genes reportedly involved in cancer development. The differential expression of a subset of genes was confirmed in additional larynx carcinoma samples and in carcinomas from a distinct head and neck subsite. This result suggests the existence of potential common biomarkers for prognosis and targeted-therapy development in this heterogeneous type of tumor.

Background

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies in humans, affecting distinct head and neck topologies including oral cavity, oropharynx, hypopharynx, larynx and nasopharynx. HNSCC is associated with high alcohol and tobacco consumption, and represents a major international health problem with approximately 650,000 cases and 90,000 deaths per year worldwide [1]. In Brazil, over 13,000 new cases are expected in 2008 [2]. Currently, advances in both surgical and nonsurgical therapeutics have led to increased local tumor control. However, overall mortality rates have not improved due to tumor recurrences in regional and distant sites of the aerodigestive tract [3]. When detected early, HNSCC has a 75% 5-year survival rate, but most patients present metastatic disease at the time of diagnosis, which reduces survival rate to 35% [4]. This 5-year survival rate is one of the lowest among aggressive cancers and has shown no significant improvement in recent years [5,6].

Currently, there are very few molecular markers that can be used with accuracy and reliability as indicators of head and neck carcinomas with potential for metastatic progression, and therefore as indicators of a more aggressive tumor behavior. A pre-operative marker, for example, could significantly help in determining the most appropriate treatment for a particular patient [7]. Moreover, changes in the gene expression profile arising exclusively or preferentially in cancer can be used as molecular markers [8]. In fact, these markers may provide us with new means for the early detection of cancer and cancer risk assessment, as discussed by Hunter *et al.* (2005) [9] for HNSCC.

In order to investigate molecular markers that may be relevant for prognosis and therapy in cancer disease, large-scale transcriptomic approaches such as SAGE and microarrays have been extensively reported in the literature [10-12]. In the present study, we decided to use SAGE since

this technique allows an unbiased global view of all the transcripts expressed in a tissue sample at a given time point. Despite its appropriateness for such studies, SAGE is an expensive and complex technique, thus commonly involving few and often rare biological samples.

We generated individual SAGE libraries from metastatic (N+) and non-metastatic (N0) larynx carcinomas, and from normal mucosa samples. A database was created to provide absolute frequency tags for each gene in metastatic and non-metastatic tumors, and for the normal tissues. For the statistical analysis of differentially expressed tags, the Poisson distribution was used as the basic probabilistic model. The Cox partial likelihood combined with Dempster p-values allowed us to consider an efficient significance test to compare the Poisson means of the three groups. Also, the choice of critical level depended on the expression power of the tag been tested. The analysis of the data by our statistical approach revealed subsets of differentially expressed genes between tumor and normal tissues, and between metastatic and non-metastatic carcinomas. These differentially expressed genes deserve further consideration as potential biomarkers for metastatic progression, and therefore as indicators of a more aggressive tumor behavior.

Methods

Sample preparation for SAGE and real time PCR experiments

Samples were frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using TRIzol Reagent and treated with DNase (Invitrogen Corporation, Carlsbad, CA, USA). cDNA synthesis was performed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer.

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.

SAGE

SAGE was carried out using the I-SAGE™ Kit (Invitrogen Corporation, Carlsbad, CA, USA). Briefly, mRNA was captured from total RNA by binding to oligo (dT) magnetic beads, and reverse transcribed with SuperScript™ II reverse transcriptase and *E. coli* DNA polymerase. Bound cDNA was cleaved with *Nla* III (anchoring enzyme), divided in two fractions and ligated to adapters A and B, both containing a *Bsm*F I restriction site followed by a CATG 3' overhang, with different primer anchoring sequences at the 5' end. Adapter linked cDNA from both fractions were cleaved with *Bsm*F I (tagging enzyme) to generate adapter linked tags that were filled in by Klenow polymerase and then mixed and ligated to form adapter linked ditags. This mixture was used as template, in three 96-well 50 µl PCR reactions using primers complementary to the adapters, and the ~100-bp products were PAGE purified. Adapters were eliminated by digestion with *Nla* III and PAGE purification of the 26 bp ditags that were ligated to form concatamers. Concatamers were submitted to polyacrylamide gel electrophoresis and regions ranging from 300–500 bp, 500–800 bp and 800–1000 bp were purified and ligated to pZero®-1 cloning vector. Ligation reactions were used to transform One Shot® TOP10 Electrocomp™ *E. coli* cells using 0.2 cm cuvettes and a Gene Pulser II electroporator (Bio-Rad Laboratories, Hercules, CA, USA) set at 2.5 kV, 25 mF and 200 Ω. Cells were plated on low salt LB agar containing Zeocin®, in plates compatible with the automated colony picker QPix2 (Genetix, New Milton, Hampshire, UK). Picked colonies were grown separately on 96-well plates containing 2XYT media. An aliquot of each well was then used directly in a PCR reaction, with forward and reverse M13 primers. Amplified inserts were checked and sequenced with forward M13 primer in a MegaBACE™1000 sequencer (Amersham Biosciences, Piscataway, NJ, USA) and the DYEnamic ET Dye Terminator Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA), or alternatively, an ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA) and the ABI PRISM® BigDye™ Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Three SAGE libraries were generated using two larynx cancer samples (one with lymph node metastasis or N+ and one with no lymph node metastasis or N0) and a normal control library pooled from two normal samples (surgical margins from one N+ and one N0 larynx cancer). For each library, 6,000 sequencing reactions were performed and tags were extracted to yield approximately 100,000 tags per library.

The raw data files are available at the Gene Expression Omnibus database (GEO) under the accession numbers: GSM303325 (pool of normal samples); GSM303340 (N0 tumor), GSM303349 (N+ tumor).

SAGE database

Tag frequency tables, composed of a "tag" column (10 bp sequences) and a "count" column (number of times that the tag appears in the library) were obtained by the SAGE™ Analysis 2000 Software 4.0, with minimum tag count set to 1 and maximum di-tag length set to 28 bp, whereas other parameters were set on default. A relational MySQL database [13] was developed to store data from SAGE experiments. The datasets contained information on: gene name, accession number, UniGene code, gene symbol, absolute frequency tags in metastatic and non-metastatic tumors and normal tissues. Other tables were generated to store information on metabolic pathways and gene ontology. Scripts developed in Perl [14] integrated with the MySQL database allowed the identification of genes and their respective frequencies in the three libraries which were used as input data in the program that performed statistical analysis. A schematic representation of databases, data analysis, and experimental validation representing our approach is shown in Figure 1.

Statistical Method

Before starting the statistical analysis, we decided to exclude very low expression tags from the study. The inclusion criterion considered only tags with total normalized frequencies larger than 3. In order to obtain normalized frequencies, the absolute frequencies in each library were divided by the total number of tags of this library and multiplied by the total number of tags of the smallest library. For each tag, we observed the sum of its three normalized frequencies. If this sum was larger than 3, it was kept in the study; otherwise, it was excluded. The remaining tags, after this exclusion procedure, are the object of our study (Additional file 1, Supplementary Table 1).

Returning to the absolute frequencies of the remaining tags, we observed that all frequencies were low in relation to the size of the libraries. In such rare cases, the Poisson distribution is the adequate statistical model for the analysis. In fact, the three absolute frequencies for each tag are considered independent Poisson distributed variables. The statistical objective at this point, for a specific tag, is to decide whether there are differences in expression among the libraries. We should perform statistical tests for every tag in the data bank.

Comparing Poisson distribution is not an easy task. We then used the Partial Likelihood method as developed by Cox (1975) [15]. Briefly, the procedure considers the three frequencies of a specific tag as forming an observation of a trinomial distribution, where the sample size is now the total tag abundance, S . Representing now the unknown trinomial probabilities of a specific tag by (p_1, p_2, p_3) and the total library sizes by (N_1, N_2, N_3) , homoge-

Figure 1
Fluxogram showing the strategy used in SAGE data analysis.

neity among the original three Poisson averages can be tested by testing, in the trinomial model, the hypothesis

$$H: (p_1, p_2, p_3) = (N_1, N_2, N_3)/N \text{ where } N = N_1 + N_2 + N_3.$$

Again, we have a difficult task to compute a *p-value* in a tri-dimensional sample space. Since we have distinct tag abundances, which can go from 4 to more than one thousand tags, we have to be very precise in defining the *p-values*. For this task, we decided to use the method developed by Dempster (1997) [16]. The method consists of ordering the sample space by the likelihood ratios. To compute

the *p-value*, the tail area was considered as the set of all points that have likelihood ratios smaller than those of the observed frequencies.

Finally, as mentioned before, the tag abundances can be very different, and considering the same significance level would be inappropriate for the tags with low frequencies. Following the recommendations of DeGroot (1975) [17], we used the decision theory optimum procedure that minimizes the risk function $a\alpha + b\beta$. Here, α and β are the first and second kind of errors. In our case, we decided to choose $a = 4$ and $b = 1$ since we believe that the first type of error (deciding in favor of differentially expressed when it is not) is more dangerous than the second type of error (deciding against differentially expressed when it is). Using simulated samples, we found that the level of significance is a function of S , the tag abundance: $\alpha = 0.07S^{-1/2}$.

A detailed description of the statistical method is presented in Varuzza and Pereira (2008) [18].

Functional classification of differentially expressed genes and online gene expression analysis

Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes. This task was performed using terms from the Gene Ontology database [19].

Additionally, we used the Oncomine database [20] in order to search for a previous association of differentially expressed genes found in this study with head and neck cancer.

Real Time PCR

Three genes displaying down (*KRT31*) or upregulation (*BST2*, *MFAP2*) were selected for validation in additional tissues using real-time polymerase chain reaction. One gene (*GNA15*) that did not present differential expression was also selected for this validation. Their expression was checked in 26 larynx SCC samples (15 N0 and 11 N+) relative to matched normal samples and in 36 tongue SCC samples (18 N0 and 18 N+). The primers were manually designed using the following parameters: 19–23 bp length, 30–70% GC content, a short amplicon size (66–110 bp), and at least one primer of each pair flanking an intron-exon boundary to prevent genomic amplification (Table 1). All primers were purchased from Invitrogen (Brazil).

Real time PCR was carried out in a 7500 Real-Time PCR System (Applied Biosystems). Reactions were performed in 20 μ l with 10 μ l of Power SYBR® Green PCR Master Mix (Applied Biosystems), and 400 nM of each primer. For every experiment, 10 ng cDNA were used, and each sam-

Table 1: Primers for real time PCR, amplicon size, values of slope, PCR efficiency and linearity (R2).

Genes	Primers	5' → 3'	Amplicon size (bp)	Slope	PCR Efficiency	R2
GNAI5	Forward	GAGAACCGCATGAAGGAGAG	84	-3.312	100.0	0.991
	Reverse	AAAGAGGATGACGGATGTGC				
KRT31	Forward	TGAGCAGGAGGTCAATACCC	110	-2.913	120.4	0.990
	Reverse	GACTCCTGGTCTCGTTCAGC				
BST2	Forward	GGAGGAGCTTGAGGGAGAG	75	-3.476	93.9	0.991
	Reverse	CTCAGTCGCTCCACCTCTG				
MFAP2	Forward	GCCGTGAGGAACAGTACCC	91	-3.152	107.6	0.990
	Reverse	CGGAGGCTGTAGAAGCAGAC				
TUBA6	Forward	TCAACACCTTCTTCAGTAAACG	101	-3.341	99.2	0.991
	Reverse	AGTGCCAGTGCGAACCTTCATC				
GAPDH	Forward	ACCCACTCCTCCACCTTTGA	101	-3.392	97.1	0.990
	Reverse	CTGTTGCTGTAGCCAAATTCGT				
ACTB	Forward	GGCAGCCAGCACAATGAAG	66	-3.255	102.8	0.994
	Reverse	CCGATCCACACGGAGTACTTG				

ple was tested in triplicate. The PCR conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, 60°C for 1 min, 65°C for 34 sec. Following the PCR, dissociation curve analysis was performed to confirm the desired single gene product.

For each primer set, the efficiency of the PCR reaction (linear equation: $y = \text{slope} + \text{intercept}$) was measured in triplicate on serial dilutions of the same cDNA sample (a pool of 10 samples).

The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/\text{slope})}]$ and ranged from 1.96 to 2.02 in the different assays. The slope and R2 values for target and reference genes are shown in Table 1.

Initially, five control genes were used (*TUBA6*, *ACTB*, *GAPDH*, *BCR*, *HPRT*). The GeNorm program [21] calculated stability and assumed that three genes (*TUBA6*, *ACTB*, *GAPDH*) were the most appropriate.

The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl (2001) [22]. Sta-

tistical analysis was calculated by a two-tailed unpaired *t* test using GraphPad prism software.

Results and discussion

Statistical analysis of SAGE data

We constructed three SAGE libraries from two larynx carcinoma samples and a pooled control sample aiming to identify global events involved in tumorigenesis and potential biomarkers in HNSCC.

Given the huge amount of data generated by SAGE, events that play a consistent role in cancer phenotype may be undistinguished from those that are random events, leading to false positive and false negative results. Statistical analysis and bioinformatic tools are used to overcome these limitations and improve the identification of a gene expression signature of biological and therapeutic interest. In the present study, we propose a statistical approach to analyze SAGE data through the use of Poisson probabilistic model and the conditional test of Cox partial likelihood. A Dempster methodology for ordering the sample points of the sample spaces throughout the likelihood ratio was also considered to compute the p-values. As the

sample size differs considerably, we obtain the significance critical level as a function of the tag abundance. To order the differentially expressed genes we consider the relative distance of the p-values against the critical level.

A total of 53,898 SAGE unique tags were obtained: 8,979 were only found in the metastatic larynx carcinoma library, 17,588 only in the non-metastatic carcinoma library, 15,102 only in the control library, and 12,229 tags were expressed in at least two libraries (Additional file 1, Tables 2, 3, 4–5). The sequences were stored in a MySQL relational database and analyzed as shown in Figure 1. Statistical analysis identified subsets of 1,216 differentially expressed tags between tumor and normal libraries, and 894 differentially expressed tags between metastatic and non-metastatic carcinomas. Sixty top-up and 60 top-downregulated tags in aggressive versus non-aggressive tumors and in both these tumors versus normal tissues, as well as their normalized frequencies, and the corresponding genes according to SAGE Genie and SAGEmap databases [23,24] are presented in Supplementary Tables 6–11 (Additional file 2).

Since several authors have reported that chi-square test is the most appropriate for SAGE experiments [25–28], we compared the performance of our statistical approach (named here as Kemp method) with that of chi-square test. For this comparison, the SAGE data set was divided into two groups: the low-abundance tags with counts equal and lower than 50, and the high abundance tags expressed at higher levels (> 50). Good correspondence between the data obtained by both tests was found for the latter tag group (Figure 2), indicating that they are equivalent for the analysis of highly expressed sequences. A similar result was not observed for low-abundance tags (Figure 3).

Using a proposed tag-customized critical level for both tests, we found 341 discordant tags, which represent 4.8% of total differentially expressed tags: 100 (29.3%) were considered differentially expressed by chi-square test but not by Kemp method, and 241 (70.7%) by Kemp but not by chi-square test. Most discordant cases were low-abundance tags (Additional file 3).

A tag presenting a differential expression pattern but low counts may be considered as statistically non-significant by methods that use fixed critical levels. Although a number of these tags probably have biological relevance, their selection from the SAGE data sets remains a challenge. To circumvent this limitation, Kemp's method calculates the critical level of each particular tag taking into account its total frequency, thus making the method applicable for detecting differences in expression of tags with counts ranging from 20 to 50. In addition, the use of

a tag-customized critical level minimizes both type I and type II errors. Conversely, most of the statistical tests currently used to detect differentially expressed genes are based on asymptotic results, and perform poorly for low expression tags. Another feature of these tests is the common use of a single canonical cutoff for the significance level (p-value) of all tags, without taking into account the type II error.

Differentially expressed genes: biological functions and potential involvement in HNSCC

Information on biological processes was obtained from the Gene Ontology (GO) database [19] for the top up- and down-regulated genes identified by the statistical approach (Tables 2 and 3). The data may be helpful for evaluating their potential as drug targets and molecular markers of cancer. Although some GO terms are not directly related to tumorigenesis, as lipid metabolism process and viral genome replication, they provide evidence of some important changes in cell metabolism coupled to energy generation and cell growth [29].

The functions of up-regulated genes in tumors include signal transduction (*BST2*, *FLNB*, *GNAI2*), transcription (*NRG1*), anti-apoptosis (*ANGPTL4*, *CCL2*, *IFI6*), cell adhesion (*SAA1*), cell migration and angiogenesis (*MYH9*), epidermis development and keratinization (*COL1A1*, *COL7A1*, *KRT14*, *LAMC2*, *S100A7*), and proteolysis (*MYH9*). Down-regulated genes are also involved in signaling (*CD24*, *DBNL*, *ECM1*, *TNFSF10*, *TSPAN6*), transcription (*EHF*, *PTRF*), anti-apoptosis (*SERPINB2*), keratinocyte differentiation, keratinization and epidermis development (*EHF*, *KRT13*, *SPRR3*, *TGM3*, *CRABP2*), and inflammatory response (*ANXA1*, *S100A9*). Comparison of aggressive (N+) and non-aggressive (N0) larynx tumors also showed interesting differences, including up-regulation of *NRG1*, a ligand for the receptor tyrosine kinase ErbB3 and 4 [30,31], and down-regulation of *IGFBP3* and keratin 6A (*KRT6A*) in N+ tumor. The latter result is interesting since K6-null mice exhibit changes in the oral mucosa resembling those of congenital pachyonychia [32]. In addition, K6a/K6b double-null mice also show localized disintegration of the dorsal tongue epithelium [33]. In relation to *IGFBP-3*, which has pro-apoptotic properties [34], reduced expression has already been found in tongue SCC cases, and associated with significantly shorter disease-specific and disease-free survival [35]. The authors have suggested that its down-regulation is an early event in head and neck tumorigenesis, with adverse prognostic significance in tongue cancer, and may represent a marker of aggressive disease, reinforcing the results of the present study.

Table 2: Information on biological processes based on Gene ontology.

<i>Biological Process</i>	<i>Up-regulated genes</i>
Cell communication signal transduction cell-cell signaling	ARHGAP29, BST2, CCL2, CXCL14, CMIP, FLNB, GNAI2, LY6E BST2, CCL2
Transcription	MZF1, NRG1, RPI3-122B23.3, ZNF452
Translation	RPS15, RPS23
Apoptosis induction anti-apoptosis	INCA BID ANGPTL4, CCL2, IFI6, XAF1
Cell adhesion	AJAP1, CCL2, MSLN, SAA1
Cell migration	MYH9, SAA1, LUM
Cell cycle	PLK1
Cell division	MYH9
Cell proliferation	BOLA2, BST2, PLK1
Cellular development process cell differentiation keratinocyte differentiation	MYH9 S100A7, SPRR2F
Cellular structure morphogenesis	MYH9
Developmental process organ development epidermis development keratinization	BST2, SPRR2F CCL2, MEPE, SPARC COL1A1, COL7A1, KRT14, LAMC2, S100A7, SPRR2F SPRR2F
Response to stimulus defense response inflammatory response immune response response to stress response to oxidative stress response to external stimulus	IL1F5, SERPINA3 BST2, CCL2, IFI6, IFITM2, IL1F5, SEMA3C DTL, SGK S100A7 CXCL14, CCL2, GNAI2, S100A7, SAA1, SEMA3C, TOPBP1
Angiogenesis	ANGPTL4, MYH9
Transport	MYH9, NEFL, RBPI, SGK, SLC15A3, SLC6A8
Metabolic process protein metabolic process protein modification process lipid metabolic process carbohydrate metabolic process DNA metabolic process nucleic acid metabolic process RNA processing	NADK INCA, LEPREL1, MYH9, NRG1, PRSS21, PSMC1 CCL2, DTL, FKBP9L, HSPE1, ISG15, SGK, TOR3A APOC1, APOLI, CEL, PLA2G4E, PTGS1, SERPINA3 NANS DTL, H3F3B OERH LSM4, SNRPD3
Cytoskeleton organization	FLNB, MYH9, NEFL, PLEK2
Extracellular structure organization	LUM

Table 2: Information on biological processes based on Gene ontology. (Continued)

Viral genome replication	<i>CCL2</i>
Cellular homeostasis	<i>CCL2, IFI6, SAA1, SELT</i>
No classification	<i>BASPI, CCNYL1, F8A1, FGFBP2, GRAMD1B, IFI27, KIAA1467, KIAA1799, KRTDAP, MFAP2, MSMB, NOL6, OLFML2A, SNCG</i>
Down-regulated genes	
Cell communication signal transduction cell-cell signaling	<i>ANXA1, ARHGAP27, CD24, CRABP2, DBNL, ECM1, GPR126, IL6R, MAL, TNFSF10, TSPAN6, TYRO3, CD24, MAL, S100A9, TNFSF10</i>
Transcription	<i>CRABP2, EHF, HOP, PTRF</i>
Apoptosis induction anti-apoptosis	<i>CLU, MAL, TNFSF10, ANXA1, SERPINB2</i>
Cell adhesion	<i>CLDN4, TYRO3</i>
Cell migration	<i>ANXA1, CD24, PRSS3</i>
Cell proliferation positive regulation	<i>IL6R, EHF, CLU, TSPAN31, CD24</i>
Cellular development process cell differentiation keratinocyte differentiation epithelial cell differentiation	<i>CLU, HOP, KRT19, MAL, AZML1, ANXA1, SPRR3, TGM3, EHF</i>
Developmental process organ development ectoderm development epidermis development epidermal cell differentiation keratinization	<i>EHF, IL6R, MAL, CLU, HOP, MAL, KRT6A, CRABP2, KRT13, SPRR3, TGM3, CNFN, PPL, SPRR3</i>
Response to stimulus defense response inflammatory response immune response response to stress response to external stimulus	<i>NCF1, ANXA1, LYZ, MGLL, S100A8, S100A9, CLU, CRI, GBP6, IL1RN, IL6R, CD24, CLU, CAT, CD24, CSTB, KRT8, PDE6B, SPRR3</i>
Transport	<i>ALDH3A1, AQP5, ARHGAP27, CAT, CD24, KIF1C, PGD, PLLP, RHCG, SPNS2</i>
Metabolic process protein metabolic process protein modification process lipid metabolic process carbohydrate metabolic process	<i>ALDH3A1, CD24, ECHDC3, TPI1, PRSS3, RANBP9, TMPRSS11E, UBR4, USP10, ANXA1, PRSS3, TGM3, USP10, AKR1C2, ANXA1, APOD, CLU, LTB4DH, MGLL, PIGF, TPI1, PGD, TPI1</i>
Lymphocyte activation	<i>CD24</i>
Homeostasis	<i>RHCG, CD24</i>

Table 2: Information on biological processes based on Gene ontology. (Continued)

No classification	<i>C20orf149, C6orf205, C9orf58, CAPNS2, CRCT1, DIS3L2, FAM129B, GPRASP2, HPCAL1, IER2, IGHA1, KRT78, LOC342897, LOC643008, LYPD2, LYPD3, MGC59937, MUC1, NUCKS1, PRH1, TMEM59, TPPP3, ZFAND1</i>
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Top up- and down-regulated genes selected from SAGE in tumor samples compared to normal samples.

Table 3: Information on biological processes based on Gene ontology.

Biological Process	Up-regulated genes
Cell communication signal transduction cell-cell signaling	<i>CXCL14, OR4S2, RPS6KA1, TNFRSF18, TNFSF10, TOLLIP</i>
Transcription	<i>NRG1, SUMO1</i>
Apoptosis induction anti-apoptosis	<i>INCA, TNFSF10, PRKCZ, TNFRSF18</i>
Cell-adhesion	<i>MSLN</i>
Cell cycle	<i>CCND1, UBE2C</i>
Cell proliferation negative regulation	<i>EMP3</i>
Cellular development process cell differentiation	<i>KRT19</i>
Developmental process organ development epidermis development	<i>KRT19</i>
Response to stimulus defense response inflammatory response response to stress response to oxidative stress response to external stimulus	<i>SERPINA3, GPX2, CXCL14, OR4S2</i>
Transport	<i>HBB</i>
Metabolic process protein metabolic process protein modification process lipid metabolic process carbohydrate metabolic process RNA metabolic process RNA processing	<i>NADK, DKFZP586H2123, INCA, NRG1, SULF2, UBE2C, USP9X, CCND1, POMT2, PRKCZ, SUMO1, USP14, SERPINA3, DCXR, PCBP2, RBM17</i>
DNA repair	<i>SUMO1</i>
No classification:	<i>ANXA7, BRD9, C6orf148, CMIP, FLJ23577, LOC283516, LOC283731, LOC388796, MFAP2, RRP15, SNCG, TMEM109, ZC3H7B</i>

Table 3: Information on biological processes based on Gene ontology. (Continued)

Down-regulated genes	
Apoptosis	
induction	KLK8
anti-apoptosis	IGFBP3
	ANGPTL4
Cell adhesion	SAA1
Cell migration	SAA1
Cell cycle	
Negative regulation	KLK10
Cell proliferation	
Negative regulation	FGFBP1
keratinocyte proliferation	KLK8
Cellular development process	
cell differentiation	IGFBP3, KLK8, SPON2
keratinocyte differentiation	SPRR2E, SPRR2F, SPRR3
Developmental process	SPRR2E, SPRR2F
organ development	
ectoderm development	KRT6A
epidermis development	SPRR2E, SPRR2F, SPRR3
keratinization	SPRR2E, SPRR2F, SPRR3
Response to stimulus	
defense response	NCF1
inflammatory response	PI3, S100A8, S100A9,
immune response	DEFB4, HLA-A, PI3, TAPBP
response to stress	HIG2, KLK8
response to external stimulus	KLK8, SAA1
Angiogenesis	ANGPTL4
Transport	ALDH3A1, HBA2, PGD
Metabolic process	ALDH3A1, TPI1
protein metabolic process	TAPBP
protein modification process	CCT3, FKBP9L, HSPE1, IGFBP3
lipid metabolic process	PLA2G4E, TPI1
carbohydrate metabolic process	PGD, TPI1
Cellular homeostasis	SAA1
No classification	C10orf99, C9orf58, CAPNS2, FAM129B, GPRASP2, IGHA1, LMNA, LOC645960, LYPD2, MUC1, NOL6, PSME2, SLFN13, SNHG8, TJP2, TncRNA

Top up- and down-regulated genes selected from SAGE in N+ tumor sample compared to N0 sample.

Potential molecular markers identified by SAGE:

Validation by Real-Time PCR

The selection of genes for validation by real-time RT-PCR was carried out after an extensive literature analysis of gene expression studies of head and neck carcinomas [3,4,36-66]. The following criteria were used for gene selection: (i) potential involvement in cancer development or aggressiveness and a yet unclear role in HNSCC

tumorigenesis, and (ii) similar expression pattern in data reported in the literature as well as in our SAGE experiments.

Using these criteria as guidelines, four genes were selected: two with a pronounced overexpression in SAGE tumor libraries (*BST2* and *MFAP2*), one with an intermediate downregulation profile (*KRT31*, also referred to as

Figure 2
Chi-square p-value versus Kemp value for high-abundance tags.

KRTHA1) and one with a non-significant differential expression pattern (*GNA15*).

According to the statistical analysis performed, *BST2* and *MFAP2* tags were expressed at high levels in tumors compared to normal tissues (at least 13-fold or higher), the latter also exhibiting a remarkable overexpression in N+ samples in relation to N0 samples. The normalized frequencies of *BST2* tags showed N+ tumor/normal and N0 tumor/normal ratios of 15.8 and 24.3, respectively. For *MFAP2*, N+ tumor/N0 tumor and N+ tumor/normal ratios were 25.3 and 13.5, respectively. In contrast to these genes, *GNA15* showed no differences in gene expression between samples analyzed by SAGE and was selected as a negative control. Although classified as a relevant under-expressed candidate marker in tumors by the statistical analysis of SAGE data, *KRT31* displayed less expressive differences between N+ or N0 tumors and normal tissues. The normalized frequencies of tags are shown in Supplementary Tables 6–11 (Additional file 2). Similar expression patterns of *BST2*, *MFAP2*, *KRT31* and *GNA15* tags were observed by using a chi-square test.

The expression data for the selected genes were validated in 15 pairs of tumor and matched normal tissues from N0 LSCC and 11 pairs from N+ LSCC. The data were also validated in another head and neck subsite by using 36 pairs of tumor and matched normal tissues from tongue squamous cell carcinomas (18 N+ and 18 N0). *MFAP2* was upregulated (≥ 2 fold) and *KRT31* was downregulated (≥ 2 -fold) in both N+ and N0 laryngeal tumors versus normal samples, the former also in tongue tumors. *BST2* gene was also upregulated but only in N0 tumors versus normal tissues. No difference between N+ and N0 carcinomas was detected for these genes, except for *MFAP2* in tongue samples. According to SAGE expression profiles, *GNA15* exhibited a non-significant differential expression pattern in carcinomas versus normal tissues, except between N+ and N0 tumors (Figure 4).

The results of the real time PCR experiments were, therefore, in agreement with SAGE data. However, as PCR experiments were performed using a larger number of cases than SAGE, we observed high variability of gene expression among the samples. This finding suggests

Figure 3
Chi-square p-value versus Kemp value for low-abundance tags.

molecular heterogeneity in HNSCC, as previously stated by Mendez et al. (2002) [55].

The selected genes present intriguing functions related to normal and neoplastic development. *KRT31* gene, for example, encodes a type I hair keratin, which is specifically expressed in hair and nails but has been previously observed in normal keratinocytes from buccal mucosa [67]. In cancer, loss of differentiation-specific hair keratins was found in late-stage pilomatrixoma, a skin tumor of follicular origin [68]. Since, keratin 31 has been detected in normal oral mucosa, a similar change in its expression pattern may occur in mucosa-derived squamous carcinomas.

The *BST2* gene encodes the bone marrow stromal cell antigen 2, a transmembrane glycoprotein potentially involved in interactions between cancer cells and bone marrow stromal cells and related to angiogenesis, cell proliferation and chromosomal instability [69]. The *BST2* promoter region contains putative *cis* elements for

GATA1, STAT 3 and 1 transcription factors, the latter over-expressed in HNSCCs [70]. *BST2* up-regulation has been observed in multiple myeloma, non-Down syndrome (DS) acute megakaryocytic leukemia, tamoxifen-resistant breast cancer [71-73] and, in the present study, was up-regulated in HNSCC samples. Stromal cells prevent chemotherapy-induced apoptosis of leukemia cells [74]. The findings of Ge et al. (2006) [73] suggest that *BST2* could potentially participate in the leukemia-cell protection from ara-C-induced cytotoxicity mediated by bone marrow stromal cells. These data and the findings of Becker et al. (2005) [72] on *BST2* overexpression in tamoxifen-resistant breast cancer indicate that *BST2* may possibly represent a new therapeutic target for leukemia as well as for other types of cancer, including HNSCC.

The *MFAP2* or *MAGP-1* gene encodes the microfibrillar-associated protein 2, a small molecular weight component of extracellular microfibrils, which are structural elements of elastic tissues in the lungs, skin, and vasculature. Miyamoto et al. (2006) [75] showed that *MAGP-1* protein

Figure 4

Gene expression in metastatic (N+) and non metastatic (N0) tumors. Expression of BST2, MFAP2, KRT31 and GNA15 was determined by real-time PCR in (A) 26 pairs of larynx tumors and matched normal tissues (15 N0 and 11 N+) and (B) 36 pairs of tongue tumor and matched normal samples (18 N+ and 18 N0). Relative quantitation of target gene expression for each sample was calculated according to Pfaffl [22]; GAPDH was used as the internal reference and normal sample as the calibrator. Values were Log2 transformed (y-axis) so that all values below -1 indicate down-regulation in gene expression while values above 1 represent up-regulation in tumor samples compared to normal samples. Differences in gene expression between groups (N0 and N+) were calculated by unpaired t test using GraphPad prism software and were considered statistically significant at $P < 0.05$ (*). The error bar represents the mean $\pm$ S.E.M (standard error of the mean).

can bind to the Notch1 receptor, leading to a subsequent signaling cascade. In self-renewing tissues and during tumorigenesis, Notch signaling may inhibit differentiation, lineage specification at developmental branch points and induction of differentiation. For example, Notch signaling regulates binary cell fate decisions in the development of the peripheral nervous system in flies. Equipotent precursors give rise to two alternative cell fates: epidermal or neuronal, depending on whether a progenitor cell receives a strong or weak Notch signal. In the skin, Notch induces terminal differentiation of keratinocytes. Therefore, the Notch pathway may lead to different and sometimes opposing outcomes. One explanation is that Notch function is context-dependent [76]. Abnormal Notch activation has been observed in different tumors [77-80] although growth suppression has also been noticed after constitutively over-expressed active Notch1 [81]. Thus, Notch signaling can function as both an oncogene and a tumor suppressor, even within a single tumor, supporting the idea that the Notch1 pathway is cell-type specific and context-dependent [82].

It is noteworthy that all these three genes (*BST2*, *KRT31*, *MFAP2*) and 27 differentially expressed genes referred to above (*ANGPTL4*, *ANXA1*, *CCL2*, *CD24*, *COL1A1*, *COL7A1*, *CRABP2*, *DBNL*, *ECM1*, *EHF*, *FLNB*, *GNAI2*, *IFI6*, *KRT13*, *KRT14*, *LAMC2*, *MYH9*, *NRG1*, *PTRF*, *S100A7*, *S100A9*, *SAA1*, *SERPINB2*, *SPRR3*, *TGM3*, *TNFSF10*, *TSPAN6*) presented the same expression pattern in Oncomine data sets and in our analysis, except two genes which exhibited a different pattern (*NRG1*, *TNFSF10*) and five genes with no information in head and neck data sets available through Oncomine (*ANGPTL4*, *FLNB*, *DBNL*, *IGFBP3*, *PTRF*).

Overall, the results of the real time PCR experiments showed consistent patterns in HNSCC patients and were in agreement with SAGE analysis. However, little is known about changes at the protein level, and the relationship between gene expression and tumor phenotype as well as the potential value of these genes as biomarkers for HNSCC tumorigenesis should be evaluated in future studies.

Conclusion

To the best of our knowledge, this is the first study reporting SAGE data in head and neck squamous cell tumors. The analysis of SAGE data by our statistical approach was effective in identifying differentially expressed genes reportedly involved in cancer development. In agreement with our statistical analysis, three genes (*BST2*, *MFAP2* and *KRT31*) selected for validation experiments were differentially expressed in an independent subset of HNSCCs compared to normal tissues or in metastatic versus non-metastatic samples. The selected genes have not been pre-

viously implicated in head and neck tumorigenesis. In addition, our data suggest a role for Notch signaling in HNSCC tumorigenesis, together with factors involved in keratinocyte differentiation, keratinization and epidermis development. The confirmation of the differential expression of this subset of genes selected from LSCC SAGE libraries in other HNSCC sites reinforce the existence of potential common biomarkers for prognosis and targeted therapy of such tumors.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NJFS participated in the design of the study and analysis of the data, developed the expression database and bioinformatic tools and drafted the manuscript. LV participated in the analysis of the data, developed the expression database and statistical tools. AM-L, ML, PS carried out the analysis and interpretation of the data and drafted the manuscript, DGP carried out the SAGE database. RVR performed the real time PCR experiments and data analysis. FGN and GENCAPO team members were responsible for sample and data collection and initial on-site sample processing, sequencing SAGE libraries, provided the pathological analysis of the cases, obtained informed consent and discussed the findings. WASJ carried out the SAGE experiments and analysis and helped in the interpretation of data. CABP participated in the study design and coordination, carried out the analysis and interpretation of the data and drafted the manuscript. EHT participated in the study design and coordination, was responsible for sequencing SAGE libraries, carried out the analysis and interpretation of the data and drafted the manuscript. All authors read and approved the final manuscript.

Appendix

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Additional material

Additional file 1

Supplementary Table 1. Tags with different frequencies between larynx SAGE libraries according to the criteria described in the Materials and Methods section. **Supplementary Table 2.** A total of 8,979 tags expressed in the N+ tumor SAGE library. **Supplementary Table 3.** A total of 17,588 tags expressed in the N0 tumor SAGE library. **Supplementary Table 4.** A total of 15,102 tags expressed in the control SAGE library. **Supplementary Table 5.** A total of 12,229 tags expressed in at least two SAGE libraries. Click here for file
[<http://www.biomedcentral.com/content/supplementary/1755-8794-1-56-S1.xls>]

Additional file 2

Supplementary Table 6. Sixty top-up regulated tags in aggressive versus non-aggressive larynx library. **Supplementary Table 7.** Sixty top-down regulated tags in aggressive versus non-aggressive larynx library. **Supplementary Table 8.** Sixty top-up regulated tags in aggressive versus normal larynx library. **Supplementary Table 9.** Sixty top-down regulated tags in aggressive versus normal larynx library. **Supplementary Table 10.** Sixty top-up regulated tags in non-aggressive versus normal larynx library. **Supplementary Table 11.** Sixty top-down regulated tags in non-aggressive versus normal larynx library. Click here for file
[<http://www.biomedcentral.com/content/supplementary/1755-8794-1-56-S2.xls>]

Additional file 3

Supplementary Table 12. Discrepancies between Kemp and chi-square analysis of SAGE data set. Click here for file
[<http://www.biomedcentral.com/content/supplementary/1755-8794-1-56-S3.xls>]

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CAPÍTULO 5

INTRODUÇÃO

As proteínas RIP (*receptor-interacting proteins*) compreendem uma família de cinases de serina/treonina que integram sinais de estresse extra e intracelulares causados por diferentes fatores, incluindo infecções, inflamação e lesões no DNA. Respostas pró-sobrevivência, inflamatórias e imune, bem como padrões de sinalização indutores de morte celular dependem das cinases RIP.

A proteína RIP2 (também conhecida como RICK ou CARDIAK), assim como outras RIPs, ativa o NF- κ B (*nuclear factor κ B*) interagindo com os fatores associados ao receptor do fator de necrose tumoral (TNFR). A RIP2 mostra isoformas geradas por *splicing* alternativo, cujas funções e significados ainda não foram completamente esclarecidos. Essa proteína interage especificamente com o domínio CARD da caspase-1, uma caspase pró-inflamatória, e ativa diretamente o padrão de sinalização ERK 1 / 2. Existem também evidências de sua participação em imunidade inata e adaptativa e em sinais iniciados por receptores de ambas as cascatas imunes: receptores *toll-like* (TLRs) e intracelulares NOD (*nucleotide-binding and oligomerization domain*) para imunidade inata, e receptores antígeno-específicos para imunidade adaptativa.

Nosso grupo identificou previamente uma variante do gene *RIPK2* gerada por *splicing* alternativo (AY562996), combinando análise por ferramentas de bioinformática e pela reação em cadeia da polimerase via transcriptase reversa (RT-PCR) de sequências ORESTES (*open reading frame expressed sequence tags*). Essa nova variante apresenta remoção do exon 2, que gera um código de parada prematuro e, conseqüentemente, um produto truncado com a perda dos domínios CARD e cinase.

A utilização de sítios de *splicing* alternativo difere entre tipos celulares, tecidos e fases do desenvolvimento e parece ser afetada por condições fisiológicas e estresse, tais como choque térmico, oxidativo e osmótico. Dado que as cinases RIP integram sinais de estresse extra e intracelulares e participam do início de diferentes cascatas de sinalização relacionadas tanto com proliferação como com morte celular, mudanças ambientais afetando sua expressão podem ter importantes implicações na fisiologia normal e em doenças.

MATERIAIS E MÉTODOS

No presente estudo, a expressão de duas isoformas RIP2 derivadas de *splicing* alternativo foi investigada em células normais humanas procedentes de autópsia e em amostras de carcinoma epidermóide de cavidade oral. Para avaliar se a variante menor é conservada entre espécies, o cDNA de tumores de mama caninos foi amplificado utilizando condições de baixa estrigência.

Com a finalidade de determinar se ambas as variantes são reguladas no nível pré-traducional por condições de estresse, sua expressão foi analisada sob exposição ao frio e ao calor e sob choque osmótico e ácido em três linhagens celulares tumorais humanas (*FaDu*/HTB-43, *SiHa*/HTB-35 and *HeLa*/CCL-2).

As linhagens celulares foram descongeladas e colocadas em meio MEM EARLE suplementado com soro fetal bovino, aminoácidos não essenciais, piruvato de sódio, penicilina e estreptomicina, sendo mantidas a 37°C em atmosfera de 5% de CO₂. Quando a densidade celular mostrava-se próxima ao estado de confluência, o material dos frascos foi tripsinizado e subdividido em réplicas. Para choque ácido, as células foram submetidas, imediatamente após a troca do meio, a uma atmosfera com CO₂ 10% por 24h e 72h, e mantidas a 37°C em atmosfera de CO₂ 5% por 24h e 72h. O pH do meio foi rigorosa e repetidamente verificado a cada 6h. Para choque osmótico, as culturas foram alimentadas com meio MEM completo contendo glicerol 0,4M e 0,8M (soluto eletricamente neutro), enquanto os respectivos controles foram mantidos em ausência desse soluto. O bloqueio das culturas foi efetuado duas ou cinco horas após iniciado o experimento. Para choque térmico, as linhagens celulares foram submetidas, após a troca do meio, a temperaturas de 17°C ou 5°C por períodos de 64 h, ou a 39-40°C por aproximadamente 3h. Os controles foram mantidos por períodos idênticos a 37°C. Um segundo experimento de choque térmico foi realizado com dez réplicas, duas para cada condição.

RESULTADOS E DISCUSSÃO

Foi observado que as duas variantes RIP2 são expressas em todos os tecidos analisados e que a razão variante 1 / variante 2 é regulada em resposta à temperatura. A variante 2 também foi detectada em amostras de tecidos caninos, evidenciando sua conservação entre espécies relativamente distantes.

Como as cinases RIP possuem um papel crítico na integração de sinais de estresse, a elucidação dos fatores que atuam na regulação dessas proteínas é importante.

A mudança de expressão das isoformas RIP pode levar a respostas opostas - proliferação ou morte celular - com consequências importantes em diferentes condições patológicas. Seria interessante determinar o papel fisiológico de cada isoforma na adaptação ao estresse em células normais e tumorais. Entretanto, o desafio é integrar os achados *in vitro* e *in vivo*.

Trabalho em fase de submissão**TEMPERATURE REGULATES ALTERNATIVE SPLICING IN *RIPK2*
GENE**

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RUNNING TITLE

Alternative splicing in *RIPK2* gene

KEY WORDS

Alternative splicing, *RIPK2*, temperature regulation, gene expression.

ABSTRACT

Receptor-interacting proteins (RIP) are a family of serine/threonine kinases which integrate extra- and intracellular stress signals caused by different factors, including infections, inflammation and DNA damage. Pro-survival, inflammatory and immune responses as well as death-inducing signaling pathways can all be initiated by the RIP kinases.

RIP2 is an important component of the nuclear factor κ B (NF- κ B) activation by tumor necrosis factors and shows isoforms generated by alternative splicing, whose precise functions and significance remain to be determined. In the present study, the expression of *RIPK2* transcripts was investigated in normal human tissues and in human and canine tumor samples. In order to determine if both variants are regulated at the pre-translational level in response to stress conditions, we analyzed their expression upon heat/cold exposure and acidic and osmotic shock in three human tumor cell lines.

We observed that both variants are expressed in all tissues analyzed and demonstrated that the variant 1/variant 2 ratio is regulated in response to temperature stress. As the RIP have a critical role in the integration of stress signals, the elucidation of factors involved in the regulation of these proteins is important. The change in RIP2 isoforms expression may lead to opposite responses - proliferation or cell death - with important consequences in different pathological conditions. It would be interesting to determine the physiological role of each isoform in the adaptation to stress in normal and tumor cells. However, the challenge is to integrate the findings *in vitro* and *in vivo*.

INTRODUCTION

Receptor-interacting proteins (RIP) are a family of serine/threonine kinases, which integrate extra and intracellular stress signals caused by different factors, including infections, inflammation, cellular differentiation and DNA damage. Pro-survival, inflammatory and immune responses as well as death-inducing signaling pathways can all be initiated by the RIP proteins (Meylan and Tschopp 2005).

RIP1, the best-characterized member of the RIP family, is an important component of the nuclear factor κ B (NF- κ B) activation by tumor necrosis factors (TNFs). TNFs and related cytokines initiate different signaling cascades that can result in opposite responses, both cell proliferation and cell death. Binding of TNF to its receptor leads to the recruitment and assembling of a signaling complex containing death domain proteins and the TNF receptor-associated factor 2 (TRAF2), triggering pro-apoptotic responses. The additional recruitment of RIP1 to this complex as well as its polyubiquitination through the lysine at position 63 (Lys63) of ubiquitin activate the mitogen-activated protein kinases (MAPKs) and the I κ B kinase (IKK), which phosphorylates NF- κ B inhibitors, targeting these proteins for proteasomal degradation. NF- κ B transcription factors are thereby released and may induce expression of proinflammatory, prosurvival and immunomodulatory genes (Zhang, Kovalenko et al. 2000; Ea, Deng et al. 2006; O'Donnell, Legarda-Addison et al. 2007).

Besides its pro-survival role, RIP1 can act as a pro-death molecule, forming a complex with caspase-8 to initiate apoptosis (Lin, Devin et al. 1999; Kim, Choi et al. 2000; Martinon, Holler et al. 2000; O'Donnell, Legarda-Addison et al. 2007). RIP1 interacts with RIP3, another member of the RIP family, which promotes apoptosis in various cell types when overexpressed (Sun, Yin et al. 2002).

RIP4, unlike RIP3, induces the activation of NF- κ B and AP-1 pathways when overexpressed. This member of the RIP kinase family is a modulator of keratinocyte differentiation and, as RIP5, is characterized by the presence of ankyrin repeats in their C-terminal domain (Meylan, Martinon et al. 2002; Zha, Zhou et al. 2004; Meylan and Tschopp 2005; Adams, Pankow et al. 2007). RIP6 (or LRRK1) and RIP7 (or LRRK2) are additional RIP kinases that have a leucine-rich repeat (LRR) motif and may be involved in pathogen-, damage- or stress-associated molecular patterns (Festjens, Vanden Berghe et al. 2007).

RIP2 (also called RICK or CARDIAK), like other RIPs, activates NF- κ B, interacting with the TNFR-associated factors. This RIP specifically interacts with the CARD of caspase-1, a pro-inflammatory caspase, and directly activates ERK1/2 signaling (Inohara, del Peso et al. 1998; Thome, Hofmann et al. 1998; Navas, Baldwin et al. 1999). Some reports claim the participation of RIP2 in innate and adaptive immunity, and in transducing signals from receptors of both immune cascades: Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD) intracellular receptors for innate immunity and antigen-specific receptors for adaptive immunity (Chin, Dempsey et al. 2002; Kobayashi, Inohara et al. 2002). Otherwise, the studies of Park et al (Park, Kim et al. 2007) and Hall et al (Hall, Wilhelm et al. 2008) indicated that the absence of RIP2 causes reduced cytokine production and are defective in their responses to NOD but does not affect T cell proliferation, T helper differentiation or TLR responses. In addition, RIP2 has been demonstrated to have an important role for the healing process, either as a regulator of keratinocyte proliferation, migration or differentiation (Adams, Valchanova et al. 2010).

Similar to RIP1 in the TNF signaling cascade, Lys63-linked polyubiquitination of RIP2 induces NF- κ B activation after NOD stimulation. The polyubiquitin chains are linked at lysine 209 in RIP2 kinase domain (Hasegawa, Fujimoto et al. 2008) whereas, in RIP1, are linked at lysine 377, in an intermediate region between the N-terminal kinase and C-terminal death domain (Ea, Deng et al. 2006; Li, Kobayashi et al. 2006). In NOD signaling pathway, the interaction of RIP2 and NEMO, a regulatory subunit of IKK, is also essential for NF- κ B activation (Inohara, Koseki et al. 2000). Recently, Clark and collaborators (Clark, Marinis et al. 2008) demonstrated that MAP3K4, a MAPK kinase kinase that is a major mediator of environmental stress, sequesters RIP2 from the NOD signaling inhibiting NF- κ B cascades.

We previously described a new alternative splicing variant of the *RIPK2* gene (AY562996), using a combined bioinformatic and reverse transcription polymerase chain reaction (RT-PCR) analysis of open reading frame expressed sequence tags (ORESTES) (Mancini and Tajara). This new variant presents skipping of exon 2 and is characterized by extensive truncation of the the C-terminus resulting in a frameshift that generates a premature termination codon (PTC). Recently, Krieg et al (2009) (Krieg, Le Negrate et al. 2009) showed the loss of the CARD domain and intermediate region as well as truncation of the kinase domain make this shortened form RIPK2 unable to activate NF- κ B and caspase pathways, and loss of autophosphorylation and kinase activity.

The use of alternative splice sites may differ among cell types, tissues and phases of development (Clarke, Jordan et al. 2000; Sun, Yin et al. 2002; Zeng, Sharpe et al. 2006) and has been shown to be affected by physiologic and stress conditions, such as heat shock, oxidative and osmotic stress (Shen, Beall et al. 1993; Takechi, Hosokawa et al. 1994; Tomasini, Samir et al. 2001; Hashimoto, Zhang et al. 2002; Gray, Iglesias et al. 2003; Matsuzaki, Manabe et al. 2004; Nurmi, Puolakkainen et al. 2005; Wakasugi, Nakano et al. 2005; Bond, Patel et al. 2006; Kim and Gladyshev 2006; Fiol, Mak et al. 2007). For example, Fujikake and collaborators (Fujikake, Nagai et al. 2005) identified three new variants of the *Drosophila* heat shock transcription factor gene (*Hsf*) regulated in response to heat/cold stress. In mouse, one alternatively spliced mRNA of the *HSP47* (heat shock protein 47) gene is temperature-induced (Takechi, Hosokawa et al. 1994). A similar event was observed for the “readthrough” transcript (type R) of the acetylcholinesterase gene (*Ache*) in mouse neuroblastoma N18TG2 cells (Perrier, Salani et al. 2005).

In humans, an interesting case of temperature-dependent alternative splicing was observed in fibroblasts of a patient with Ehlers–Danlos Syndrome type VII (Weil, D'Alessio et al. 1989). The patient showed a mutation in the last nucleotide of exon 6 of the *COL1A1* gene, which produced an aberrant spliced transcript not detectable at 31°C but present at 39°C. The insertion of a cryptic exon in the neurofibromatosis type 1 mRNA induced by cold-shock conditions was also observed by Ars and collaborators (Ars, Serra et al. 2000).

RIPK1, 2, 3 and 5 genes show variants generated by alternative splicing of the primary transcripts whose precise functions and significance remain to be determined. Since RIP kinases integrate extra and intracellular stress signals and initiate different signaling cascades that can result both in cell proliferation and cell death, environmental changes may affect their expression with important implications.

In the present study, the expression of two *RIPK2* isoforms was investigated in normal and tumor cells. In order to determine if both variants are regulated at the pre-translational level in response to stress conditions, we analyzed their expression upon heat/cold exposure and acidic and osmotic shock in human tumor cell lines.

MATERIALS AND METHODS

Samples

Nine samples of normal human tissues removed at autopsy (brain, testis, heart, lung, stomach, kidney, larynx, liver and tongue) and samples from 32 patients with squamous cell carcinoma of the oral cavity and 4 dogs (*Canis lupus familiaris*) with mammary tumors (matched tumor/surgical margins) were used to evaluate the expression of two splicing variants of the *RIPK2* gene using RT-PCR and quantitative PCR.

For the stress experiments, we utilized the human ATCC cell lines HeLa/CCL-2, (adenocarcinoma of the cervix), FaDu/HTB-43 (squamous cell carcinoma of the pharynx), and SiHa/HTB-35 (squamous cell carcinoma of the cervix). All three cell lines were cultured in Modified Eagle's Medium (Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (Cultilab), 10mM of non-essential amino acids (Invitrogen), 0.11g/L of sodium pyruvate, 100 units/ml penicillin (Cultilab), and 90µg/ml streptomycin (Cultilab), in a humidified atmosphere with 5% CO₂ at 37°C.

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.

Heat, osmotic and acidic shock

Prior to stress treatment, replicas from each cell line were allowed to grow to 80-90% confluence. The medium was replaced with 15mL of fresh medium at 0 hour. For the heat shock experiments, cultures were maintained at 40°C for 3 h, and at 17°C or 5°C for 64 h. Osmotic shock was achieved by incubation of cell line cultures in medium containing 0.4M or 0.8M ultrapure glycerol (hypertonic stress) for 2 or 5 h at 37°C. Acidic shock was performed by maintaining the cultures in an atmosphere with elevated CO₂ (10% CO₂) for 24 h or 72 h. Ten replicas were cultured under each stress condition, and ten control replicas were cultured without addition of glycerol, in a humidified atmosphere with 5% CO₂ at 37°C for the same time periods.

Cells were immediately lysed after the incubation period by adding TRIzol (Invitrogen), and stored at -80°C until RNA extraction.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNAs from tissue samples and cell lines were obtained using TRIzol reagent and treated with DNase. One microgram of total RNA was converted to cDNA using the ThermoScript™ RT-PCR System or SuperScript™ III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Integrity of the RNA was confirmed by agarose gel electrophoresis, and the concentration was determined by obtaining the ratio of absorbance values at 260 and 280 nm, following standard procedures.

Detection of RIPK2 variants by semiquantitative PCR and sequencing

The PCR amplification of both longer and shorter *RIPK2* variants was performed using the oligonucleotides 5'-CGCCTCTGGCACTGTGTCGT-3' (sense) and 3'-CGTGACTGTGAGAGGGACAT-3' (anti-sense). The total reaction volume was 25 µl and contained 1X PCR buffer, 1 mM MgCl₂, 2 µM of each *RIPK2* primer, 2 µM of *GAPDH* primers, 5 mM dNTPs mix, 1U Taq DNA polymerase (Invitrogen) and 50ng of cDNA. After pre-incubation for 5 min at 94°C (initial denaturation), amplification was carried out through 35, 28 or 21 cycles at 94°C for 50 s, 58°C for 40 s, 72°C for 50 s, and 72°C for 10 min, using a thermal cycler (9700 GeneAmp PCR System – Applied Biosystems, Foster City, CA, USA). PCR amplification of the endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), was carried out using sense (5'-CTGCACCACCAACTGCTTA-3' or 5'-ACCCACTCCTCCACCTTTGA-3') and anti-sense (5'-CTAGACGGCAGGTCAGGTC-3' or 5'-CTGTTGCTGTAGCCAAATTCGT-3') primers. The PCR products from *RIPK2* variant 1 (457 bp), variant 2 (303 bp), and *GAPDH* (296 bp and 101 bp) were separated on 2% agarose gels in the presence of ethidium bromide and quantified by an imager system (EDAS 290, Kodak, New Haven, CT). The PCR bands were cut out of the agarose gel and directly sequenced in both directions with sense and antisense primers, using a 377 ABI Prism Sequencer (Applied Biosystems). The sequences were analyzed using BLAST similarity search against a Nucleotide Database (www.ncbi.nlm.nih.gov/BLAST/).

Splice variant-specific real time PCR

The expression of *RIPK2* variant 1 and variant 2 following stress treatment of FaDu cells was investigated by real time PCR. The reaction was performed in quadruplicate using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The primers were manually designed using the following parameters: 19-24 bp length, 30-70% GC content and a short amplicon size (66-104 bp), as follows, *RIPK2* variant 1, sense (5'-

AGAAGCTGAAATTTTACACAAAGC-3') and antisense (5'-CCATTTGGCATGTATTCAGTAAC-3'); *RIPK2* variant 2, sense (5'-TGCTCGACAGAAACTGAATATC-3') and antisense (5'-AAGGAGGAGTCATATTGTGCAG-3'); *GAPDH*, sense (5'-ACCCACTCCTCCACCTTTGA-3') and antisense (5'-CTGTTGCTGTAGCCAAATTCGT-3'); *TUB6A*, sense (5'-TCAACACCTTCTTCAGTGAAACG-3') and antisense (5'-AGTGCCAGTGCGAACTTCATC-3'); *ACTB*, sense (5'-GGCACCCAGCACAATGAAG-3') and antisense (5'-CCGATCCACACGGAGTACTTG-3'). All primers were purchased from Invitrogen. Briefly, the reactions were carried out in a total volume of 20 µl, with 10 µl of *Power SYBR Green PCR Master Mix* (Applied Biosystems), 250 nM of each primer and 10 ng cDNA. The PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 10 sec, 60°C for 1 min. Following the PCR, dissociation curve analysis was performed to confirm the desired single gene product.

The gene expression stability over different samples was analyzed using the geNorm software (<http://medgen.ugent.be/~jvdesomp/genorm>). Each transcript level was normalized by division with the expression values of *GAPDH*, *ACTB* and *TUBA6*, used as internal controls.

The transcript level was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The fold difference (relative abundance) was calculated using the formula $2^{-\Delta\Delta C_t}$ and was plotted as means $\pm$ SD, with $n=3$ technical replicates. Fold differences of >3 were considered to be significant.

All reactions were performed with a negative control. One sample without treatment was chosen to be used as the calibrator control for the reactions. Experiments were repeated whenever the coefficient of variation was higher than 5%. After each reaction, the products were analyzed on a 2% agarose gel stained with ethidium bromide.

RESULTS

Identification and Expression Patterns of RIPK2 variants

The open reading frame expressed sequence tags (ORESTES, (Dias Neto, Correa et al. 2000) generated by the Human Cancer Genome Project (HCGP); (Camargo, Samaia et al. 2001; Brentani, Caballero et al. 2003); were matched to the RefSeq and mRNA public data sets (www.ncbi.nlm.nih.gov). Genomic mapping of the ORESTES sequences revealed the existence of a putative new *RIPK2* splice variant. RT-PCR and cDNA sequencing were

performed to confirm the splicing event, using normal and tumor tissues. This approach revealed the expression of both longer *RIPK2* variant 1 (NM_003821) and shorter variant 2 (AY562996) in different samples. The latter is generated by skipping of exon 2 (154bp), which alters the reading frame, producing several premature stop codons.

Normal tissue samples from brain, testis, heart, lung, stomach, kidney, larynx, liver and tongue, and matched tumor/surgical margins samples from 32 patients with oral cancer co-expressed *RIPK2* variants 1 and 2 (Figure 1A and B). The co-expression of both isoforms was reinforced by database analysis (UCSC Genome Browser - <http://genome.ucsc.edu/>) of ESTs covering the exon 2 of *RIPK2*. This analysis showed the skipping of exon 2 in 33 of the 80 ESTs covering this transcript region. To investigate whether splice variant 2 is conserved across species, we amplified cDNA from canine mammary tumors by using low stringent conditions. Again, a putative splice variant was detected (Figure 1C).

Splicing patterns of RIPK2 under stress conditions

To investigate whether alternative splicing of *RIPK2* is induced or inhibited by stress conditions, three cell lines (FaDu, SiHa and HeLa) were exposed to severe heat shock (40°C, 17°C or 5°C), osmotic shock (hypertonic medium) or acidic shock (atmosphere of 10% CO₂). Only heat shock altered the splicing pattern. The semi-quantitative RT-PCR results showed an increase in the transcription of variant 1 at 40°C and an increase in the transcription of variant 2 at 17°C compared to that at 37°C (Figure 2 B). In all replicas of the Fadu cell line at 40°C, the result was confirmed by quantitative PCR, and in most replicas at 17°C, variant 2 showed higher expression than variant 1 (Figure 2 A). These results suggest that alternatively spliced exon 2-containing mRNA may be transcribed more efficiently at higher temperatures than the variant without exon 2.

DISCUSSION

In the present study, we detected the expression of two *RIPK2* variants in normal tissues and tumor cells. We found evidence that transcript 2 is conserved across species, since it was also detected in canine mammary tissue. At the protein level, a western blot band, which may correspond to isoform 2, was detected in the samples analyzed.

We observed a shift in the expression of *RIPK2* variants 1 and 2 as a response to temperature stress *in vitro*. Hyperthermia accompanies some pathophysiological processes, such as infection, injury and inflammation, and may lead to enhancement of immune

responses to the causal stimuli. A beneficial effect of hyperthermia has also been observed when used therapeutically together with cancer treatment (Wust, Hildebrandt et al. 2002; Yoo and Lee 2007). However, the molecular mechanisms that mediate the effects of hyperthermia are not completely known so far.

Yan and collaborators (Yan, Xiu et al. 2007) found evidence that hyperthermia induces TLR expression and TLR signaling-mediated activation of NF- κ B and MAPK pathways, resulting in increased synthesis of pro- and anti-inflammatory cytokines. Zhao et al (Zhao, An et al. 2007) also observed TLR activation and significantly increased cytokine production when TLR stimulation was accompanied by a hyperthermia pretreatment. These data suggest that fever may modulate innate immune responses by TLR pathway and, although the role of RIP2 in this signaling remains controversial (Chin, Dempsey et al. 2002; Kobayashi, Inohara et al. 2002; Park, Kim et al. 2007; Hall, Wilhelm et al. 2008), provide a possible link to *RIPK2* expression changes depending on the temperature variation.

RIP2 integrates extra and intracellular stress signals and is an important component of the NF- κ B signaling cascade, transducing signals from innate immune system proteins, including cytoplasmic NOD proteins, which are involved in detecting pathogens in intracellular compartments. In addition, RIP2 has been suggested to be required for T-cell-receptor signaling and therefore may also participate of the adaptive immune response (Kobayashi, Inohara et al. 2002).

Hasegawa and collaborators (Hasegawa, Fujimoto et al. 2008) demonstrated that Lys63-linked polyubiquitination at Lys209 within the kinase domain of RIP2 is essential for NOD-mediated NF- κ B activation. The authors also showed that the interaction between NEMO and the intermediate region spanning residues 293–319 of RIP2 is required for NOD signaling. Otherwise, the residue critical for kinase activity of RIP2 (Lys47) is important but not essential for NF- κ B activation (Inohara, del Peso et al. 1998; Inohara, Koseki et al. 2000) and apparently does not participate in polyubiquitination and interaction with NEMO. The kinase domain may mediate other functions, such as regulation of ERKs, p38 kinases, and own degradation (Navas, Baldwin et al. 1999; Chin, Dempsey et al. 2002; Hasegawa, Fujimoto et al. 2008). Considering these data, it is reasonable to suppose that isoform 2 of RIP2 may exhibit some impaired functions, other than polyubiquitination and interaction with NEMO, since its kinase domain is partially deleted.

In the present study, *RIPK2* variants 1 and 2 were observed in all normal and tumor tissues investigated, including human and canine samples. Variant 2 showed a tendency to have increased expression at low temperatures than variant 1, whereas the opposite occurred at 40°C.

This variability – a shift in the expression of *RIPK2* variants 1 and 2 as a response to temperature changes – suggests that stress signals caused by conditions such as infections and inflammation may affect the expression and splicing of *RIPK2*. We hypothesize that, under stress conditions, a putative mechanism would induce higher or lower expression of the exon 2-containing *RIPK2* product, which clearly affects the sequence of the encoded protein. If isoform 2 is a non-functional protein, the balance of isoforms 1 and 2 would change, with consequences on signaling both NF-κB and caspase pathways related to RIP2. For instance, hyperthermia would increase the turnover rate of RIP2 or affect ERK pathways, whereas hypothermia might trigger proapoptotic responses. However, this hypothesis obviously requires experimental confirmation.

Recently, Dasgupta and collaborators (Dasgupta, Agarwal et al. 2008) described a C-terminal fragment of another member of RIP family, RIP1, which can activate signaling events, including NF-κB and TNF pathways, and consequently, induce survival and apoptotic responses. They concluded that short RIP1 affects the long isoform levels and may represent a new regulation mechanism. Albeit exhibiting some differences, RIP1 and 2 participate in the same regulatory net and are probably derived from a common ancestry. Therefore, it is tempting to hypothesize that short RIP2 may reduced production of long RIP2 under different conditions.

As RIP kinases play a critical role in integrating stress signals, the elucidation of the factors that take part in the regulation of these proteins is of major importance. The change in expression of RIP isoforms may lead to opposite responses - cell proliferation or cell death - with important consequences in different pathological conditions. It would be interesting to determine the physiological roles of each isoform for stress adaptation of normal and cancer cells. However, the challenge will be to link in vitro and in vivo findings.

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APPENDIX

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Figure 1. Expression of *RIPK2* mRNA and RIP2 protein in normal and tumor tissues. RT-PCR products from *RIPK2* variant 1 (456bp), variant 2 (302bp), and *GAPDH* (305 bp) in (A) samples from patients with oral cancer, (B), normal human tissues and (C) canine mammary tumor samples. T=tumor; M=surgical margin; 1=brain; 2=testis; 3=heart; 4=lung; 5=stomach; 6=kidney; 7=larynx; 8=liver; 9=tongue; L=100 bp fragment size marker. *GAPDH* mRNA was included as a control.

Figure 2. A. Splicing patterns of *RIPK2* under temperature stress. *RIPK2* splice variants in FaDu cell line cultures maintained at 40°C for 3 h and at 17°C or 5°C for 64 h. **A.** Quantification of *RIPK2* splice variant 1 (white) and 2 (black) expression by real time PCR. **B.** RT-PCR products from *RIPK2* variant 1 (456 bp), variant 2 (302 bp), and *GAPDH* (101 bp). Arrows indicate shifted bands.

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