Alessandra Vidotto

MARCADORES PROTÉICOS DO CARCINOMA EPIDERMÓIDE DE CABEÇA E PESCOÇO COM FENÓTIPO INVASIVO

Tese apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no Curso de Pós-Graduação em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientadora: Profa. Dra. Eloiza Helena Tajara da Silva

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SUMÁRIO

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"Se vi mais longe foi por estar sobre os ombros de gigantes"

(Isaac Newton)

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(Mahatma Gandhi)

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Lista de Abreviações

CECP : carcinoma epidermóide de cabeça e pescoço

FABP: fatty acid binding protein

HNSCC: head neck squamous cell carcinoma

MALDI-Q-TOF: matrix-assisted laser desorption ionisation-quadrupolo-time of-flight

N0: linfonodos negativos

PLUNC: palate lung and nasal epithelium clone protein

TEM: transição epitélio-mesenquimal

TGF-beta: fator de crescimento transformante beta

Introdução: O comprometimento de linfonodos regionais por células neoplásicas é atualmente o indicador mais utilizado para prognóstico em pacientes com carcinoma epidermóide de cabeça e pescoço (CECP). Apesar disso, a compreensão detalhada dos mecanismos envolvidos na formação de metástases linfáticas ainda não foi completamente atingida. **Casuística e Método:** Foi avaliado o perfil protéico de linfonodos metastáticos e não metastáticos, bem como de amostras de saliva e soro de 62 pacientes em diferentes estágios da doença e de 29 controles, utilizando eletroforese bidimensional, espectrometria de massas por MALDI-Q-TOF e experimentos de validação por *Western blot*. **Resultados:** Os resultados mostraram várias proteínas com expressão elevada em linfonodos metastáticos em relação aos não metastáticos, como stratifina, glutathiona S-transferase pi, apoliproteína A-I, alfa-1-microglobulina, dissulfeto isomerase, galectinas, citoqueratinas, imunoglobulinas, transtirretina e proteínas de ligação ao cálcio (família S100) e a ácidos graxos (FABP). De forma inversa, as proteínas calrreticulina, tropomiosina 3, triofosfato isomerase, piruvato quinase, anidrase carbônica, gama actina, peroxirredoxina 2, profilina 1, gliceraldeído 3-fosfato desidrogenase e proteínas de choque térmico mostraram níveis reduzidos em linfonodos metastáticos. Essas proteínas estão envolvidas em processos de desenvolvimento epidérmico, proliferação, migração e adesão celular, apoptose, resposta inflamatória e metabolismo de xenobióticos. Os dados relacionados à expressão de proteínas de choque térmico e enzimas da via glicolítica sugerem um efeito do ambiente dos linfonodos e no controle da progressão do tumor ou na reprogramação das células metastáticas. Em saliva, 13 proteínas exibiram um padrão alterado nas amostras de pacientes com câncer, incluindo expressão elevada de queratinas, imunoglobulinas, alfa-amilase, PLUNC e zinc-alfa-2-glicoproteína e

expressão reduzida de miosina. Em amostras de soro, seis proteínas apresentaram expressão aumentada (albumina, alfa-1-microglobulina/bikunina precursor, apolipoproteína A-I, haptoglobina, serotransferrina e transtirretina) e duas estavam com expressão diminuída (hemoglobina alfa e hemoglobina beta), quando comparadas com o grupo controle. **Conclusão:** Os resultados obtidos revelaram novos marcadores potenciais, como profilina 1 e E-FABP, PLUNC e transtirretin que podem ser úteis na definição do fenótipo invasivo e no rastreamento e diagnóstico desse grupo de neoplasias.

Palavras-chave: 1. Neoplasias de cabeça e pescoço; 2. Metástase Neoplásica; 3. Linfonodos; 4. Saliva; 5. Soro; 6. Proteômica; 7. Eletroforese em Gel Bidimensional; 8. Espectrometria de Massas; 9. Marcadores Biológicos.

Introduction: The regional lymph nodes play a pivotal role in diagnosis, staging and management of head and neck squamous cell carcinomas (HNSCC). Despite their importance, detailed understanding of the probable mechanisms of lymphatic metastases has not been completely achieved. **Subjects and Methods:** We analyzed metastatic and normal lymph node tissues, as well as saliva and serum from sixth-two patients with HNSCC, and twenty-nine controls using two-dimensional electrophoresis, MALDI-Q-TOF and western blot. **Results:** Several proteins were found to be significantly increased in metastatic nodes, such as stratifin, glutathione S-transferase pi, apoliproteín A-I, alpha-1-microglobulin, disulfide isomerase, galectin, citokeratins, immunoglobulins, transtirretin, calciun-binding protein (família S100) and fat-binding protein (FABP). Among the down-regulated proteins in metastatic lymph nodes are calreticulin, tropomiosin 3, triosephosphate isomerase, piruvate quinase, anidrase carbonic, gamma actin, peroxiredoxin 2, profilin 1, gliceraldeyde 3-fosfato desidrogenase and heat shock proteins. These proteins are involved in epidermis development, cell proliferation, migration and adhesion, apoptosis, defense and inflammatory response and xenobiotic metabolism. Our data on the expression of heat shock proteins and enzymes of the glycolytic pathway suggest an effect of the lymph node environment in controlling tumor progression or in metabolic reprogramming of the metastatic cell. In saliva, 13 proteins showed an altered pattern of expression in samples patient, including over-expression of keratins, immunoglobulins, alpha-amylase, PLUNC and zinc-alpha-2-glycoprotein and down-regulation of myosin. In serum samples, six proteins were over-expressed (serum albumin, alpha-1-microglobulin/bikunin precursor, apolipoprotein A-I, haptoglobin, serotransferrin, transthyretin) and two were under-expressed (hemoglobin subunit alpha, hemoglobin

subunit beta) compared to the control group. **Conclusion:** New potential markers, such as profilin-1 and E-FABP, were identified and may be proved useful for defining the invasive phenotype of head and neck carcinomas.

Keywords: 1. Head and neck neoplasm; 2. Metastasis, 3. Lymph node; 4. Saliva; 5. Serum; 6. Proteomics; 7. Two-dimensional electrophoresis; 8. Mass spectrometry; 9. Biological Markers.

INTRODUÇÃO

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1.1. O processo de formação de metástases

As metástases compreendem a principal causa de morte em pacientes com câncer.[1] O poder que as células malignas possuem para matar seu hospedeiro reside na sua habilidade de deixar o tumor primário, disseminar-se e sobreviver em outros sítios, assim como de exibir um crescimento incontrolável e auto-renovação, produzindo tumores secundários mais agressivos e incuráveis. Nos últimos anos, muitos dados têm revelado fatores envolvidos nesta cascata destrutiva, que incluem uma lista crescente e extensa de eventos epigenéticos e de mutações.[2,3]

Nas etapas de iniciação do processo de metastatização, sinais liberados pelas células mesenquimais do estroma ou mutações em diferentes membros de vias de transdução de sinais auxiliam na perda de adesão célula-célula e promovem a migração celular, que são características de um evento de diferenciação reversível e importante, presente na embriogênese e denominado transição epitélio-mesenquimal (TEM).[4] Bons exemplos de reguladores da TEM incluem pequenos RNAs não-codificantes e membros das vias de sinalização do fator de crescimento transformante beta (TGF-beta), do receptor de proteína tirosina-quinase, Notch e Wnt.[5-10] A atividade anormal de vários mediadores dessas cascatas é importante para os estágios iniciais do desenvolvimento tumoral e, além de tornar a célula dependente ou sensível aos seus efeitos, pode conferir vantagens para as etapas seguintes da tumorigênese.[11,12] De fato, os resultados de experimentos de microarranjos de cDNA demonstram que tumores primários e metástases de um mesmo indivíduo compartilham modificações genéticas, que são conservadas durante a evolução do tumor.[13] A análise de amostras não pareadas

também sugere que a assinatura da expressão gênica metastática já pode estar presente em fases iniciais do processo neoplásico.[14]

Com o crescimento tumoral, uma baixa tensão de oxigênio estimula uma resposta pró-angiogênica.[15] Graças a microRNAs (miRs)[16] e citoquinas secretadas por células estromais e neoplásicas,[17] as células endoteliais de vasos sanguíneos pré-existentes sintetizam moléculas de adesão e proteases, que permitem sua migração até o tumor, através do estroma degradado.[18] Essas células endoteliais se proliferam e geram novos vasos, fornecendo oxigênio e nutrientes para manter o crescimento tumoral e representam uma rota importante para o processo de metastatização. A formação de vasos linfáticos, comum em várias condições inflamatórias, também é estimulada em alguns tumores humanos[19,20] e compreendem a principal rota de disseminação de células neoplásicas nesses casos, nos quais receptores de citoquinas e seus ligantes possuem um papel crítico e podem ser responsáveis pela formação de metástases em linfonodos.[21] Na verdade, a rede linfática é mais permissiva à disseminação metastática que o sistema vascular sanguíneo porque seus capilares exibem uma única camada de células endoteliais não circundadas por pericitos, possuem estruturas semelhantes a válvulas intercelulares que facilitam a entrada de células e as membranas basais dos vasos são incompletas.[22] O carcinoma epidermóide de cabeça e pescoço (CECP) é um exemplo de tumores que freqüentemente disseminam para os linfonodos regionais[23] e está quase sempre associado à inflamação crônica.

A chegada a outro sítio anatômico não é garantia de sucesso para a maioria das células metastáticas. Os processos de extravasamento e disseminação requerem características específicas do tumor bem como condições receptivas locais. Para aumentar as chances de obter um resultado favorável, tem sido sugerido que os sítios

alvos são preparados antecipadamente pela interação à longa distância com o tumor primário.[24] Dependendo do tumor, um crescimento importante é observado antes do extravasamento, ainda dentro dos vasos sanguíneos.[25] Em outros casos, as células permanecem dormentes no novo sítio até tornarem-se apropriadamente estimuladas,[26] quando a razão proliferação:apoptose e sua habilidade de induzir angiogênese aumentam[27] ou superam a resposta imune local.

O padrão de disseminação e colonização não é aleatório e, dependendo do sítio primário, determinados tumores disseminam-se para um órgão alvo específico mais freqüentemente que para outros. O mecanismo envolvido nesse tropismo não é completamente entendido, mas quimiocinas e seus receptores, assim como padrões de circulação e características estruturais dos capilares no sítio secundário devem ser importantes.[28] Um tropismo diferencial ocorre em câncer de mama, que freqüentemente espalha-se para pulmão, osso, cérebro e fígado, enquanto, em carcinomas de cabeça e pescoço, os linfonodos regionais são sítios preferenciais e metástases distantes são encontradas mais raramente ou tardiamente.[29] Por que os CECPs possuem esse comportamento se carcinomas de células pequenas de cabeça e pescoço[30] e vários tumores de glândulas salivares,[31,32] localizados no mesmo sítio anatômico, freqüentemente desenvolvem metástases distantes? A resposta provavelmente está nas características das células do estroma e do tumor, assim como na rede linfática. Por exemplo, além da estrutura permissiva do sistema linfático, como mencionado anteriormente, a pressão hidrostática nesses vasos é mais baixa que a do sistema sanguíneo, facilitando, conseqüentemente, o processo de disseminação tumoral. Além disso, a linfa é mais rica em fatores de resposta imune que, embora insuficientes

para destruir células tumorais,[33] podem ter um papel importante na seleção de fenótipos de resistência imune.

1.2. Tumores epidermóides de cabeça e pescoço

O CECP é uma doença anatomicamente heterogênea relacionada ao consumo de álcool e tabaco, que surge na mucosa de sítios distintos da cabeça e do pescoço, incluindo cavidade oral, faringe e laringe. Embora esse grupo de carcinomas seja considerado uma única doença, dados moleculares e clínicos sugerem uma superposição de entidades diferentes.[33] De fato, a análise de sua expressão gênica mostra que mesmo subsítios orais possuem perfis moleculares distintos.[34] Divergências no comportamento são igualmente bem conhecidas; por exemplo, os tumores da hipofaringe, supraglote e da base de língua são geralmente agressivos enquanto os de glote apresentam um melhor prognóstico.[33,35] Tais diferenças podem ser explicadas em parte pela influência de fatores do microambiente, incluindo a rede linfática deste sítio anatômico.

As metástases em linfonodos ainda representam um dos fatores de prognósticos mais importantes em CECP e são críticas no delineamento do tratamento.[36] Entretanto, há uma incidência elevada de metástases ocultas mesmo nos pacientes que apresentam linfonodos negativos (N0) e não existem métodos sensíveis para detectá-las,[37] o que torna importante o entendimento dos mecanismos moleculares envolvidos na disseminação linfática. As ferramentas proteômicas permitem uma abordagem poderosa na identificação de novas proteínas ou grupos de proteínas envolvidos nesse processo, especialmente aquelas que apresentam modificações pós-traducionais, como fosforilação, glicosilação e clivagem proteolítica.[38]

1.3. A importância da análise de fluidos corporais em CECP

Diferentemente dos linfonodos, mas potencialmente valiosos no estudo de CECP, fluidos corporais como a saliva e o soro têm sido utilizados no monitoramento e no diagnóstico desse tumor. Tais fluidos fornecem vantagens importantes em comparação a biópsias, incluindo baixa invasividade e custo, fácil coleta e processamento da amostra, assim como redução da ansiedade e do desconforto dos pacientes, especialmente quando um monitoramento por longo período de tempo é necessário.[39,40]

Composta de uma mistura de fluidos orais procedentes de glândulas salivares maiores e menores e do sangue,[41] a saliva é provavelmente o fluido do nosso corpo mais facilmente acessível. Possui um papel importante na manutenção da saúde e da homeostase oral, participando da remineralização do esmalte dos dentes, defesa contra microorganismos, lubrificação, digestão inicial dos alimentos, modulação de pH e no processo de percepção do paladar.[41-44] Estes atributos são derivados de diferentes componentes, que incluem proteínas, hormônios, eletrólitos como o cálcio, bicarbonato, fosfato e fluoreto, e pequenas moléculas como a uréia. As proteínas salivares têm sido estudadas por técnicas bioquímicas tradicionais ou por abordagem proteômica de alto desempenho e mais de mil já foram identificadas na saliva total e em secreções de glândulas individuais, embora aquelas expressas em baixos níveis certamente ainda não foram detectadas[45-69] [http://www.biosino.org/bodyfluid/fluid.jsp?bf=Saliva; http://hspp.dent.ucla.edu/cgi-bin/hspmscgi-bin/search_pro_c.cgi]. As proteínas que compõem a saliva são mucinas, imunoglobulinas, aglutininas, proteínas ricas em prolina, cistatinas, histatinas, defensinas, peroxidases, amilase, albumina, lipase, lisozima, lactoferrina e várias outras. Muitas delas formam complexos e apresentam

modificações pós-traducionais como glicosilação, acetilação, deamidização, sulfatação e fosforilação. Em relação à distribuição em categorias funcionais, as proteínas salivares atuam principalmente em resposta imune, citoesqueleto, processo metabólico, comunicação e proliferação celular, bem como em processos relacionados com doenças neurodegenerativas, diabetes e câncer[59].

O interesse crescente na saliva como um fluido diagnóstico tem conduzido à padronização de processos de coleta e estocagem, principalmente porque diversos fatores podem afetar seu fluxo e a composição. Diferentes métodos de coleta do fluido total estimulado ou não, da secreção de uma única glândula salivar, do fluido crevicular e de transudato da mucosa são disponíveis, cada um deles projetado para objetivos distintivos.[70] Além da contribuição das glândulas salivares e do sangue, assim como da estimulação e do bloqueio da salivação, outros fatores como ritmo circadiano, status fisiológico, medicação, ingestão de alimentos, gênero e idade afetam o fluxo e a composição da saliva.[69,71] Essas características têm sido exploradas no monitoramento de hormônios[72] e níveis de droga,[73] exposição a poluentes ambientais[74] e infecções.[75] A análise do fluxo e dos constituintes da saliva também possui uso potencial como ferramenta valiosa para monitorar diversas condições patológicas, dado que mudanças substanciais em ambos os parâmetros têm sido associadas com doenças sistêmicas e locais, incluindo periodontite,[76] diabete mellitus,[77] fibrose cística,[78] artrite reumatóide, síndrome de Sjögren,[79] doenças de glândulas salivares,[80] câncer de mama,[81,82] ovário[83] e oral.[84-87]

No que diz respeito ao câncer oral, os primeiros relatos na utilização na saliva para seu rastreamento e diagnóstico são relativamente recentes.[88,89] A proximidade dos tumores da cabeça e pescoço oferece à saliva uma vantagem importante em relação a

outros fluidos e tecidos, além da característica não invasiva e da compatibilidade com abordagens proteômicas. Realmente, a proteômica da saliva pode ser explorada para detecção precoce de tumores, predição de agressividade e prognóstico.[68] Uma comparação entre amostras de indivíduos saudáveis e pacientes pode revelar níveis únicos ou elevados de proteínas específicas, assim como a presença de isoformas derivadas de modificações pós-traducionais.[55]

Da mesma forma que a saliva, o soro e o plasma também são uma fonte muito importante de marcadores biológicos e, embora com composições diferentes, ambos podem fornecer informações ricas sobre processos fisiológicos e patológicos. Entretanto, diferentemente da saliva, a análise do soro e do plasma em diagnóstico é amplamente conhecida.[90-92] Esses fluidos têm origem extra e intracelular e refletem metabolismo ou morte celular. A maioria das proteínas é sintetizada pelo fígado, endotélio, sistema nervoso central e células sanguíneas e suas principais funções incluem balanço hídrico e salino, transporte e defesa, como a vasopressina, a transferrina e as imunoglobulinas.[39] Em função dos níveis protéicos no soro e no plasma exibirem variações intra e inter-individuais e também variarem no tempo e no espaço, a padronização no processo de coleta e estocagem é um ponto crítico para resultados consistentes.[40]

Recentemente, as abordagens proteômicas de alto desempenho têm sido utilizadas para identificar biomarcadores sensíveis e específicos no soro e no plasma.[93,94] Um grande número de proteínas diferentes tem sido identificado por estudos colaborativos,[95] embora aproximadamente 20 das mais abundantes no plasma correspondam a 99% da massa total de proteína. Entre elas, estão apolipoproteínas, transtirretina (prealbumina) e a proteína de ligação a retinol.[96]

O perfil protéico sérico de muitas doenças tem sido estudado, como em diabetes,[97] doenças auto-imunes[98] e cardíacas[99] e infecciosas.[100] Dados promissores têm sido relatados para uma variedade de cânceres, entre eles os de cólon;[101,102] ovário;[103] mama;[104] cabeça e pescoço[105-107] e outros[108].

1.4. Objetivos

O presente trabalho teve como objetivo geral a pesquisa de marcadores de diagnóstico, prognóstico e classificação do câncer de cabeça e pescoço. Os seus objetivos específicos compreenderam:

1. Implantar a metodologia de eletroforese bidimensional no Laboratório de Marcadores Moleculares e Bioinformática Médica, FAMERP, e difundir os conhecimentos adquiridos na fase de implantação da técnica;
2. Investigar o perfil protéico de metástases em linfonodos regionais procedentes de pacientes com carcinoma espidermóide de cabeça e pescoço e sua relação com diferentes parâmetros clínicos e laboratoriais, incluindo sítio anatômico, tamanho do tumor e evolução;
3. Investigar o perfil protéico de saliva e soro de pacientes com carcinoma epidermóide de cabeça e pescoço antes e após a radioterapia;
4. Validar, por técnicas de *Western blot*, marcadores potenciais do processo de metastatização do carcinoma epidermóide de cabeça e pescoço;
5. Investigar, por técnicas de proteômica, o papel do estroma na tumorigênese de cabeça e pescoço.

ARTIGOS CIENTÍFICOS

2. ARTIGOS CIENTÍFICOS

Os resultados estão apresentados em forma de artigos científicos. No total foram apresentados cinco artigos, dois deles já publicados, um artigo submetido para avaliação pela revista BMC Medical Genomics e dois outros em fase de submissão.

Artigo I

Título: Solubilization of Proteins from Human Lymph Node Tissue and Two-Dimensional Gel Storage.

Autores: Alessandra Bernadete Trovó de Marqui, Alessandra Vidotto, Giovana Mussi Polachini, Cláudia de Mattos Bellato, Hamilton Cabral, Andréia Machado Leopoldino, José Francisco de Góis Filho, Érica Erina Fukuyama, Flávio Aurélio Parente Settanni, Patrícia Maluf Cury, Gustavo Orlando Bonilla-Rodriguez, Mario Sergio Palma and Eloiza Helena Tajara.

Periódico: Journal of Biochemistry and Molecular Biology - JBMB.

Artigo II

Título: Purification, Biochemical and Functional Characterization of Miliin, a New Thiol-Dependent Serine Protease Isolated from the Latex of *Euphorbia milii*.

Autores: L.P. Moro, M.T. Murakami, H. Cabral, A. Vidotto, E.H. Tajara, R.K. Arni, L. Juliano and G.O. Bonilla-Rodriguez.

Periódico: Protein & Peptide Letters.

Artigo III

Título: Genomics and Proteomics Approaches to the Study of Cancer-Stroma Interactions.

Autores: Flávia Cristina Rodrigues-Lisoni, Paulo Peitl Jr, Alessandra Vidotto, Giovana M. Polachini, José V. Maniglia, Juliana Carmona-Raphe, Caique Fernandes de Souza, Rodrigo Antonio Parra Teixeira, Erica Erina Fukuyama, Pedro Michaluart Jr, Marcos Brasilino de Carvalho, Sonia Maria Oliani, Head and Neck Genome Project GENCAPO, Eloiza H. Tajara.

Periódico: BMC Medical Genomics (submetido).

Artigo IV

Título: Protein Profile in Head and Neck Squamous Cell Carcinomas by Tandem Mass Spectrometry Analysis: Evaluating the Invasive Phenotype.

Autores: Alessandra Vidotto, Andréia Machado Leopoldino, Patricia Maluf Cury, Giovana Mussi Polachini, Marcos Brasilino de Carvalho, Head and Neck Genome Project GENCAPO, Eloiza H. Tajara.

Periódico: Proteomics (a ser submetido)

Artigo V

Título: Salivary and serum proteomics in head and neck carcinomas – before and after treatment.

Autores: Alessandra Vidotto, José Victor Maniglia, Eloiza H. Tajara.

Periódico: Oral oncology (a ser submetido)

ARTIGO CIENTÍFICO I

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JOURNAL OF Biochemistry and Molecular Biology

Short communication

Solubilization of Proteins from Human Lymph Node Tissue and Two-Dimensional Gel Storage

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In the present study, we compared six different solubilization buffers and optimized two-dimensional electrophoresis (2-DE) conditions for human lymph node proteins. In addition, we developed a simple protocol for 2-D gel storage. Efficient solubilization was obtained with lysis buffers containing (a) 8 M urea, 4% CHAPS (3-|(3-cholamidopropyl) dimethylammonio|-1-propanesulfonate), 40 mM Tris base, 65 mM DTT(dithiothreitol) and 0.2% carrier ampholytes; (b) 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10 (N-decyl-N, N-dimethyl-3-ammonio-1-propanesulfonate), 40 mM Tris base, 65 mM DTT and 0.2% carrier ampholytes or (c) 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT and 0.2% carrier ampholytes. The optimal protocol for isoelectric focusing (IEF) was accumulated voltage of 16,500 Vh and 0.6% DTT in the rehydration solution. In the experiments conducted for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), best results were obtained with a doubled concentration (50 mM Tris, 384 mM glycine, 0.2% SDS) of the SDS electrophoresis buffer in the cathodic reservoir as compared to the concentration in the anodic reservoir (25 mM Tris, 192 mM glycine, 0.1% SDS). Among the five protocols tested for gel storing, success was attained when the gels were stored in plastic bags with 50% glycerol. This is the first report describing the successful solubilization and 2D-electrophoresis of proteins from human lymph node tissue and a 2-D gel storage protocol for easy gel handling before mass spectrometry (MS) analysis.

Keywords: Gel storage, Human lymph node tissue, Protein solubilization, Two-dimensional gel electrophoresis

Introduction

The analytical potential of 2-DE is dependent on good sample preparation, in order to obtain reproducibility, good resolution and a great spot number of proteomic maps. In many cases, the proteins of the sample need to be solubilized, disaggregated, denatured and reduced (Shaw and Riederer, 2003). For this purpose, mixtures of chaotropic compounds, detergents or surfactants, reducing agents and carrier ampholytes are employed (Molloy, 2000; Garfin, 2003).

The role of chaotropes, such as urea and thiourea, is to disrupt hydrogen bonding, leading to protein unfolding and denaturation. Surfactants such as CHAPS, SB 3-10, ASB-14 (amidosulfobetaine-14) and SDS act synergistically with chaotropes. Reducing agents, such as DTT and TBP (tributyl phosphine), are used to break intramolecular and intermolecular

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disulfide bonds. Carrier ampholytes enhance protein solubility by minimizing protein aggregation due to charge-charge interactions (Herbert, 1999; Molloy, 2000; Berkelman and Stenstedt, 2002; Shaw and Riederer, 2003).

Due to variable protein expression from one tissue to another, conditions of protein solubilization that are optimal for one particular tissue type may not hold for others. In addition, several 2-DE steps must be optimized for each tissue in order to obtain good results. In the present study, a comparison was made of six previously described solubilization methods for obtaining proteomic maps of human lymph node tissue. We also describe a simple 2-D gel storage protocol for easy gel handling prior to MS analysis.

Materials and Methods

Thirty-one lymph nodes were collected from head and neck squamous cell carcinoma patients at the Cancer Hospital "Arnaldo Vieira de Carvalho", São Paulo, Brazil. Tissue samples were obtained immediately after the removal of the surgical specimen, snap-frozen and stored in liquid nitrogen. The Ethics Committee approved the research, and written informed consent was obtained from all patients.

One lymph node sample was cut into six pieces of about 5 mm^3. 500 µL of one out of six different lysis buffers were added to each piece (Table 1). The specimens were disrupted by sonication 12 times at intervals of 10 s at 10°C and vortexed for 2 min. The lysates were centrifuged at 10,000 rpm for 3 min at 4°C. The supernatants were transferred to other tubes, the insoluble pellets were washed with 200 µL lysis buffers and the second supernatants were collected. The protein concentration of the supernatants was determined by the Bradford method (Bradford, 1976). The protein samples were stored at –70°C.

2-DE was performed using IPGphor and SE 600 Ruby (GE Healthcare). For IEF, 500 µg protein were diluted with rehydration solution (8 M urea, 2% CHAPS, 0.6% DTT, 0.5% IPG buffer, bromophenol blue trace) to a total volume of 250 µL. IPG strips (pH 3-10 L, 13 cm) were rehydrated in this solution for 12 h under mineral oil. IEF was performed at 20°C, with the following parameters: 500 V (1 h), 1,000 V (1 h), 8,000 V (2 h or 3 : 30 h or 5 h), 500 V (0 h or 1 h), until 16,500 Vh, 26,500 Vh or 41,500 Vh were attained. The current was limited to 50 µA/strip. After IEF, the IPG strip was stored at –70°C until analysis by SDS-PAGE.

The individual strips were incubated, at room temperature, in the equilibrium solution A (2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, bromophenol blue trace and 1% DTT), followed by solution B (solution A except that DTT was replaced by 2.5% iodoacetamide), for 15 min each. When the proteins were solubilized using solutions containing TBP, the strips were only incubated in solution C (solution A except that DTT was replaced by 5 mM TBP) for 30 min. The IPG strips were washed in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), placed on top of 12.5% SDS-PAGE and sealed in place with sealing solution (0.5% low-melting agarose in SDS electrophoresis buffer). The electrophoresis conditions were 15 mA/gel for 30 min, followed by 30 mA/gel for 5 h at room temperature.

Proteins were detected by Coomassie Blue staining. Briefly, gels were incubated overnight in fixing solution (50% ethanol, 10% acetic acid), followed by a destaining solution (50% ethanol, 5% acetic acid) for 3 min, and incubated for 90 min with 0.05% Coomassie Brilliant Blue R-250 solution (0.125 g Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid). Subsequently, the gels were washed four times with destaining solution, for 15, 45, 120 and 120 min, respectively, and incubated in preserving solution (5% acetic acid) for approximately 72 h.

For gel storing, five protocols were tested.

- Protocol 1. The gel was washed twice in solution D (30% ethanol) for 30 min, followed by solution E (30% ethanol, 3.5% glycerol) for 60 min.
- Protocol 2. The gel was incubated in 4.3% glycerol for 5 min.
- Protocol 3. The gel was washed four times in water for 4 h.
- Protocol 4. The gel was incubated in 8.7% glycerol for 60 min.

Table 1. Lysis buffer composition. Composition of six lysis buffers tested for protein solubilization efficiency

	Buffer 1	Buffer 2	Buffer 3	Buffer 4	Buffer 5	Buffer 6
Chaotropes	8 M Urea	5 M Urea 2 M Thiourea	5 M Urea 2 M Thiourea	7 M Urea 2 M Thiourea	5 M Urea 2 M Thiourea	7 M Urea 2 M Thiourea
Detergents	4% CHAPS	2% CHAPS 2% SB 3-10	2% CHAPS 2% SB 3-10	4% CHAPS	2% CHAPS 1% SB 3-10 1% ASB -14	2% CHAPS 0.5% ASB -14
Salts	40 mM Tris base	40 mM Tris base	40 mM Tris base			
Reducing agents	65 mM DTT	65 mM DTT	2 mM TBP	65 mM DTT	2 mM TBP	65 mM DTT
Carrier ampholytes	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)	0.2% (pH 3-10)
References	(Herbert, 1999; Molloy *et al.*, 1998)	(Rabilloud *et al.*, 1997)	(Rabilloud *et al.*, 1997; Molloy *et al.*, 1998; Tachibana *et al.*, 2003)	(Molloy, 2000; Berkelman and Stenstedt, 2002; Rabilloud *et al.*, 1997; Görg *et al.*, 2000)	(Garfin, 2003)	(Castellanos-Serra and Paz-Lago, 2002)

• Protocol 5. The gel was incubated in 10% methanol for 48 h. The gels was placed between two cellophane sheets PT (Coopercel) previously embedded in solution E (protocol 1), in water (protocol 2), in 8.7% glycerol (protocol 3 and 4) or stored in a clear plastic bag with 2 ml of 50% glycerol solution applied over both surfaces of the gel. The bag was sealed.

No gel drying equipment was used. Stained and stored gels were scanned with an ImageScanner (GE-Healthcare), and spot detection was manually performed with the Melanie 3.0 software (GeneBio).

One protein spot from 2-DE gel was selected, excised, digested with trypsin and submitted to MALDI-TOF-TOF (Matrix Assisted Laser Desorption - Time of Flight - Time of Flight) 4700 Proteomics Analyser (Applied Biosystems) operated in positive ion reflectron mode to identify the peptides. Such spot was manually cut out from the gel in a clean-air cabinet, to prevent contamination. The protein spot was placed into 0.5 mL tube previously washed with 50% methanol and deionized water. The gel pieces were destained in 250 µL of 50% acetonitrile (ACN)/50 mM ammonium bicarbonate under constant agitation to complete colourlessness. The gel pieces were then dehydrated with 200 µL of ACN for 15 min; acetonitrile was discarded and the gel pieces dried in Speed Vac for 30 min. For rehydration and digestion, each gel piece was rehydrated with 20 µL of a trypsin solution (0.4 µg modified trypsin in 50 mM acetic acid and 50 mM ammonium bicarbonate). After 30 min incubation at room temperature, a volume of 50 µL of 50 mM ammonium bicarbonate or the sufficient amount to cover the gel pieces was added, and the sample was incubated for 24 h at 37°C in a water bath, for enzymatic cleavage. Peptides were extracted with 50 µL 1% trifluoroacetic acid/TFA (first extraction: overnight) and 50 µL 1% TFA/50% ACN (second extraction: 2 h). The resulting supernatants were mixed and concentrated in a vacuum centrifuge to 10 µL.

About 1 µL of this solution was eluted in 1 µL matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid, 0.1% TFA in 50% ACN). Then, 0.5 µL of the mixture was spotted on a sample plate and introduced into the mass spectrometer after drying. The instrument was calibrated externally using 4700 standard kit (Applied Biosystems).

Proteins were identified by MASCOT MS/MS Ions Search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH = MIS). The search parameters were set up as follows: MSDB (Mass Spectrometry Protein Sequence Database); taxonomy *Homo sapiens*; 1 missed cleavage; carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modification, respectively; peptide mass and MS/MS tolerance of 1 and 0.8 Da, respectively; the peptide ion MH^+ and monoisotopic masses.

In the present study, all chemicals used were of highest quality (Merck, Calbiochem, GE Healthcare, Sigma and Bio-Rad).

Results and Discussion

Initial extraction and solubilization is a key factor for proteomic analysis. In the present study, we tested six buffers for solubilization of protein from human lymph node samples, which differed in one or more components, including chaotropes, detergents and reducing agents (Table 1).

Extraction buffer 1 is a standard solution for protein solubilization. About 285 spots were visualized in the gel using this solution (Fig. 1A). Buffers 2 and 3 have similar compositions, except for the reducing agent, and about 281 and 113 spots were visualized in the gels, respectively (Fig. 1B, C). In buffer 4, Tris base was not used for protein solubilization. The results showed 283 spots (Fig. 1D). In lysis buffers 5 and 6, ASB-14 was used as detergent and the gels exhibited poor resolution and streaking (Figs. 1E, F). In conclusion, buffers 1, 2 and 4 were the best solutions for protein solubilization of human lymph node tissue with regard to gel quality. Also, these solutions resulted in high protein concentration (9.8 µg/µL, 8.0 µg/µL and 8.9 µg/µL, respectively), higher than buffer 3 (6.6 µg/µL), but much higher than buffers 5 and 6 (0.3 µg/µL and 1.2 µg/µL). Sharp differences are seen comparing Fig. 1C with 1A, 1B and 1D and reflect the results of the Bradford assay. Although this assay is sensitive to various components of lysis buffers, the reagents of buffers 1-6 probably had no effect on protein quantification, and the gel quality showed be consequence of the buffer efficiency. Thirty other human lymph node samples were also submitted to buffer 4 and all gels showed excellent quality.

In the buffers tested, urea varied from 5 to 8 M, CHAPS from 2 to 4%, SB 3-10 from 1 to 2%, and ASB-14 from 0.5 to 1%. The carrier ampholytes were used at a low concentration (0.2%), in order to avoid extended running times, because they contribute to the initial conductivity of the sample solution (Garfin, 2003). Tris base was added to three buffers (1, 2 and 3). This compound is used when basic conditions are required for full solubilization or to minimize proteolysis (Rabilloud, 1996). However, addition of ionic compounds in buffers for protein solubilization can result in first-dimension disturbances. Therefore, salts must be removed after the solubilization step or maintained at as low a concentration (lower than 10 mM) in the rehydration solution and IEF (Berkelman and Stenstedt, 2002; Shaw and Riederer, 2003). In our experiments, Tris base was added to the buffers at 40 mM, but the final salt concentration during rehydration was maintained at approximately 10 mM.

Thiourea was introduced in combination with urea, to increase the solubility of proteins, mainly of membrane proteins. The use of this component inhibits the adsorption of protein to the gel matrix, when IEF is conducted in IPG. This efficient chaotrope is poorly soluble in water and requires high concentrations of urea for solubility (optimal condition is 2 M thiourea in 5-7 M urea). This reagent improved the solubility of proteins as compared to urea alone, as already stated by other authors (Rabilloud *et al.*, 1997; Pasquali *et al.*, 1997; Musante *et al.*, 1998; Rabilloud, 1998; Giavalisco *et al.*, 2003; Méchin *et al.*, 2003; Taylor and Pfeiffer, 2003).

We also examined three zwitterionic detergents (CHAPS, SB 3-10, ASB-14). The sulfobetaine CHAPS is the most commonly used detergent for 2-DE, from 2% to 4% in high concentrations of urea. Sulfobetaines with long linear tails (i.e., SB 3-10, ASB-14) have been shown to possess a greater

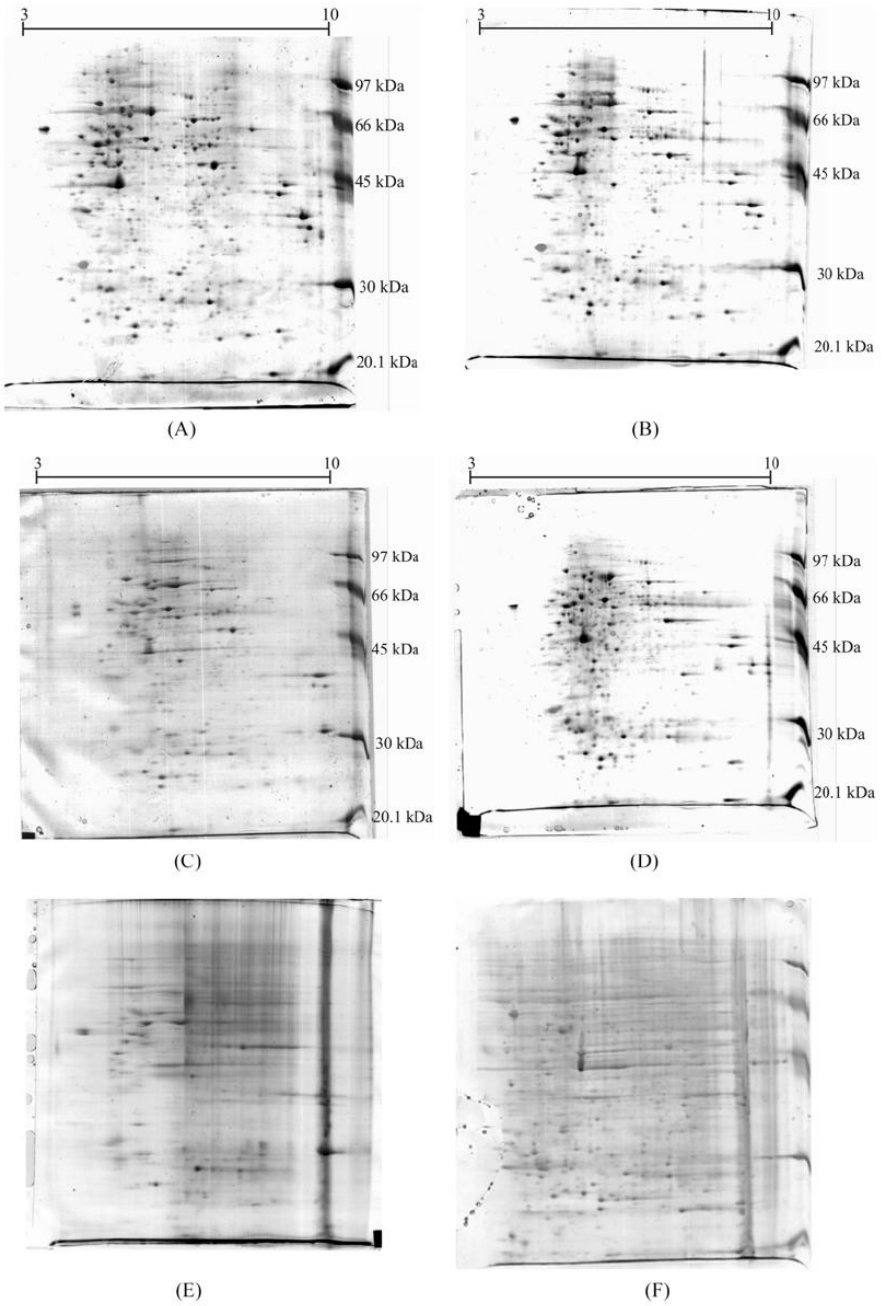

Fig. 1. Comparison of solubilization conditions. Lysis and solubilization from human lymph node tissue proteins were performed using six different buffers (1-6): (A) Buffer 1 (285 spots); (B) Buffer 2 (281 spots); (C) Buffer 3 (113 spots); (D) Buffer 4 (283 spots); (E) Buffer 5 and (F) Buffer 6. Composition of buffers as in Table 1. Proteins were separated on a 13 cm pH 3-10 IPG, 12.5% SDS-PAGE and stained with Coomassie Blue.

ability to solubilize membrane proteins. However, SB 3-10 has poor solubility in high concentrations of urea (Herbert, 1999; Görg and Weiss, 1999). In contrast, ASB-14 is compatible with 9 M urea, but results in large horizontal streaking towards the basic end of the strip. This streaking appears to be an interaction between ASB-14 and the cathode rather than purely an issue with basic proteins, as the effect was independent of the pH range of the IPG strip (pH 3-10 or pH 4-7) (Stanley *et al.*, 2003). Our gels also showed increased streaking in all pH ranges when ASB-14 was used in the extraction solution (Fig. 1E, 1F). Studies using three different detergents (CHAPS, ASB-14 or NP-40) in the solubilization solution also showed more streaking in the second dimension with ASB-14 than with CHAPS (Carboni *et al.*, 2002). The ionic detergent SDS is very effective for protein solubilization, however it is incompatible with IEF (Zuobi-Hasona *et al.*, 2005). For this reason, it was not selected for our optimization experiments.

As for reducing agents, TBP and DTT are commonly used in extraction solutions. When the proteins are solubilized using reagents containing free thiol such as DTT, IPG equilibration requires two steps: reduction (by DTT) and alkylation (by iodoacetamide). DTT has been the standard reducing agent for 2-DE for many years (Molloy, 2000; Görg *et al.*, 2000) and is effective for reducing protein disulfide bonds prior to SDS-PAGE, while iodoacetamide eliminates artifacts of disulfide formation during electrophoresis, for less streaking and better resolution. However, DTT is charged, especially at alkaline pH, and thus migrates out of the pH gradient during IEF, which results in loss of solubility for some proteins. In contrast, TBP lacks a free thiol group, making the second equilibration of the IPG strips unnecessary. In addition, TBP is neutral and does not migrate during IEF, thus, the reducing conditions are maintained over the entire focusing process. On the other hand, TBP has a low solubility, is unstable, volatile and toxic (Rabilloud, 1996; Herbert *et al.*, 1998; Molloy *et al.*, 1998; Berkelman and Stenstedt, 2002). In the present study, the buffers 2 and 3, used for protein solubilization, had similar compositions, except for the reducing agent (DTT in buffer 2 and TBP in buffer 3). Nevertheless, the numbers of spots visualized were very different (281 vs 113 spots), probably due to the reducing agent. Therefore, DTT showed significant improvements in the resolution of proteins by 2-DE. Similar results were reported for myelin proteins (Taylor and Pfeiffer, 2003).

The solubilization of proteins was performed in the absence of protease inhibitors, but using 2 M thiourea, and the sample was processed at 4 to 10°C. According to literature, proteolysis can be inhibited by preparing the sample at such a low temperature, in the presence of Tris base, 2 M thiourea and in strong denaturants such as 8 M urea (Rabilloud, 1996; Carboni *et al.*, 2002; Castellanos-Serra and Paz-Lago, 2002; Berkelman and Stenstedt, 2002).

IEF and SDS-PAGE were also developed using duplicate extracts from buffers 1, 2 and 4. Extracts from other buffers

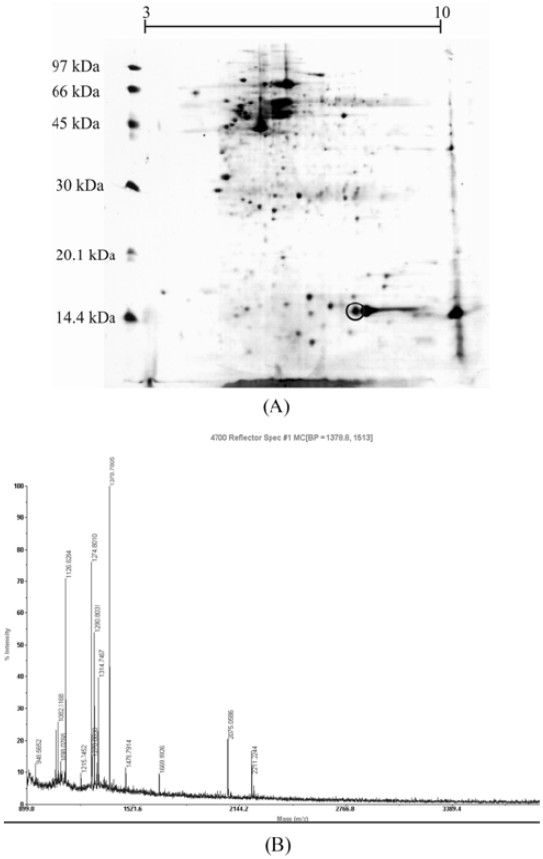

Fig. 2. A one-year-old archived SDS-PAGE and mass spectrum of tryptic peptides derived from hemoglobin. (A) A one-year-old archived SDS-PAGE of a sample from a patient with head and neck squamous cell carcinoma. Highlighted in circle is the spot excised from the gel. (B) Mass spectrum corresponding to a spot with apparent pI and MW of 7.1 and 14.5 kDa, respectively.

were sufficient to perform only one experiment.

In the lysis buffer tests, IEF was carried out with total focusing for 16,500 Vh. After lysis buffer optimization, other IEF conditions were tested in 30 samples (16,500 to 41,500 Vh). The focusing settings are known to be critical for protein separation. In particular, total voltage and slow sample entry have a pronounced effect on the spot pattern quality (Görg *et al.*, 2000). The most remarkable results were the decreasing in horizontal streaking and the well-rounded spots in 16,500 Vh focused gels and by using double concentration of DTT (0.6%) in the rehydration solution (Hoving *et al.*, 2002). DTT is negatively charged at alkaline pH, and migrates towards the anode, causing depletion of DTT at the cathode. In this region the formation of new disulfide bridges could occur, due to oxidation of sulphydryl groups.

Experiments conducted in 30 samples for the SDS-PAGE

step tested the SDS electrophoresis buffer at a doubled concentration (50 mM Tris-base, 384 mM glycine, 0.2% SDS) in the cathodic reservoir as compared to the anodic reservoir. This condition resulted in decreased protein smearing in the gel, which is usually caused by buffer depletion during electrophoresis (data not shown). The most important component of the typical second-dimension buffer system must be SDS, which binds protein at a relatively constant ratio, thereby allowing for the size-based separation imparted by the sieving matrix. It was observed that buffer depletion during electrophoresis causes dissociation of the SDS from the protein and, consequently, elongated protein patterns or smearing (Werner, 2003).

Five protocols for gel storing were tested and success was attained when the gels were stored in plastic bags with 50% glycerol solution (Protocol 5). This procedure permitted the easy handling of the gel, without the risk of breakage and without harming the image and MS analysis. The gels were submitted to spot excision and MS analysis up to one year after SDS-PAGE. Figure 2A shows a one-year-old archived SDS-PAGE of a sample from a patient with head and neck squamous cell carcinoma. The MS spectrum corresponding to a spot with apparent pI and MW of 7.1 and 14.5 kDa, respectively, is presented in Figure 2B. This protein spot was identified as hemoglobin. The remaining protocols resulted in cracking gels. Preservation of gels for a long time after electrophoresis is frequently desirable, mainly if their transport to another laboratory is necessary. Several methods for drying polyacrylamide gels have been described. These protocols have been routinely applied in thin gels (0.8 mm). However, cracking gels were observed when these methods were performed in thick 2-D gels (1.5 mm).

Progress in sample preparation methodology for 2-DE has focused on improvements in sample buffer constituents to achieve better representation of the proteome. However, it must be taken into account that a variety of factors influence the sample preparation steps, some of which have been reported in this manuscript. We highlighted that this is the first report describing the successful solubilization and 2-DE of protein from human lymph node tissue and a simple protocol for long time preservation of 2D gels.

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ARTIGO CIENTÍFICO II

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Purification, Biochemical and Functional Characterization of Miliin, a New Thiol-Dependent Serine Protease Isolated from the Latex of *Euphorbia milii*

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Abstract: Miliin, a new thiol-dependent serine protease purified from the latex of *Euphorbia milii* possesses a molecular weight of 79 kDa, an isoelectric point of 4.3 and is optimally active at 60 ºC in the pH range of and 7.5-11.0. Activity tests indicate that milliin is a thiol-dependent serine protease.

Keywords: Medicinal plant, Latex, *Euphorbia milii*, Serine protease, Purification, Characterization.

INTRODUCTION

Proteolytic enzymes that hydrolyze the peptide linkages are ubiquitous in prokaryotes and eukaryotes and have found extensive use in various aspects of food processing such as meat tenderization, cheese processing, baking and as important additives in the production of detergents [1,2]. Proteases have also been used as model systems to improve our understanding of structure-function relationships, and protein folding pathways [3-6].

Plant proteases play crucial roles in a wide range of intracellular and extracellular process such as fruit development and ripening [7], serve as nutritional reserves, participate in storage protein degradation in germinating seeds [8, 9], activation and degradation of proteins [10]. Furthermore, proteases encountered in latex are also involved in protecting the plant against predator attacks [11, 12].

Due to their potential applications, proteases from plant latex, fruits and seeds [11, 13], representing several families such as Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae, and Euphorbiaceae [11] have been isolated and characterized.

Euphorbia milii, commonly known as "Crow-of-Thorns" (referred to as "Coroa-de-Cristo" in Brazil), a cosmopolitan flowering plant that belongs to the Euphorbiceae plant family originally from Africa, is primarily used in gardens and as living fences as well as an indoor ornamental plant [14]. Recently, medicinally important applications have been reported for the latex of *E. milii*, which is a potent plant molluscicide and a promising alternative to niclosamide (NCL). Its latex is mostly used as a molluscicidal compound [15, 16], but in China its stem; roots and latex form part of the herbal medicine repertoire used for the treatment of hepatitis and abdominal edema [17]. In Brazil, the milky sap is extensively used as an active ingredient in cosmetic products for skin peeling [18].

We present here the purification, biochemical properties, and a mechanistic classification of a new protease from the latex of *Euphorbia milii*, named miliin.

MATERIALS AND METHODS

Materials

Sephacryl S-100 HR was purchased from Pharmacia. Bovine serum albumin (BSA), azocasein, DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid), CAPS, 3-(cyclohexyl-amino)-1-propanesulfonic Acid, TAPS, *N*-tris(hydroxy-methyl) methyl-3-aminopropanesulfonic acid, DTT (dithiothretiol), β-mercaptoetanol, HEPES, iodoacetic acid (IAA), urea, EDTA (ethylene diamine tetraacetic acid), 2,2 dithio-pyridine, $HgCl_2$ (mercuric chloride), pCMB (*p*-chloro-mercuric benzoate), PMSF (phenylmethansulfonyl fluoride), benzamidine, acrylamide, *N,N*-methylene bisacrylamide, Coomassie brilliant blue R-250, standard proteins were obtained from Sigma Chemical Co. (United States). Coomassie brilliant blue G250 was from Eastman Kodak. Trichloroacetic acid was obtained from Applied Biosystems. All other chemicals used were of analytical grade.

Purification

Several small incisions (1 cm) were made in the young stems of *E. milii* and the fresh latex was collected and suspended in 0.03 M sodium acetate buffer, pH 4.5, containing 0.01 M $NaHSO_3$. This latex suspension was stored in the dark at 4 °C and was submitted to acetone precipitation (1:4

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mixture of acetone and latex suspension) in order to remove gum and other undesired chemical compounds and maintained overnight at 0 °C. The solution was centrifuged at 20,000 xg for 10 min in a *Jouan* CR3i centrifuge and the pellet was re-dissolved in a 0.03 M Na-acetate pH 4.5 buffer maintained at 4 °C. The clear supernatant was injected onto a Resource 15S cation-exchange column (3 x 15 cm) previously equilibrated with 0.03 M Na-acetate pH 4.5 at a flow rate of 3 mL/min using a Pharmacia FPLC system. A non-linear NaCl gradient from 0 to 2.0 M (total elution volume 150 mL) was used to elute the bound proteins (Fig. **1A**). Fractions of 4 mL were collected and tested for the presence of proteolytic activity and samples from peak "A" were pooled, concentrated using Amicon micro-concentrators with a molecular cutoff of 50 kDa and stored at 4 °C. This fraction was then applied to a Sephacryl S-100 HR column (1.8 x 200 cm) pre-equilibrated with 0.03 M Na-acetate, 0.2 M NaCl buffer, pH 4.5. Fractions (Fig. **1B**) obtained were pooled, dialyzed against a 0.03 M Na-acetate buffer, pH 4.5 and tested for proteolytic activity.

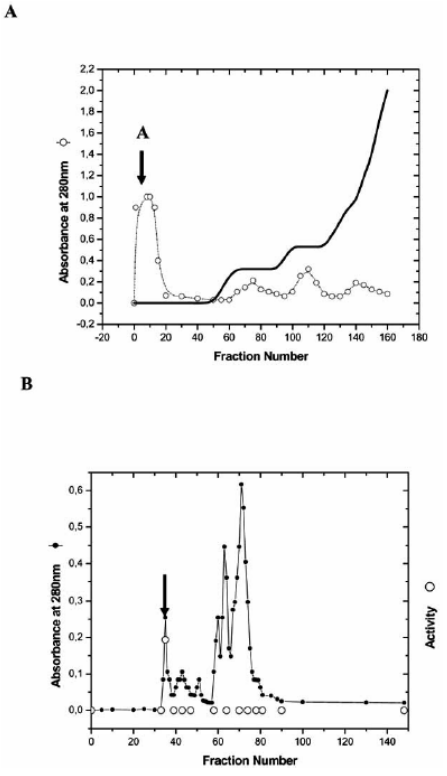

Figure 1. Chromatography of latex from E. milii. (A) Cationic-exchange on Source 15S (●) non linear NaCl gradient which ranged from 0 to 2.0 M eluted with 0.05 M acetate buffer, pH 4.5 at flow rate of 3 mL/min. (B) Gel filtration of Sephacryl S-100 was equilibrated with 0.03 M Na-acetate, 0.2M NaCl buffer, pH 4.5. Fractions were collected at a flow rate of 0.25 mL/min and assayed for protein content (A_{280}) (●) and proteolytic activity using casein (○). The arrow shows the position of the peak displaying proteolytic activity.

Protein Concentration

Protein concentrations were measured by two methods: (a) by measuring absorption at 280 nm utilizing a *Varian* Cary 100 spectrophotometer and (b) by the Bradford [19] assay where BSA (Sigma) was used as a standard to generate the calibration curve.

Assay for Proteolytic Activity

The proteolytic activity of the enzyme was monitored using (1%, w/v) either casein or azocasein as a substrate as previously described [20, 21], but without the inclusion of β–mercaptoethanol in the assay mixture.

Effect of pH and Temperature

The effect of pH on the activity of the purified enzyme was investigated using the substrates casein and azocasein in 0.05 M buffers of Na-acetate (pH 5.0-5.5); ADA (pH 6.0-7.0); HEPES (pH 7.5); Taps (pH 8.0-8.5); Glycine (pH 9.0-9.5); Caps (pH 10.0-10.5-11.0) and sodium carbonate (pH 11.5-12.0). 50 µL and 100 µL of the miliin samples were equilibrated in 100 µL of the buffers at the desired pH at 37 °C for 10 min and the substrate solution previously prepared in the same buffer was added. Similarly, the temperature optimum was determined by incubating the protein samples in the buffer representing the pH optimum for each substrate in the temperature range of 25 and 85 °C in steps of 10 °C and activity was determined at these temperatures.

Electrophoresis

Assessment of homogeneity of the enzyme, at different stages of purification as well as the molecular mass determination of the purified enzyme, was carried out using 12.5% SDS-PAGE following the method of Laemmli [22] and stained using silver nitrate. After obtaining the pure enzyme, two samples of the purified protease, were applied under non-reducing and reducing conditions, respectively. Under reducing conditions, the protein sample was boiled in a sample buffer containing 0.05 M β-mercaptoethanol and SDS whereas both β-mercaptoethanol and SDS were absent under the non-reducing condition. Globular proteins used as M_r standards were: phosphorylase b (97 kDa), bovine albumin (66 kDa), ovoalbumin (45 kDa), bovine carbonic anhydrase (30 kDa), bovine pancreas trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.4 kDa). A plot of the logarithm of the molecular mass of each standard vs. mobility was generated to estimate the molecular mass of the purified enzyme.

Zymogram

Zymograms were prepared based on the protocol of García-Carreño *et al.* [23], with modifications. After running SDS-PAGE under non-reducing conditions or PAGE under reducing conditions, the gels were immersed in 0.1M Tris-HCl buffer, pH 8.0 for 5 min at 4 °C (Solution A). Subsequently the gels were immersed in a 2.5 % Triton X-100 solution for 30 min at 4 °C (Solution B), following which the first immersion step was repeated. Next, the gel was immersed in 3 % casein prepared in solution A for 30 min. at 4 °C in order to allow the substrate to diffuse into the gel. The

temperature was then raised to 25 °C and the gels were incubated for an additional 60 min for digestion by the active fractions. Finally, the gel was washed with solution A and stained with 0.1 % Coomassie brilliant blue R-250 and washed with a 40 % ethanol and 10 % acetic acid solution to destain and improve the contrast.

pH and thermal stability. To determine the effects of pH (5.0-12.0) and temperature (25-85°C), on the proteolytic activity, the enzyme was incubated in the presence of casein under the above-specified conditions of pH for 1, 4 and 24 h and for the temperature experiments, incubation was performed for 2 h and analyzed as described previously. Acetone and ethanol (67%), cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), triton X-100 (0.25M) and urea (5.5 M) were used to determine the effect of chemical denaturants and organic solvents and assayed using azocasein. The assay was repeated after 24 h for samples under the same experimental conditions, in order to evaluate the effects on miliin stability.

Mechanistic Classification

The effect of various compounds, including divalent ions and thiol reagents, was analyzed following the approach proposed by Dunn [24]. The enzyme was incubated for 15 min with these chemicals at concentrations of 10 mM for divalent ions and 2 mM for β-mercaptoethanol, DTT and cysteine hydrochloride. A sample was assayed by the method describe above for azocasein. Other compounds also used to characterize the protease were: iodoacetamide, PCMB, DTNB, 2.2'-dithiopyridine, mercuric chloride, PMSF, benzamidine and EDTA. The enzyme was incubated for 15 min. with these compounds at a concentration of 2 mM, and samples was assayed by the method describe earlier for casein.

Determination of the Isoelectric Point

The isoelectric point of the purified enzyme was determined by isoelectric focusing (IEF) on polyacrylamide gel as described [25, 26, 27]. IEF was performed using an Ettan IPGphor II Isoelectric Focusing System (*GE Healthcare*). For IEF, approximately 200 µg protein was diluted with a rehydration solution (8 M urea, 2 % CHAPS, 0.6 % DTT, 0.5 % IPG buffer, traces of bromophenol blue) to a total volume of 250 µL. IPG strips (pH 3-10 L, 13 cm) were rehydrated in this solution for 12 h under mineral oil. IEF was performed at 20 °C, with the following parameters: 500 Vh (1 h), 1,000 Vh (1 h), and 8,000 Vh until 26,500 Vh was attained. The current was limited to 50 µA/strip [28]. After IEF, the IPG strip was stored at –80 °C. The pI of the band was estimated by the software ImageMaster 2D platinum (*GE Healthcare*).

RESULTS AND DISCUSSION

A new protease from the latex of *Euphorbia milii*, named miliin was successfully purified with 26.7 % recovery (Table **1**) by utilizing a combination of acetone precipitation followed by ion-exchange and size-exclusion chromatography. Under non-reducing conditions (PAGE and Zymogram), the purified enzyme was present as a single band (Fig. **2A** and **B**). As presented in Fig. (**2C**), the zymogram (performed after SDS-PAGE) displayed the presence of a single band with high proteolytic activity. Using the zymogram and SDS-PAGE, the molecular mass was estimated by plotting the relative mobility of Mr protein markers vs. the logarithm of their molecular masses (not shown). These results suggest that the enzyme is monomeric, with a molecular mass around 79 kDa. Isoelectric focusing of miliin exhibited a single spot with an estimated pI of 4.3 (Fig. **3**), different from another protease isolated from the same source with a similar name: milin, with an estimated pI of 7.2 [29]. Fig. (**2D**) demonstrates that when the sample is boiled in the absence of β-mercaptoethanol, the band of 79 kDa disappears and at least four other bands, of lower Mr, show up (lane 2). In the presence of that thiol reagent at least three bands are evident (lane 4). Lane 3 shows that, when the sample is not boiled and/or is treated with β-mercaptoethanol, the band of 79 kDa appears, along with others of lower Mr. It is likely that the SDS present in the sample buffer and during the run, also results in some protein modification. Modifications could also arise from heating and the presence of β-mercaptoethanol, which is able to disrupt disulfide bonds. Our attempts to crystallize purified miliin were not successful likely due to the glycosylation.

The presence of the protein sample as a single band in the electrophoresis and IEF experiments prove its purity. The degree of purity is similar to that reported for the cucumisin-like serine protease from the latex of the *Euphorbia supine* [30] and is greater than that reported for serine proteases from the latex of *Cucumis trigonus* Roxburghi [31], *Cryptolepis buchanani* [32] and for a vacuolar serine endopeptidase induced by glucose starvation in maize roots [33].

The M_r of the isolated proteases from euphorbains and hevains range from 33 to 117 kDa. [34], however, the major-

Table 1. Purification of Miliin from the Latex of *Euphorbia milii*

Step	Activity (U/mL)	Total Protein (mg/mL)	Specific activity (U/mg)	Total Volume (mL)	Total Units (U)	Purif. factor (fold)	Recovery (%)
Crude extract	22.3	5.7	3.9	27	603	1.0	100.0
Precip.extract	42.1	4.8	8.9	27	1,137	2.3	188.0
Íon exchange	11.1	0.8	13.9	92	1,024	3.5	169.0
Gel filtration	6.5	0.3	18.9	25	161	4.8	26.7

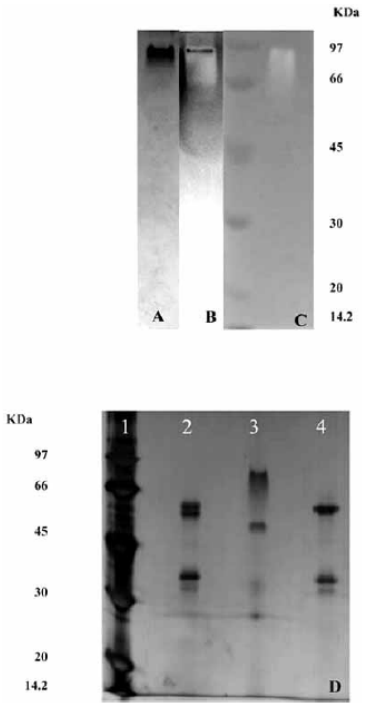

Figure 2. (A) Non-denaturating PAGE under non-reducing conditions: the gel was stained with silver nitrate. (B) Zymogram PAGE under non-reducing conditions: the gel was stained with coomassie brilliant blue R-250. Lane 1, Protein markers; Lane 2, purified miliin. (C) Zymogram under denaturing conditions (D) SDS-PAGE under denaturing conditions of samples submitted to different pretreatments. Lane 1, Protein markers; Lane 2, enzyme boiled without β-mercaptoethanol; Lane 3, sample not submitted to heating nor to β-mercaptoethanol; Lane 4 sample with β-mercaptoethanol.

ity display molecular weights of between 60 and 80 kDa. [35]. In contrast, the animal serine proteases display lower molecular masses when compared with plant serine proteases [36]. The comparison of physiological and biochemical properties of miliin with other serine proteases is presented in Table (**2**), which indicates that the optimal pH of this enzymes lies in the alkaline range.

The enzyme retained its proteolytic activity over a broad pH and temperature ranges tested. Hydrolysis of casein and azocasein tested in the 5.0 to 12.0 pH range indicated the presence of two pH optima (pHs at 7.5 and 11.0) for the first substrate and a maximum value around 5.5 for the second substrate (Fig. **4A**). Drapeau *et al.* [37] and Vallés *et al.* [1] reported a similar behavior for a purified extracellular protease from *Staphylococcus aureus*, which showed two peaks of maximum activity, at pH 4 and 7.8, using hemoglobin and proteolytic enzymes from ripe fruits of *Bromelia antiacantha* Bertol respectively. The fact that Azocasein was maximally degraded at pH 5.5-6.0, might be due to some modification of its sulphanilamide chromophore, as proposed by Shannon and Wallace [38]. Concerning the effect of temperature, the catalytic activity was assayed from 25 to 85 °C with maximal activity around 60 °C (Fig. **4B**). When the protease was incubated at 85 °C, more than 20 % of the activity was retained.

Figure 3. Isoelectric focusing of miliin on a strip. The band has an apparent pI between 4.5 and 5.0. The pI was estimated as 4.3 by analysis with ImageMaster 2D platinum (GE Healthcare).

The activity and stability of plant proteases over broad temperature and pH ranges has received much attention especially from pharmaceutical and biotechnological industries [39].

Miliin hydrolyses natural substrates such as casein (high activity) and azocasein (low activity). This enzyme did not display either amidolytic or esterase activity on substrates such as 3-N-alpha-benzoyl-DL-arginine p-nitroanilide (BApNA), N-Acetyl-L-Tyrosine Ethyl Ester (ATEE) and N-benzoyl-L-arginine ethyl ester (BAEE).

Since proteolytic enzymes can be used for cleaning protein stains, we tested the effects of tensoactives, organic solvent and urea, over a period of 24 hours (Fig. **6**). The enzyme retained 85% activity in presence of the nonionic detergent (Triton X-100) and 35%, 32% and in presence the ionic detergent SDS and CTAB respectively. The effect of acetone was weaker than for ethanol, causing a decrease to 27.5% and 47.5%, respectively. Urea at a high concentration (5.5M) resulted in only 10 % inhibition. After 24 hours the effects of acetone, ethanol and CTAB were more pronounced.

Surfactant-enzyme interactions in aqueous solutions have been extensively studied for technical applications, such as drug delivery, cosmetics and detergents, as well as for studying interactions between membrane proteins and lipids [40].

The enzymatic activity was significantly inhibited by all cysteine protease inhibitors we tested and also by PMSF (Table **3**). PMSF inhibition was not reverted by DTT (not shown), and another serine protease inhibitor used for trypsin-like proteases, benzamidine, did not affect the enzymatic activity. James *et al.* [33] reported a similar behavior for a serine protease from maize roots.

Table 2. Physicochemical Properties of Miliin (Molecular Mass, Optimum Temperature and Optimum pH) in Comparison with Other Plant Serine Proteases

Source	M_r (kDa)	T (°C)	pH	Substrate
Euphorbia milii (this report)	79	60	7.5-11.0	Casein
Euphorbia milii [29]	51	60	8.0	Casein
Ipomoea carnea [42]	80.2	65	6.5	Casein
Cucumis trigonus Roxburghi [31]	67	70	11	Casein
Cucumis melo [43]	67	70	10.5	Casein
Zea mays [33]	60	N/A	6.0-6.5	Casein
Cucurbita ficifolia [44]	60	55	9.2	Azocasein
Euphorbia dupifera [34]	117	N/A	6.3 and 7.8	Azocollagen
Benincasa hispida [45]	67	60	10.0	Casein
Euphorbia pulcherrima [46]	74	N/A	7.0	Azocasein
Trichosantus kirrilowi [47]	50	70	10.0	Casein
Trichosanthes bracteata [48]	67	N/A	11.0	Casein
Euphorbia supine protease [30]	80	N/A	8.0	Casein

N/A, no data available

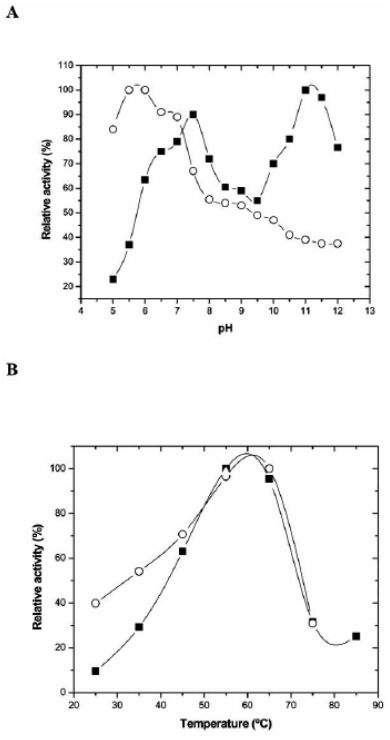

Figure 4. Effects of pH (A) and temperature (B) on miliin activity towards casein (■) and azocasein (○).

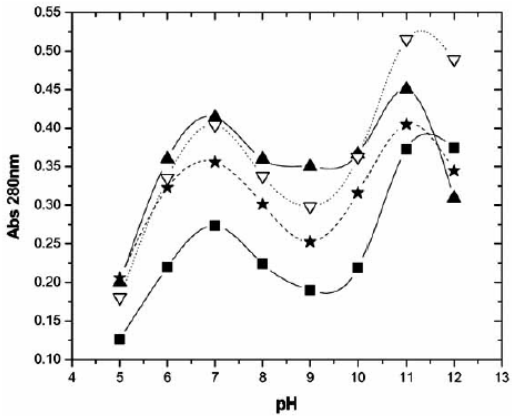

Figure 5. Effects of pH on miliin stability using casein (★) Control (▲) 1h (▽) 4h and (■) 24h. The enzyme was equilibrated at a determined pH, and assays were carried out at the same pH as discussed in Material and Methods.

Some divalent ions such as $ZnCl_2$, $NiCl_2$, $HgCl_2$, KCl, $AlCl_3$, inhibited the catalytic activity, however, $CaCl_2$, NaCl, $FeCl_3$, $CuCl_2$, $MnCl_2$, did not alter the catalytic activity. When tested against 2 mM thiol reagents, the protease activity decreased by about 5 % in the presence of cysteine hydrochloride and DTT and was unaffected by the presence of β-mercaptoethanol (Table **4**). EDTA, a metallo-protease inhibitor did not alter the activity of the enzyme.

In conclusion, we have isolated a novel protease, miliin from the latex of *Euphorbia milii* and the preliminary characterization of this enzyme suggests that it is a thiol-dependent

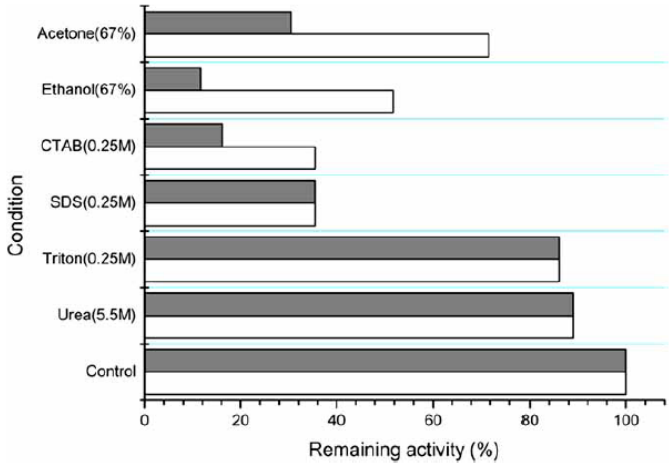

Figure 6. Effects of denaturants and organic solvents on the proteolytic activity of miliin. The temperature used was 37°C at pH 5.5 using 0.05 M acetate buffer. The enzyme was incubated for 15 min. with surfactants and a sample was assayed by the method described above with azocasein. The white bars represent the enzymatic assay (time zero), and the gray bars the assay performed for the same experimental set after 24 hours. The control was assayed in the absence of denaturants and organic solvents.

Table 3. Effect of Various Protease Inhibitors (at 2mM Final Concentration) on the Activity Of Miliin Using Casein as Substrate

Inhibitor class	Inhibitor	Residual activity (%)
Cysteine proteases	Iodoacetic acid	82.0
Serine proteases	pCMB	51.2
Metallo-proteases	DTNB	48.8
	2,2 dithiopyridine	21.0
	$HgCl_2$	39.0
	PMSF	37.8
	Benzamidine	99.7
	EDTA	100.0

[a] inhibitor final concentration: 2mM.

Table 4. Effect of Various Compounds (at 10 mM Final Concentration) on the Azocaseinolytic Activity of Miliin

Compound	Relative activity (%)
$MnCl_2$	102.0
Control	100.0
$CaCl_2$	100.0
NaCl	100.0
$FeCl_3$	100.0
$CuCl_2$	100.0
*β-mercaptoethanol	100.0
$MgCl_2$	97.0
$BaCl_2$	97.0

(Table 4) contd….

*DTT	96.0
*Cysteine hydrochloride	95.3
KCl	94.3
$AlCl_3$	68.5
$NiCl_2$	66.0
$ZnCl_2$	57.0
$HgCl_2$	46.0

* final concentration: 2mM.

serine protease either due to a cysteine residue in close vicinity to the active site or due to the presence of an essential thiol group in the protein structure. Similar observations were reported in the case of cucumisin, a serine protease from the princemelon fruits [41] and a protease from the latex of *Euphorbia milii* [29].

ACKNOWLEDGMENTS

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ARTIGO CIENTÍFICO III

Genomics and Proteomics Approaches to the Study of Cancer-Stroma Interactions

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Keywords: head and neck cancer, stroma-tumor interaction, conditioned medium, subtractive hybridization, proteomics.

Abstract

Background: The development and progression of cancer depend on its genetic characteristics as well as on the interactions with its microenvironment. Understanding these interactions may contribute to diagnostic and prognostic evaluations and to the development of new cancer therapies. Aiming to investigate potential mechanisms by which the tumor microenvironment might contribute to a cancer phenotype, we evaluated soluble paracrine factors produced by stromal and neoplastic cells which may influence proliferation and gene and protein expression.

Methods: The study was carried out on the epithelial cancer cell line (Hep-2) and fibroblasts isolated from a primary oral cancer. We combined a conditioned-medium technique with subtraction hybridization approach, quantitative PCR and proteomics, in order to evaluate gene and protein expression influenced by soluble paracrine factors produced by stromal and neoplastic cells.

Results: We observed that conditioned medium from fibroblast cultures (FCM) inhibited proliferation and induced apoptosis in Hep-2 cells. In neoplastic cells, 41 genes and 5 proteins exhibited changes in expression levels in response to FCM and, in fibroblasts, 17 genes and 2 proteins showed down-regulation in response to conditioned medium from Hep-2 cells (HCM). Nine genes were selected and the expression results of 6 down-regulated genes (*ARID4A*, *CALR*, *GNB2L1*, *RNF10*, *SQSTM1*, *USP9X*) were validated by real time PCR.

Conclusions: A significant and common denominator in the results was the direct or indirect potential induction/inhibition of an immune or inflammatory response in the absence of a specific protein.

Background

Solid tumors are characterized by the presence of two major components: neoplastic cells and a specialized nonmalignant stroma in which they are immersed and are essential for their survival and proliferation. In carcinomas, a basement membrane is usually present between these components [1, 2].

The tumor stroma is distinguished by an enrichment of microvessel density, abundance of endothelial cells and precursors, inflammatory cells including lymphocytes, neutrophils, macrophages, dendritic and mast cells, and a connective tissue with fibroblasts, myofibroblasts and histiocytes responsible for remodeling and deposition of extracellular matrix (ECM) components - fibronectin, collagens, elastin, and glycosaminoglycans [2-4]. Although these cells are nonmalignant, they have a unique gene expression pattern, compared to stroma cells in normal tissues [5, 6].

Substantial evidence indicates that the development and the progression of cancer not only depend on its genetic characteristics but also on interactions with its microenvironment [4, 7, 8]. In fact, tumor cells may alter the surrounding stroma through direct cell contact or via the secretion of paracrine soluble factors, inducing cell differentiation or extracellular matrix modifications [9]. In it turn, stromal cells may promote cancer progression and acquisition of invasiveness [10-12]. It is possible that such interactions contribute to the neoplastic cell phenotype and behavior as observed during the normal development process and function of organs and tissues [13, 14]. As Albini and Sporn (2008) appropriately propose, the microenvironment may be more than a partner but also an essential component of the cancer, and both should be considered as a functional whole [15].

In this context, inflammation and infection have gained special attention. Well known examples connecting infection-related or -unrelated chronic inflammation and increased risk for cancer development are described in the literature [16], and probably more than 15% of cancers are linked to these factors [17]. TNF-alpha and NF-κB transcription factor should play a central role in this process, modulating transcription of genes encoding angiogenic and growth factors, inflammatory cytokines and anti-apoptotic proteins [16]. In fact, many inflammatory mediators may influence cell proliferation and tumor development, as demonstrated by our recent studies on annexin A1 [18-20].

Macrophages represent one of the main inflammatory regulators in tumor stroma and are responsible for proliferation, invasion and immunosuppressive signaling, with the production of angiogenic and growth factors, chemokines, cytokines and matrix metalloproteases [21]. The key partners of macrophages in this network are fibroblasts, the so-called carcinoma-

associated fibroblasts (CAFs), which significantly increase the growth of neoplastic or normal cells [22, 23] and can enhance tumor engraftment and metastasis in animal models [24]. Recently, Hawsawi et al. (2008) [25] observed well-defined differences in gene expression and proteomic profiles between activated CAFs and fibroblasts from normal stroma, emphasizing their importance in the cancer process.

Regardless of the fact that they are easily identified by their morphology, specific cellular markers for fibroblasts remain unknown, presumably because of their large diversity [26]. In tumor stroma, fibroblasts present a phenotype similar to those associated with wound healing, with a large and euchromatic nucleus and prominent rough endoplasmic reticulum [27, 28]. These signals mediating the transition of normal to reactive fibroblasts are still not completely defined.

Many studies have analyzed the role of fibroblasts in cancer initiation and progression. To address this issue, several approaches have been used, as co-culture of cancer cells and fibroblasts and cultures with conditioned medium, combined or not with *in vivo* experiments. The data have shown that these cells, similar to macrophages, overexpress chemokines, interleukines, growth factors and matrix metalloproteinases, promoting inflammatory responses and facilitating angiogenesis, cancer-cell invasion and proliferation [29-31]. In head and neck cancer, for example, *in vitro* experiments have suggested that the presence of fibroblasts is essential for invasive features either because cancer cells express higher levels of matrix metalloproteases in the presence of fibroblasts [32, 33] or because cancer-associated fibroblasts themselves synthesize these proteins [34, 35].

Much of the answer to the question of tumor-stroma interactions lies in the identity of ligands, receptors and effectors of signaling patterns expressed by stroma and tumor cells. Numerous growth factors, cytokines, chemokines, hormones, enzymes and cells responsible for their expression have been characterized but the cross-signaling between pathways in this complex network is far from solved [7, 36]. Adding complexity to the scenario, the chemomechanical environment of the extracellular matrix may also act in concert with signaling pathways and affect the cancer process [37].

An important perspective in the study of tumor stroma is the potential use of the gene expression pattern of their cells for diagnostic or prognostic evaluation and as a target for therapy. Supporting this idea are the results from studies on outcome prediction and molecular marker analysis of the stroma [6, 38], drugs targeting inflammatory cells [39] and mediators of angiogenesis [40, 41].

In order to investigate potential mechanisms by which the tumor microenvironment might contribute to cancer phenotype, we asked whether soluble paracrine factors produced

by stromal and neoplastic cells *in vitro* may influence proliferation, and gene and protein expression. For these purposes, we exploited purified fibroblasts isolated from a primary oral cancer and an epithelial cancer cell line linked by conditioned medium and genomic and proteomic approaches. Both cells were treated with the conditioned medium of each other and submitted to analysis by rapid subtraction hybridization methodology, bidimensional electrophoresis and mass spectrometry. Based on the results of the rapid subtraction hybridization (RaSH) approach, a comparative quantitative real-time PCR was performed to validate the expression of several genes, focusing on those involved in tumorigenesis and inflammation. The results pointed to the participation of several inflammatory mechanisms that might have biological significance in epithelial tumors.

Methods

Primary tumor samples

For conditioned medium experiments, a primary epidermoid (squamous cell) carcinoma of the retromolar area was obtained from a 49-year-old male patient, prior to radiation and/or chemotherapy. Twenty-four laryngeal and 23 oral tongue squamous cell carcinoma (SCC) samples from patients undergoing tumor resection were used for gene expression analysis. All carcinoma samples were reviewed by senior pathologists and exhibited the presence of at least 70% tumor cells; the corresponding surgical margins were classified to be free of tumor cells.

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.

Epithelial cancer cell line and primary tumor cell cultures

The Hep-2 cell line, originally established from an epidermoid carcinoma of the larynx (ATCC, Rockville, Maryland, USA), was seeded at a density of 1 x 10^6 cells/mL per 75cm^2 culture flask (Corning, NY, USA) in medium MEM-Earle (Cultilab, Campinas, SP, Brazil), pH 7.5, supplemented with 20% fetal calf serum (Cultilab), 1% non-essential amino acids, 0.1% antibiotic/antimycotic (Invitrogen Corporation, Carlsbad, CA, USA), and cultured at 37°C in a humid atmosphere of 5% CO_2.

A primary carcinoma of retromolar area sample showing epithelium and adjacent connective tissues was rinsed multiple times with 100x antibiotic and antimycotic solutions (Invitrogen) and minced into 2-4 mm fragments. Single-cell suspensions were obtained by

digestion at 37°C for 1 hour with 40 mg/mL collagenase type I (Sigma Chemical, St Louis, USA). After centrifugation, the cells were washed with PBS, resuspended in DMEM medium supplemented with 20% fetal calf serum (Cultilab), 2 mM glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), and 0.1% antibiotic/antimycotic (Invitrogen). The cells were seeded at a density of 1 x 10^6 cells/mL per 75cm^2 culture flasks (Corning) and cultured at 37°C in a humid atmosphere of 5% CO_2. Cell medium was changed at 72 h intervals until the cells became confluent. Since fibroblasts were mixed with the epithelial tumor cells at the time of initial plating, fibroblasts were selected by plating the cells growing in medium supplemented with 20% serum for at least 3 weeks [42-44].

Preparation of Conditioned medium

Conditioned medium (CM) was prepared from Hep-2 cell or tumor stromal fibroblast cultures showing 80% confluence. Twenty-four, 48 and 72 hours after medium replacement, the supernatant or conditioned medium (CM24, CM48 and CM72, respectively) from three replicas was aspirated and filtered through a 0.22 μm membrane (Millipore) to remove any cell debris and stored at -80°C. Before using, the CM was diluted 1:1 in complete medium. The dilution 1:1 and CM72 were chosen to maximize the chance of detecting a cell response to soluble factors. Optimization experiments showed that dilutions lower than 1:1 resulted in higher numbers of dead cells.

Hep-2 cell-conditioned medium is referred to as HCM and fibroblast-conditioned medium is referred to as FCM.

Growth curve

Hep-2 cells were seeded at a density of 5 x 10^4 cells in plastic 6-well plates in two sets of quadruplicates. Twenty-four hours later, when cells had already adhered, Hep-2 cultures were incubated with FCMs. One replica in each set was treated with self-conditioned medium and one replica was treated with complete medium.

Medium was replaced on day 4 and cell morphology was observed every day. After 1, 3, 5 and 7 days, cells were harvested and counted using a Neubauer hemocytometer. The same experiment was repeated twice.

Immunofluorescence analysis

The Hep-2 cell line or tumor stromal fibroblasts were grown in culture chambers (Nunc, Naperville, IL, USA) and, after 3 days, the chambers were carefully removed, and the slides

with adherent cells were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, 0.1 mol/L sodium phosphate buffer, pH 7.4, for 2 hours at 4°C. The slides were washed in the same buffer and incubated with 0.1% albumin bovine and 3% normal serum in PBS (PBSA) to block nonspecific binding. The cells were immunostained with primary mouse monoclonal antibodies (Ab) anti-vimentin (NCL-VIM-V9, Novocastra, Benton Lane, Newcastle, UK) or anti-cytokeratin (M3515, antibodies to all types of cytokeratins; AE1-AE3; Dako, Carpinteria, CA, USA) diluted at 1:200 in 1% PBSA, followed by overnight incubation at 4°C. For negative controls, the cells were incubated with nonimmune mouse serum (1:200 working dilution; Sigma-Aldrich). After repeated washings in 1% PBS, a goat anti-mouse IgG (Fc fragment-specific, Dako, Glostrup, Denmark) antibody conjugated to FITC (1:50; British BioCell International, Cardiff, UK) was added, followed by 1 hour incubation at room temperature. Thus, the cells were washed thoroughly in PBS. Analysis was conducted using an Axioskop 2 light microscope (Zeiss, GR) equipped with a digital camera. Digital images were captured by using software AxioVision (Zeiss, GR).

Immunohistochemical analysis

Apoptosis was assayed using AnxA5 staining as described [45]. Fixed Hep-2 cell line or tumor stromal fibroblast in slides from culture chambers were incubated with the following reagents: 2.1% sodium citrate for 30 min at 96°C; 3% hydrogen peroxide for 15 min; 0.1% Tween 20 (Sigma-Aldrich) diluted in 0.4% PBS for 15 min; non-specific binding sites were blocked with 10% albumin bovine (BSA) diluted in TBS (20 mM Tris buffer in 0.9% NaCl, pH 8.2) for 30 min. The slides were then incubated overnight with a rabbit polyclonal antibody anti-AnxA5 (sc8300, Santa Cruz Biotechnology, California, USA), diluted 1:200. After repeated washings in 1% PBSA, a goat anti-rabbit IgG (Fc fragment specific) antibody conjugated to 5 nm colloidal gold particles (N24916, Invitrogen) was added. Silver enhancing solution (L24919, Invitrogen) was used to augment gold particle staining. At the end of the reaction, cells were washed thoroughly in distilled water, counterstained with haematoxylin and examined using an Axioskop2 microscope (ZEISS, GR).

RNA extraction for Rapid Subtraction Hybridization (RaSH) and real time PCR experiments

Hep-2 cells and stromal fibroblasts were seeded at a density of 1 x 10^6 cells/mL per 75cm^2 culture flasks in complete medium (controls) and in conditioned medium. Hep-2 cells and fibroblasts were cultured for 5 and 3 days, respectively, and harvested by addition of TRIzol Reagent, following treatment with DNase (Invitrogen). Total RNA from primary

tumor samples was also extracted using TRIzol Reagent and treated with DNase. cDNA synthesis was performed using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer.

RaSH

RaSH technique was performed as described by Jiang et al. (2000) [46]. Aliquots (20µg) of total RNA from control cells (driver) or treated cells (tester) were used for double-stranded cDNA synthesis using standard protocols [47].

The cDNA was digested with MboI (Invitrogen) at 37°C for 3h followed by phenol/chloroform extraction and ethanol precipitation. The digested cDNAs were mixed with the adaptors XPDN-14 5´-CTGATCACTCGAGA and XPDN-12 5`-GATCTCTCGAGT (Sigma Chemical, final concentration 20 µM) in 30 µl of 1X ligation buffer (Gibco BRL), heated at 55°C for 1 min, and cooled down to 14 °C within 1h. After adding 3µl of T4 DNA ligase (5U/µl) (Gibco, BRL), ligation was carried out overnight at 14°C. After phenol/chloroform extraction and ethanol/glycogen precipitation, the mixtures were diluted to 100µl with TE buffer (10mM Tris/1mM EDTA); 40µl of the mixtures were used for PCR amplification.

The PCR mixtures were set up using 10 µM XPDN-18 5´-CTGATCACTCGAGAGATC, 0.4 mM dNTPs, 10 x PCR buffer, 1.5 mM $MgCl_2$ and 1U Taq DNA polymerase (Invitrogen). Thermocycler conditions were one cycle at 72 °C for 5min, followed by 25 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1min, ending in a final extension at 72°C for 3 min. Ten µg of purified PCR product (tester) was digested with 20U XhoI (Invitrogen) followed by phenol/chloroform extraction and ethanol precipitation.

One-hundred nanograms of the tester cDNA were mixed with 5 µg of the driver cDNA in hybridization solution (0.5M Nacl, 50mM Tris/HCl, SDS2% and 40% formamide) and, after heating at 95°C, incubated at 42°C for 48h. After extraction and precipitation, the hybridization mixture (1µg) was ligated with XhoI-digested pZero plasmid and transformed into competent bacteria. Bacterial colonies were picked and used as DNA template for PCR. Clones were sequenced using an automated DNA sequencer and sequence homologies were searched using the BLAST program [48]. Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes [49].

Quantitative PCR

For validation experiments, cells were seeded at a density of 1 x 10^6 cells/mL per 75cm^2 culture flasks in two sets of quadruplicates. Twenty-four hours later, when cells had already adhered, Hep-2 culture replicas were treated with FCMs and fibroblast cultures were treated with HCMs. One replica in each set (control) was treated with self-conditioned medium. Hep-2 cells and fibroblasts were harvested after 5 and 3 days, respectively, and RNA was extracted as described above.

Nine differentially expressed genes were selected for validation by quantitative real time PCR experiments according to their direct or indirect involvement in tumorigenesis. Their expression was checked in treated samples relative to matched non-treated samples. One of these genes (*ARID4A*) was also selected for quantitative real time PCR validation in fresh tumor samples of 24 laryngeal SCC and in 23 oral tongue SCC relative to matched normal samples.

The primers were manually designed with: 19-23 bp length, 30-70% GC content and a short amplicon size (90-110 bp). Their sequences are available upon request. Real time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixture consisted of a 20ul volume solution containing 10ul of Power SYBR Green PCR Master Mix (Applied Biosystems), 500nM of each primer and 100 ng cDNA. The PCR conditions were 95°C for 10min followed by 40 cycles of 95° for 15s and 60° for 1min. Melting curve analysis was performed for each gene to check the specificity and identity of the RT-PCR products.

For each primer set, the efficiency of the PCR reaction (linear equation: y = slope + intercept) was measured in triplicate on serial dilutions of the same cDNA sample. The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/slope)}]$ and ranged from 1.96 to 2.02 in the different assays.

Three control genes (*GAPDH, ACTB* and *TUBA6*) were used as internal standards. The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl (2001) [50]. Statistical analysis was performed by a two-tailed unpaired *t* test using GraphPad prism software.

Proteomic analysis

Hep-2 cells and stromal fibroblasts were seeded at a density of 1 x 10^6 cells/mL per 75cm^2 culture flasks in complete medium and in conditioned medium, as described for RASH experiments. Hep-2 cells and fibroblasts were cultured for 5 and 3 days, respectively, and harvested by centrifugation at 3200 rpm for 5 min at 4 °C. Cells were disrupted by

sonication, proteins were isolated and two-dimensional electrophoresis (2-DE) was performed, as described by de Marqui et al. (2006) [51]. Briefly, isoelectric focusing was carried out in a IPGphor (GE Healthcare) using 13-cm immobilized pH 3-10 L gradient strips. Vertical 12.5% SDS-PAGE was performed in a SE 600 Ruby electrophoresis unit (GE Healthcare) and proteins were detected by Coomassie Blue staining. Differentially expressed proteins were excised from gel, distained, dried and in-gel tryptic-digested. Negative and positive control digests were performed on gel slices that contained no protein and on slices cut from a band of the molecular weight marker, respectively.

Samples were analyzed using MALDI Q-TOF (Matrix Assisted Laser Desorption Ionization – Quadrupole Ion Filter - Time of Flight) Premier (Waters Corporation, Milford, MA, USA) mass spectrometer (MS/MS). Duplicate or triplicate runs of each sample were made to ensure an accurate analysis.

For protein identification, the resulting MS/MS data were interpreted by MASCOT software (MS/MS Ions Search) [52] and searched against the Mass Spectrometry Protein Sequence Database (MSDB). The UniProtKB/Swiss-Prot [53] database was used for the functional classification of up- and down- expressed proteins.

Data Handling and Statistical Analysis

Quantification of apoptotic cells was performed with a high magnification objective (x40) counting cells in 100 μm^2 areas and reported as mean ±SEM per group. Densitometric analysis for the immunofluorescence staining used an arbitrary scale ranging from 0 to 255 units. Statistical differences between groups were determined by analysis of variance followed, if significant, by the Bonferroni test.

Results

Stromal fibroblasts: selection and immunofluorescence analysis

Fetal calf serum concentration and culture time provided a simple method of selecting fibroblasts from a primary carcinoma of retromolar area. Fibroblast cultures at passage 78 still showed spindle-shaped cells, which displayed the typical fibroblast markers, weak cytokeratin and intense vimentin immunoreactivity in cytoplasm, after immunofluorescence analysis (Figure 1B, E, G). Staining was obtained with both antibodies (cytokeratin and vimentin) in Hep-2 cells (Figure 1C, F, G). No labeling was detected in sections incubated with the control nonimmune mouse serum (Figure 1A, D).

Ultrastructural analysis showed that the stromal fibroblasts present large euchromatic nuclei, more granular endoplasmatic reticulum, mitochondria and nucleoli than normal fibroblasts (data not shown). Therefore, the spontaneously immortalized cell line of fibroblasts retained the characteristics of stromal cells and may correspond to cancer-associated fibroblasts (CAF).

Conditioned medium inhibits proliferation and induces apoptosis

Growth curves of Hep-2 cells treated with FCM showed decreased proliferation (Figure 2). Growth inhibition was observed as early as day 1 and was statistically significant ($P < 0.05$) at day 3 and day 5.

The immunohistochemistry reaction with AnxA5 antibody showed the presence of gold particles on the cytoplasm of the Hep-2 apoptotic cells (Figure 3). The AnxA5 immunoreactivity was found more in the apoptotic process of Hep-2 cells incubated in FCM (56%) than in cells without the treatment (24%). Apoptotic cells displayed distinctive morphology, a notable decrease in the nuclear size, irregular shape and cytoplasmic blebbing.

Genes identified using the RaSH approach

A total of 81 clones from the Hep-2 cell line and fibroblast libraries were sequenced. In the Hep-2 cell line, forty-one genes exhibited changes in expression levels in response to FCM treatment (33 down- and 8 up-regulated) and, in fibroblasts, 17 genes showed down-regulation in response to HCM treatment. These genes are involved in response to stimulus, apoptosis, cell proliferation and differentiation, signal transduction, transcription, translation and transport (Table 1 and 2).

Real-time PCR validation of differentially expressed genes

Nine genes displaying down- (*ARID4A, CALR, GNB2L1, GPNMB, RNF10, SQSTM1, USP9X*) or up-regulation (*DAP3, PRDX1*) in Hep-2 cells treated with FCM were selected and the expression data for six down-regulated genes (*ARID4A, CALR, GNB2L1, RNF10, SQSTM1, USP9X*) were confirmed by real time PCR (Figure 4A). Most results were, therefore, consistent with the RaSH data.

ARID4A expression was also analyzed in 24 pairs of tumor and matched normal tissues from laryngeal squamous cell carcinomas and in 23 pairs of tumor and matched normal tissues from oral tongue squamous cell carcinomas. *ARID4A* mRNA levels were decreased (≥2-fold) in almost half of the squamous cell carcinomas samples (-1.04 to -6.9-fold change,

23 of 47 samples, i.e., 49%) and were increased in some of these samples (1.51 to 6.26-fold change, 7 of 47 samples, i.e., 15%) (Figure 4B). In contrast, no differences in transcript levels were observed between 17 of 47 samples (36%) and normal tissue. Therefore, similarly to the Hep-2 cell line, most primary head and neck tumors (49%) showed down-regulation of *ARID4A* transcripts.

No differences were observed in respect to clinicopathological features between samples presenting up- and down-regulation of *ARID4A* transcripts (Supplementary Table 1).

Proteomics approach

Comparison between 2-DE patterns from treated cells and controls revealed approximately 80 spots with significant differences in intensity. Seven proteins (Figure 5) showing expression level changes in response to CM treatment were identified by MALDI-Q-TOF-MS mass spectrometry (Supplementary Table 2). Five proteins (alpha enolase, heterogeneous nuclear ribonucleoprotein C C1/C2, aldolase A, tubulin beta and glyceraldehyde-3-phosphate dehydrogenase) were down-regulated in Hep-2 cell line treated with conditioned medium (FCM72) and two proteins (vimentin and actin) were underexpressed in fibroblasts treated with Hep-2 cell line conditioned medium (HCM72). These proteins are involved in transcription, growth control, response to stimulus, RNA processing, glycolysis, cell motion and membrane trafficking.

Discussion

The molecular crosstalk between neoplastic and the surrounding tissue induces several stromal changes, including neoangiogenesis and inflammatory cell infiltration, as well as new extracellular matrix formation and the activation of fibroblast-like cells, a process known as desmoplasia [54], [55]. Initially, the desmoplastic response was considered a barrier against tumor invasion, but there is growing evidence that desmoplasia is an unfavorable prognostic factor. For example, Sis et al. [56] suggested that desmoplastia is related to increased risks of regional metastases, poorly differentiated primary tumors and lymphatic and venous invasion in colorectal carcinoma. Similar results were observed for head and neck squamous cell carcinomas, which show a high risk of neck recurrence in presence of a desmoplastic stromal pattern [57].

In the present study, we investigated the influence of soluble paracrine factors produced *in vitro* by stromal cells derived from an oral carcinoma and by a neoplastic epithelial cell line on proliferation and gene/protein expression. First, we noted that conditioned medium

from stromal fibroblast cultures inhibited Hep-2 cell line proliferation and induced apoptosis, suggesting that factors secreted by fibroblasts include proteins that interfere in cell growth and death of neoplastic cells. In addition, using rapid subtraction hybridization and proteomic analysis, we identified gene products generated by stromal and neoplastic cells that may influence proliferation, differentiation and apoptosis, or drive response to stimulus.

Down-regulated genes in neoplastic cells treated with FCM are involved in signal transduction (*FAS, SQSTM1, YWHAZ*), transcription (*ARID4A, CALR, MYC, PARP1, RNF10, SQSTM1*), translation (*AARS, RPLP0, RPS17, RPS23*), apoptosis (*CALR, FAS, TPT1, YWHAZ*), cell migration (*TMSB4X, GNB2L1*), cell cycle and cell proliferation (*DYNC1H1, GPNMB, LDOC1, MYC, PSM*), epidermis development (*UGCG*), response to stimulus (*EIF2AK1, LTA4H, SQSTM1*), transport (*CALR, NDUFA4, SQSTM1*) and different metabolic processes (*USP9X*). Up-regulated genes are also involved in transcription and translation (*ENO1, EIF1, TARS*), apoptosis (*DAP3, RTN3*), cell proliferation (*PRDX1, ENO1*), organ development (*PRDX1*), response to stress (*EIF1, RTN3*) and metabolic processes.

In fibroblasts treated with HCM, the biological processes of down-regulated genes include signal transduction (*S100A6, FN1*), transcription and translation (*FOSL1, RPL37A, RPL7, RPL19, RPL27A, RPLP0*), apoptosis (*CTSB, TPT1*), cell proliferation (*S100A6, FOSL1*), epidermis development (*COL1A1*), response to stimulus (*FN1, FOSL1*), transport (*ERGIC3, STX4*) and protein and RNA metabolism (*CTSB, PRPF3*).

Two genes exhibited similar patterns in both cells (*RPLP0, TPT1*), which may indicate that the transcript levels are affected by soluble paracrine factors produced by either fibroblasts or neoplastic cells or by other *in vitro* conditions. Therefore, they may not be specific to interactions between stroma and tumor.

After literature analysis, nine genes (*ARID4A, CALR, GNB2L1, GPNMB, RNF10, SQSTM1, USP9X, PRDX1 and DAP3*) showing potential involvement in signaling cascades related to tumorigenesis and/or stromal/tumor cell interactions were selected for validation by real-time RT-PCR using treated and non-treated cell lines. For six genes (*ARID4A, CALR, GNB2L1, RNF10, SQSTM1, USP9X*), the results were consistent with the RASH data. In almost half of the primary tumors analyzed, *ARID4A* transcripts also showed down-regulation, although no correlation with clinicopathological features was detected. These findings in primary tumors should reflect the complex network of a multi-cellular tissue, a situation contrasting with that of a neoplastic cell line cultured in medium conditioned by fibroblasts.

The product of *ARID4A* - AT rich interactive domain 4A (RBP1-like) - also known as *RBP1 or RBBP1* gene, interacts with the tumor suppressor retinoblastoma (pRB) and histone-modifying complexes, repressing promoters of specific genes [58]. Röhl et al. [59] detected several genes, including *ARID4A*, overexpressed in astrocytes treated with medium conditioned by activated microglia, which protected them against stress conditions. Recently, Wu et al. [60] showed that *Arid4a*-deficient mice exhibit down-regulation of several homeobox genes and of the forkhead box gene *Foxp3*, which codes a transcription factor involved in the development and function of regulatory T cells [61]. These mice also show bone marrow failure with myelofibrosis and higher frequencies of hematologic malignancies, providing evidence that *ARID4A* functions as a tumor suppressor gene and its absence is permissive for the proliferation of connective tissue elements. The study of Perez et al. [62] added data on the role of this gene in cancer. These authors detected increased mRNA levels of *ARID4A* and *RB1* in normal human epidermal keratinocytes treated with arsenic and benzo[a]pyrene *in vitro*. Since these chemicals alter proliferation and inhibit differentiation of keratinocytes [63-65], the findings may indicate that up-regulation of *ARID4A* is negatively related to epithelial differentiation. Therefore, the potential modulation of this gene by paracrine factors produced by stromal fibroblasts may represent an attempt to promote differentiation of neoplastic epithelial cells and, at the same time, their proliferation.

Calreticulin (coded by *CALR* or *CRT* gene) is a calcium-binding protein of the endoplasmic reticulum with intracellular and extracellular functions related to cellular adhesion, migration, and phagocytosis [66]. Calreticulin can be observed on the surface of stressed cells and, when bound to the plasma membrane of apoptotic cells, drives the phagocytosis by macrophages and dendritic cells [67]. In absence of this protein, the cells are not efficiently removed by phagocytes [68]. Recently, Nanney et al. [69] showed that calreticulin stimulates both migration and proliferation of keratinocytes and fibroblasts and apparently attracts monocytes and macrophages, suggesting its involvement in inflammatory response. Otherwise, fibroblasts underexpressing CARL exhibit weak adhesion and spreading [70]. Accordingly, Kypreou KP et al. [71] detected a correlation between calreticulin up-regulation and progression of fibrosis and also that TGF-beta, a contributing factor in fibrotic processes, up-regulated calreticulin in cultured human epithelial cells. In light of the data, we speculate that the low levels of this protein observed in treated Hep-2 cells inhibit proliferation, or represent a protective response of neoplastic cells to phagocytosis and antitumor immune process.

Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 or Rack1 (coded by *GNB2L1* gene) is a cytosolic protein homologous to the beta subunit of G proteins, and contains seven WD repeats, which act as sites for protein-protein interactions. Binding partners of GNB2L1 include protein kinase C, Src family kinases, components of the ERK pathway, cytokine and interferon receptors, beta integrins and many others. Many of these interactions are consistent with the participation of Rack1 in cell adhesion, movement and growth [72-75].

Sequestosome 1 or ubiquitin-binding protein p62 (coded by *SQSTM1* or *p6*0 or *p62* gene) is a 62-kDa protein that binds to the Src homology 2 (SH2) domain of $p56^{lck}$ kinase in a phosphotyrosine-independent manner [76]. It has been suggested that p62 is a signaling adaptor which links different signal transduction pathways related to cell proliferation, differentiation and death, including NF-κB pathway [77-82]. *SQSTM1* abnormal expression has been observed in hepatocellular, prostate and breast cancers [83-85] and is associated with poor outcomes in breast cancer [86].

Another gene down-regulated by fibroblast-conditioned medium is *USP9X* (Ubiquitin specific peptidase 9, X-linked), also known as *DFFRX*, *FAF* or *FAM*. This gene is a member of the peptidase C19 family and encodes a protein similar to ubiquitin-specific proteases (USPs). These proteases regulate the production and recycling of ubiquitin and are critically involved in the control of cell growth, differentiation, and apoptosis [87]. Alteration of USPs may play an important role in the pathogenesis of cancer [88] and may exert distinct growth regulatory activities by acting as oncoproteins or tumor suppressor proteins, and overexpression of certain USPs correlates with progression towards a more malignant phenotype in carcinoma of lung, kidney, breast and prostate [89, 90].

RNF10 (ring finger protein 10) is the least known gene selected for validation. The product contains a ring finger motif, which is involved in protein-protein interactions and has been described in proteins implicated in many cellular processes such as signal transduction, transcriptional regulation, ubiquination, and apoptosis [91, 92].

With respect to proteomic analysis, few differences (mostly quantitative) between treated and non-treated cells were detected. Among the proteins differentially expressed, alpha-enolase, heterogeneous nuclear ribonucleoprotein C C1/C2, aldolase A, tubulin beta and glyceraldehyde-3-phosphate dehydrogenase were down-regulated in neoplastic cells treated with FCM and vimentin and actin were down-regulated in fibroblasts treated with HCM. These proteins, produced by neoplastic cells or fibroblasts, may affect tumorigenesis. For example, the glycolytic enzyme alpha-enolase and its enzymatically inactive isoform MBP-1 (c-*myc* promoter binding protein 1) are negative regulators for *MYC* expression [93, 94].

MYC is one of the most frequently de-regulated oncogenes in cancer [95] and, in the absence of both enzymes, may become activated and accelerate tumor growth. Contrary to RaSH results, alpha enolase protein was observed underexpressed by proteomic analysis in treated Hep-2 cells, which may indicate a nonspecific finding or a post-transcriptional/posttranslational regulation of the RNA/enzyme.

Conclusions

Fibroblasts, as with other cells in tumor microenvironments, need to maintain close communication with cancer cells, promoting proliferation, recruitment of inflammatory cells and acquisition of invasive characteristics. Similarly, cancer cells may influence stromal cells to generate a favorable and supportive environment, which would supply them with nutrients and factors necessary for developing the tumor and spreading of metastasis. In the present study, we observed both positive and negative effects exerted by fibroblasts on Hep-2 cells, favoring or not the former. A significant and common denominator in the results was the direct or indirect potential induction/inhibition of an immune or inflammatory response in the absence of a specific protein. In fact, *ARID4A* down-regulation is related to low levels of the transcript factor Foxp3 [60], which in turn is linked to immune responsiveness by targeting NF-κB and CREB pathways [96]. The final effect is the inhibition of the inflammatory response and the cost is a permissive sign for fibroblast proliferation [60]. Down-regulation of *CARL* also blocks the inflammatory response but has negative effects on stroma growth [69]. In presence of low levels of Rack1, again a deficient or altered inflammatory response may occur since Rack1 underexpression has already been related to the deregulation of cytokine production [97]. Similar results have been observed in p62-deficient mice, which exhibit abnormal control of NF-κB activation and reduced inflammation in experimental conditions [98]. The opposite effect is expected for osteoactivin underexpression because this protein has been observed as a negative regulator of macrophage inflammatory responses [99].

The complexity of the tumor microenvironment is immense and much information is still necessary for better understanding how the relationship between stroma and carcinoma cells can be used for diagnostic and prognostic evaluation and a target for therapy.

Authors' contributions

FCR-L participated in the design of the study and analysis of the data, carried out cell culture, RaSH experiments and drafted the manuscript. PP helped with RaSH experiments. AV and GMP carried out proteomics analysis. JVM was responsible for sample collection and processing. JC-R carried out cloning and sequencing of the samples. BRC carried out cell culture experiments. TH helped with manuscript preparation. CFS performed the real time PCR experiments. RAPT and SMO carried out immunofluorescence and immunohistochemical analysis. EEF and PMJr carried out clinical data analysis for sample selection. MBC carried out clinical data analysis for sample selection and drafted the manuscript GENCAPO team members were responsible for sample collection and initial on-site sample processing, provided the pathological analysis of the cases, obtained the informed consent and discussed the findings. EHT participated in the study design and coordination, 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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Figure Legends

Figure 1. Immunofluorescence analysis of cytokeratin and vimentin in stromal fibroblasts and Hep-2 cell line. (A and D) Absence of immunoreactivity in sections incubated with control nonimmune mouse serum. Stromal fibroblasts (B and E) and Hep-2 cell line (C and F) were positive for vimentin and cytokeratin, respectively. (G): Densitometric analysis of immunofluorescence reaction to vimentin and cytokeratin in stromal fibroblasts and Hep-2 cell line. Scale bar, 20µm.

Figure 2. **Growth curve of Hep-2 cell line**. Hep-2 cells were cultured in complete medium, treated with self-conditioned medium (HCM) or with conditioned medium from fibroblast cultures (FCM) and collected 1, 3, 5 and 7 days after medium replacement. Data are means ± s.d. of two independent experiments in duplicates. $^{*}P < 0.05$. Error bars indicate S.D.

Figure 3. Immunohistochemistry reaction with AnxA5 antibody showed the presence of gold particles on the cytoplasm of apoptotic cells. Hep-2 cells (A) without treatment and (B) treated with conditioned medium from fibroblast culture (FCM) show AnxA5 immunoreactivity. Apoptotic cells immunolabeling for AnxA5 can be seen in Hep-2 cells treated with FCM (arrows). Staining with haematoxylin. Scale bar, 20µm.

Figure 4. Real-time PCR gene expression in a conditioned medium-treated neoplastic cell line and in primary tumors. (A) Expression of *ARID4A*, *CALR*, *DAP3*, *GNB2L1*, *PRDX1*, *RNF10*, *SQSTM1* and *USP9X* genes in Hep-2 cells treated with conditioned medium from fibroblast cultures. (B). *ARID4A* gene expression in 47 laryngeal and oral tongue carcinomas. Relative quantitation of target gene expression for each sample was calculated according to Pfaffl [50]; *GAPDH* was used as the internal reference and control 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.

Figure 5. Enlarged 2-DE gels of proteins from conditioned medium-treated Hep-2 cells and stromal fibroblasts. Five proteins (arrows), tubulin beta (A-B), alpha enolase (C-D), aldolase A (E-F), glyceraldehyde-3-phosphate dehydrogenase (G-H) and heterogeneous nuclear ribonucleoprotein C (I-J) were down-regulated in Hep-2 cell line treated with fibroblast conditioned medium (A, C, E, G and I) and two proteins (K-L),

vimentin (arrow on left) and actin (arrow on right), were underexpressed in fibroblasts treated with Hep-2 cell line conditioned medium (K).

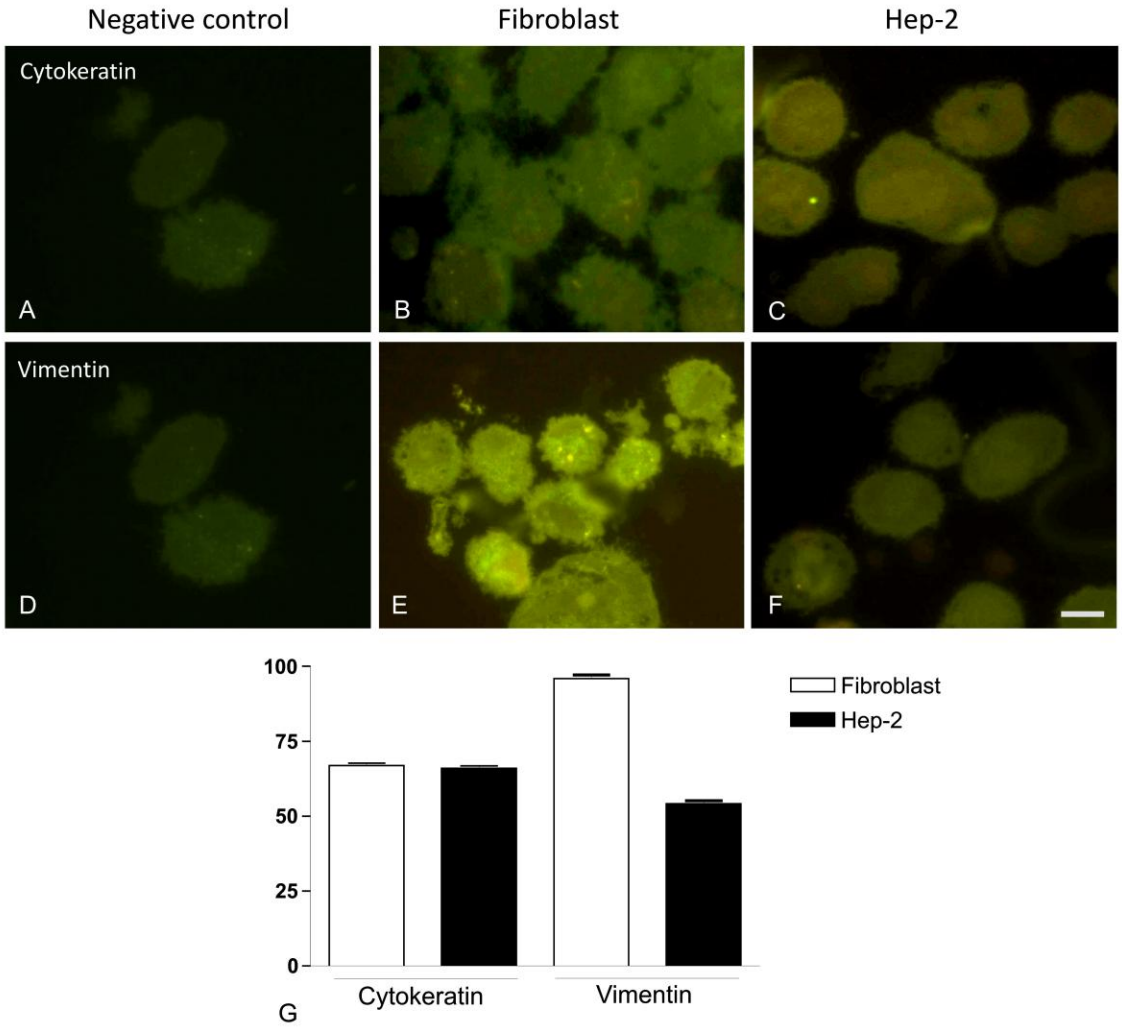
Table 1. Information on biological processes based on Gene ontology. Top down- and up-regulated genes selected by RaSH in Hep-2 samples treated with FCM.

Biological Process	***Down-regulated genes***
Cell communication	
signal transduction	*FAS, SQSTM1, YWHAZ*
Transcription	*ARID4A, CALR, MYC, PARP1, RNF10, SQSTM1*
Translation	*AARS, RPLP0, RPS17, RPS23*
Apoptosis	*CALR*
induction	*FAS*
anti-apoptosis	*TPT1, YWHAZ*
Cell migration	*TMSB4X*
Cell cycle	*DYNC1H1, MYC, PSMC6*
Cell proliferation	
negative regulation	*GPNMB, LDOC1*
positive regulation	*MYC*
Developmental process	
epidermis development	*UGCG*
Response to stimulus	
defense response	
inflammatory response	*LTA4H*
response to stress	*EIF2AK1, SQSTM1*
response to oxidative stress	
response to external stimulus	*EIF2AK1*
Transport	*CALR, NDUFA4, SQSTM1*
Metabolic process	*COX7C , OLA1,*
protein metabolic process	*PARP1, SQSTM1, USP9X*
protein modification process	*GRPEL2, HSP90AB1, PPP2R2A, PRPF4B, USP48*
lipid metabolic process	*LTA4H, UGCG*
DNA repair	*PARP1*
RNA processing	*PRPF4B, SF3B1*
Cellular homeostasis	*CALR, MYC, RPS17*
No classification	*GNB2L1, RCN1*

	Up-regulated genes
Transcription	*ENO1*
Translation	*EIF1, TARS*
Apoptosis	*RTN3*
induction	*DAP3*
Cell proliferation	*PRDX1*
negative regulation	*ENO1*
Developmental process	
organ development	*PRDX1*
Response to stimulus	
response to stress	*EIF1, RTN3*
Metabolic process	*PRDX1*
protein metabolic process	
protein modification process	*P4HB*
nucleic acid metabolic process	
RNA processing	*USP39*

Table 2. **Information on biological processes based on Gene Ontology**. Top down-regulated genes selected by RaSH in CAF samples treated with HCM.

Biological Process	*Down-regulated genes*
Cell communication	
signal transduction	*S100A6, FN1*
Transcription	*FOSL1*
Translation	*RPL37A, RPL7, RPL19, RPL27A, RPLP0*
Apoptosis	*CTSB*
anti-apoptosis	*TPT1*
Cell adhesion	*FN1*
Cell proliferation	
positive regulation	*S100A6, FOSL1*
Developmental process	
organ development	
epidermis development	*COL1A1*
Response to stimulus	
defense response	*FOSL1*
response to stress	*FN1*
Transport	*ERGIC3, STX4*
Metabolic process	
protein metabolic process	*CTSB*
RNA processing	*PRPF3*
No classification	*CIZ1, POLE4*


Negative control
Fibroblast
Hep-2
Cytokeratin
A
B
C
Vimentin
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100
75
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Fibroblast
Hep-2
Cytokeratin
Vimentin
G

Figure 1

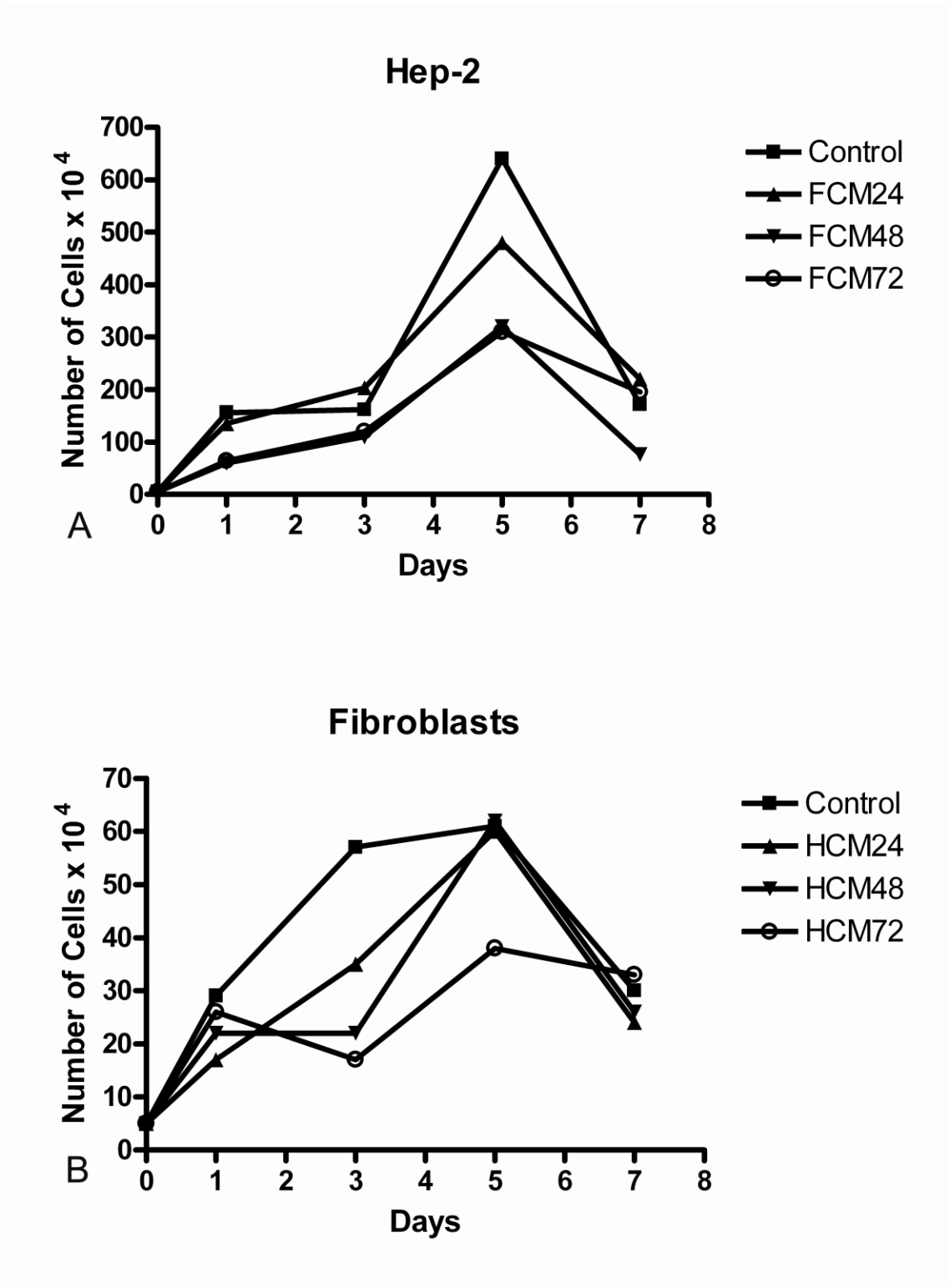

Hep-2
Number of Cells x 10 4
700
600
500
400
300
200
100
0
A
0 1 2 3 4 5 6 7 8
Days
Control
FCM24
FCM48
FCM72
Fibroblasts
Number of Cells x 10 4
70
60
50
40
30
20
10
0
B
0 1 2 3 4 5 6 7 8
Days
Control
HCM24
HCM48
HCM72

Figure 2

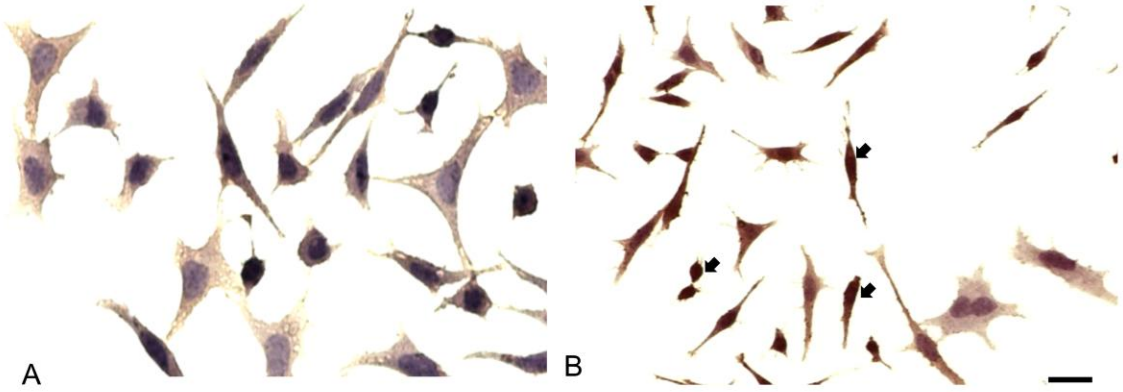


Figure 3

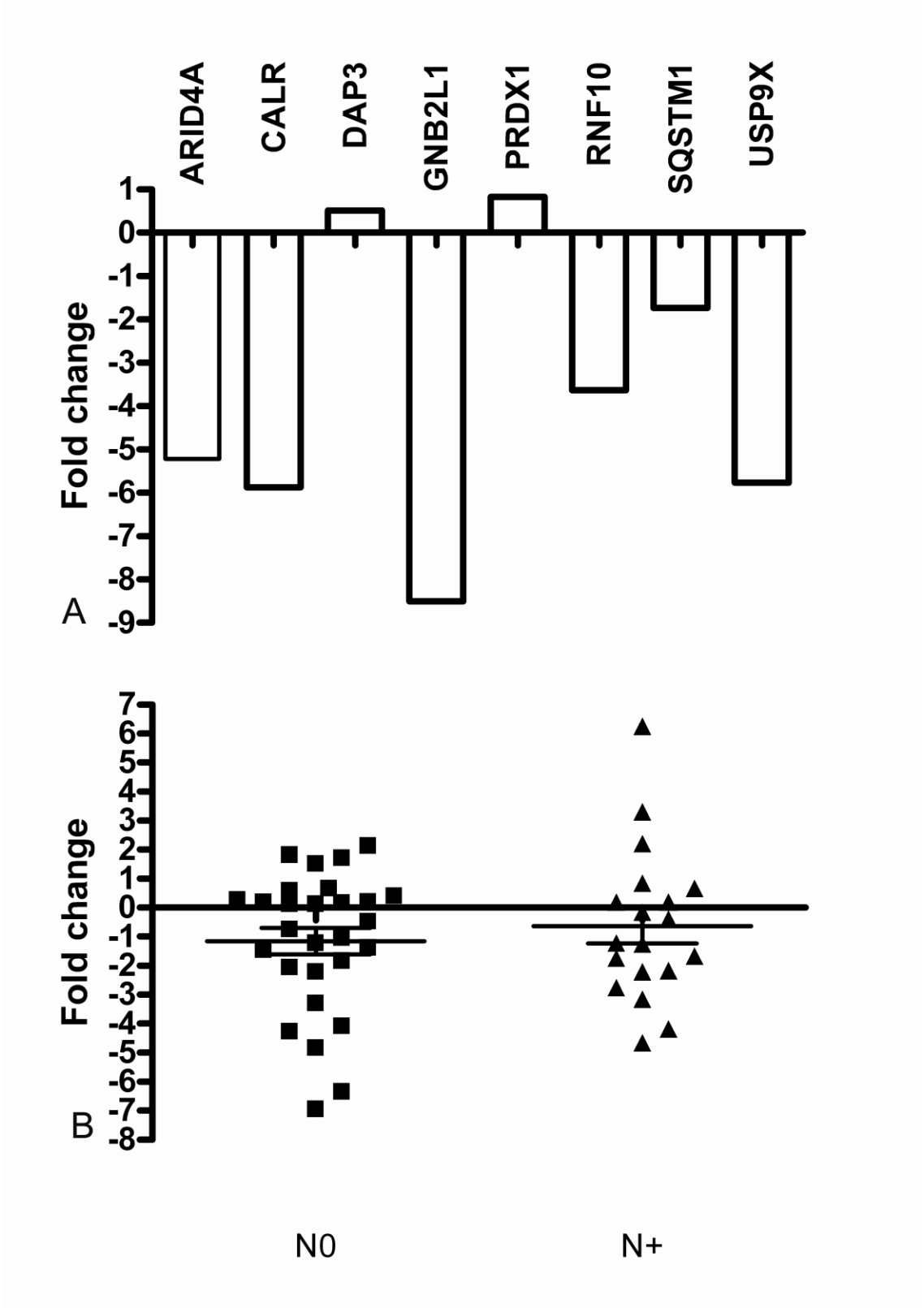

ARID4A
CALR
DAP3
GNB2L1
PRDX1
RNF10
SQSTM1
USP9X
Fold change
A
Fold change
B
N0
N+

Figure 4

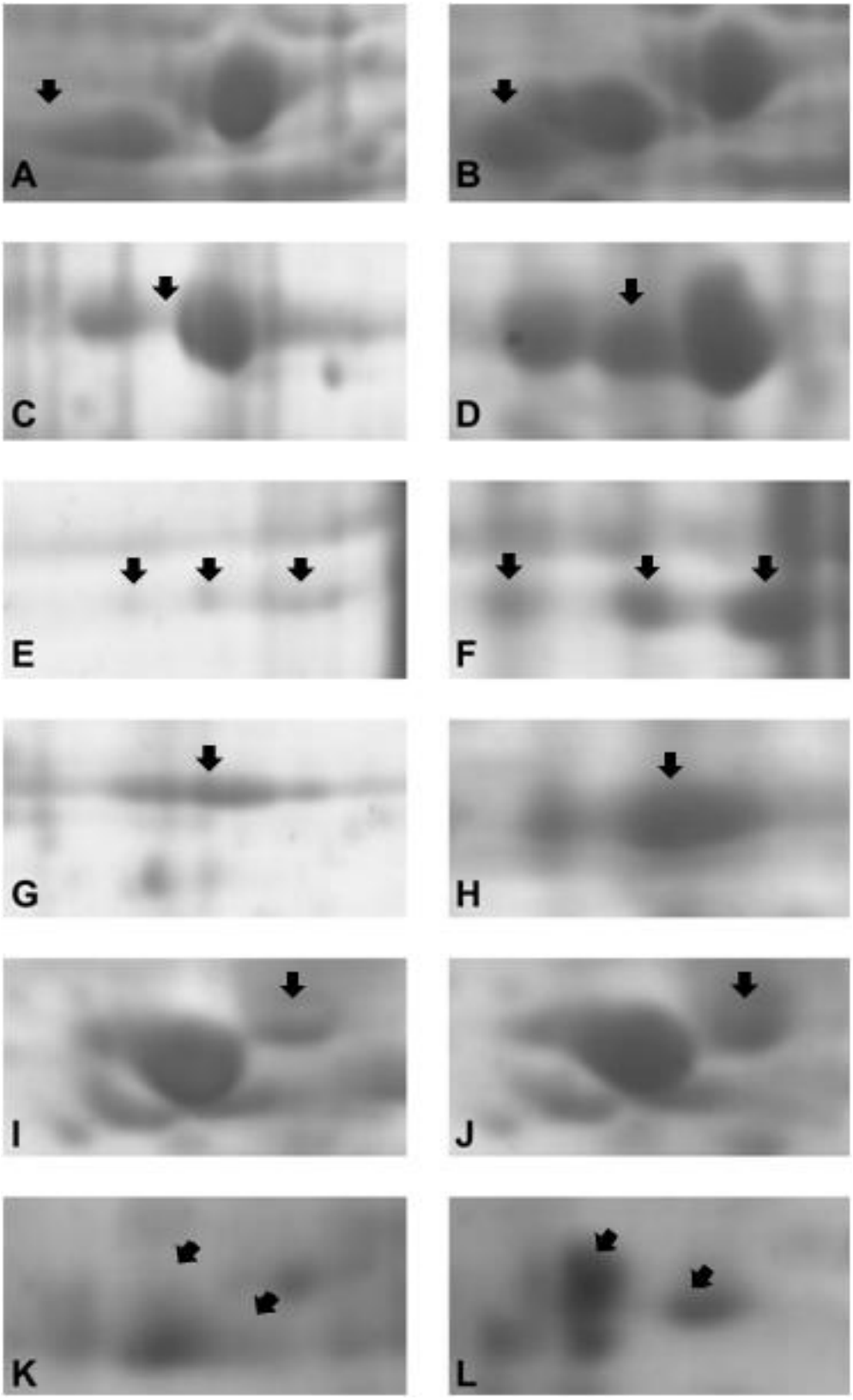


Figure 5

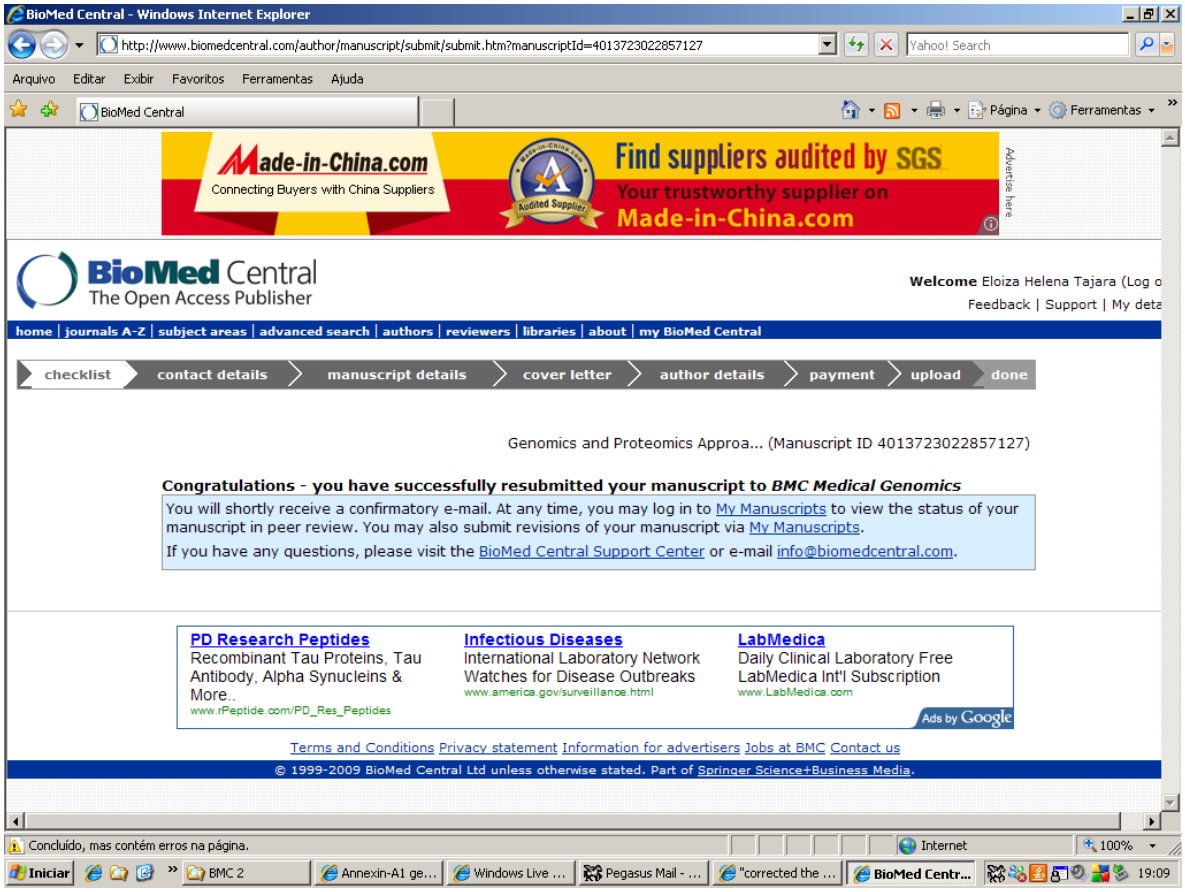
Supplementary Table 1. Clinicopathological features of 24 patients with larynx SCC and 23 patients with tongue SCC.

Case	Site	Pathologic stage	*ARID4A* expression (Fold change)	Histological differentiation	Vascular infiltration	Lymphatic infiltration	Perineural invasion
1	Tongue	T2N0M0	-6.94250	Well	No	No	Yes
2	Tongue	T2N0M0	-6.34424	Well	No		
3	Larynx	T3N0M0	-4.83139	Moderate	No	No	No
4	Larynx	T4N2BM0	-4.66025	Well	No	Yes	No
5	Larynx	TN1M	-4.27485	Moderate	No	No	No
6	Tongue	T1N2BM0	-4.18699	Well	No	Yes	No
7	Tongue	T3N0M0	-4.07653	Moderate	No	No	Yes
8	Larynx	T2N0M	-3.29721	Moderate	No	No	No
9	Larynx	T2NM	-3.15294	Moderate	No	No	Yes
10	Tongue	T2N2bM0	-2.75169	Well	No	Yes	No
11	Larynx	T4N0M0	-2.21241	Moderate	No	No	No
12	Larynx	T4N2cM0	-2.20134	Moderate	No	No	Yes
13	Tongue	T1N2bM	-2.17014	Moderate	No	No	No
14	Larynx	T3N0MX	-2.06172	Well	No	No	No
15	Tongue	T3N0M0	-1.82874	Moderate	No	Yes	Yes
16	Larynx	T2N1M0	-1.74822	Well	Yes	No	No
17	Larynx	TN3M	-1.67320	Poor	Yes	Yes	Yes
18	Tongue	T2N0M0	-1.45372	Moderate	No	No	No
19	Larynx	T4N0M0	-1.37537	Well	No	No	No
20	Tongue	T2N2bM0	-1.25641	Well	No	Yes	Yes
21	Larynx	T3N3M0	-1.21541	Moderate	Yes	Yes	Yes
22	Tongue	T1N0M0	-1.21272	Moderate	No	No	No
23	Larynx	T4N0M0	-1.04545		No	No	No
24	Tongue	T3N0M0	-0.74004	Moderate	No	No	Yes
25	Tongue	T3N0M0	-0.47105	Moderate	Yes	Yes	No
26	Tongue	T4N1M0	-0.39065	Well	No	No	No
27	Larynx	T4N2bM0	-0.15034	Moderate	No	No	No
28	Tongue	T2N0M0	0.12950	Well	No	No	No
29	Larynx	T3N0M0	0.13108	Moderate	No	No	No
30	Tongue	T3N0M0	0.16402	Well	No	No	No
31	Larynx	T2N0M0	0.18754	Well	No	No	No
32	Tongue	T4N2M0	0.19148	Poor	No	No	No
33	Larynx	T3N2cMx	0.19581	Moderate	No	Yes	No
34	Tongue	T3N0M0	0.20828	Moderate	No	No	No
35	Tongue	T2N0M0	0.27124	Moderate	No	No	No
36	Larynx	T2N0M0	0.40117	Moderate	Yes	No	Yes
37	Larynx	T3N0M0	0.59043	Moderate	No	No	Yes
38	Tongue	T4N2M0	0.66753	Poor	No	Yes	Yes
39	Larynx	Tr4N0M0	0.67217	Moderate		Yes	Yes
40	Larynx	T2N1M0	0.84555	Poor	No	No	No
41	Tongue	T3N0M0	1.51330	Moderate	No	No	Yes
42	Larynx	T4N0M0	1.71724	Well	No	No	Yes
43	Larynx	T3N0M0	1.82247	Well	No	No	No
44	Tongue	T2N0M0	2.13289	Well	No	No	No
45	Larynx	T4N2cM0	2.20806	Moderate	No	No	Yes
46	Tongue	T1N1M0	3.30854	Moderate	No	No	Yes
47	Tongue	T2N2cM0	6.26179	Moderate	No	No	Yes

Supplementary Table 2. Underexpressed proteins in Hep-2 cells and fibroblasts treated with conditioned medium from fibroblasts (FCM) and Hep-2 (HCM), respectively.

Protein	SwissProt accession	Score*	Sequence coverage (%)	Process
HEP-2 cells treated with FCM				
Alpha-enolase	P06733	84	11	transcription growth control hypoxia tolerance allergic responses
Heterogeneous nuclear ribonucleoproteins C (C1/C2)	P07910	85	10	RNA splicing
Fructose-bisphosphate aldolase A	P04075	143	12	glycolysis
Tubulin beta-1 chain	Q9H4B7	73	9	cell motion
Glyceraldehyde-3-phosphate dehydrogenase	P04406	71	10	glycolysis membrane trafficking
Fibroblasts treated with HCM				
Vimentin	P08670	82	5	cell motion
Actin, cytoplasmic 1	P60709	42	4	cell motion

*Scores greater than 40 were considered significant ($p < 0.05$).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ARTIGO CIENTÍFICO IV

Protein Profile in Head and Neck Squamous Cell Carcinomas by Tandem Mass Spectrometry Analysis: Evaluating the Invasive Phenotype

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Abstract

Lymph node metastasis is one of the most important prognostic factors in head and neck squamous cell carcinomas and critical for delineating their treatment. However, clinical and histological criteria for the diagnosis of nodal status in these tumors remain limited. In the present study, we aimed to characterize the proteomic profile of lymph node metastasis from 32 squamous cell carcinomas of the tongue, floor of the mouth and larynx using one and two-dimensional electrophoresis and mass spectrometry analysis. Thirty-four proteins were identified: 19 were overexpressed and 15 underexpressed in metastatic lymph nodes compared with non-metastatic ones. These proteins are involved in cytoskeleton organization, cell adhesion and migration, signal transduction, transcription, translation, transport, cell proliferation, apoptosis, homeostasis, metabolic processes, response to stimulus and developmental process. Five upregulated proteins (S100-A7, A-FABP, E-FABP, galectin-1 and PDI or protein disulfide-isomerase) have been associated with hypoxia and/or adhesion. Among the down-regulated proteins were γ-actin, tropomyosin alpha-3 chain and profilin-1, evoking a systemic actin cytoskeletal dysregulation, initiated probably before the tumor cells have left the primary site. Western blot analysis confirmed the results for two representative proteins of over and under expressed groups in metastasis (E-FABP and profilin-1, respectively). Our data on the expression of heat shock proteins and enzymes of the glycolytic pathway also suggested an effect of the lymph node environment in controlling tumor growth or in metabolic reprogramming of the metastatic cell. To our knowledge, this is the first study of lymph node metastasis of head and neck carcinomas using proteomic approaches. Most results were in accordance with the expected protein profile of the metastatic cell behavior. New potential markers such as profilin-1 and FABPs were identified and may prove useful for defining the metastatic phenotype of head and neck carcinomas.

Introduction

Metastases are the main cause of death in cancer patients [1]. The power of these malignant cells to kill their hosts resides in their ability to leave the primary tumor, disseminate and withstand ectopic sites, as well as to exhibit self-renewal and uncontrollable growth, leading to painful and incurable secondary tumors. In recent years, many data have revealed the determining factors mediating this destructive cascade, which include an extensive and growing list of epigenetic events and mutations [2, 3].

In the initial steps of metastatization, signals released by the stromal mesenchymal cells may cooperate to reduce cell-cell adhesion and to promote cell migration of epithelial tumor cells. The acquisition of these mesenchymal features is characteristic of an important reversible differentiation event during embryogenesis named epithelial-mesenchymal transition (EMT), which have also been implicated in tumor invasion and metastasis [4]. Typical examples of EMT regulators include small noncoding RNAs and members of the transforming growth factor beta (TGF-beta), tyrosine kinase receptor, Wnt and Notch pathways [5-10]. Abnormal activation of these pathways is important for the earlier stages of cancer development and, in addition to rendering the cell addicted or hypersensible to their effects, may provide an advantage for the next steps [11, 12]. In fact, microarray data have shown that primary tumors and metastases from the same individual share genetic changes, which are, therefore, conserved during tumor evolution [13]. The analysis of unmatched samples also suggests that the metastatic gene expression signature may already be present in the early phases of tumorigenesis [14].

As the tumor grows, low oxygen tension stimulates a proangiogenic response [15]. Due to microRNAs (miRs) [16] and cytokines secreted by neoplastic and stromal cells, [17], endothelial cells from pre-existing blood vessels synthesize adhesion molecules and proteases, allowing their migration through the degraded stroma to the tumor [18]. These migrating endothelial cells proliferate and generate new vessels which can supply oxygen and nutrients to sustain cancer growth and are an important route for metastasis. Lymphatic vessel formation, which is common in various inflammatory conditions, is also stimulated in some human cancers [19, 20] and evolves into the main route to spread tumors cells, when chemokine receptors and their ligands may play a critical role in metastasis to lymph nodes [21]. The lymphatic network is indeed more permissive for metastatic spread than the blood vascular system because their capillaries exhibit a single endothelial cell layer not surrounded by pericytes; have intercellular valve-like structures that facilitate the uptake of cells; and the basement membranes of the vessels are

incomplete [22]. Examples of tumors that frequently spread to regional lymph nodes are the head and neck carcinomas (HNSCC) [23], which are nearly always associated with chronic inflammation.

Arrival at a secondary site does not ensure success for most metastatic cells. The processes of extravasation and seeding require specific tumor characteristics and receptive conditions. To increase the chances of a favorable outcome, it has been suggested that target sites are prepared in advance by long-distance interaction with the primary tumor [24]. Depending on the tumor, substantial growth is observed before extravasation, within the blood vessels [25]. In other cases, the cells lie dormant at the new sites until appropriately stimulated [26], when the proliferation:apoptosis ratio as well as their ability to induce angiogenesis increase [27], or they overcome host immune reaction.

The pattern of metastatic seeding and colonization is not random and, depending on the primary site**,** tumor cells spread to particular organ sites more frequently than to others. The mechanism involved in this tropism is not completely known but chemokines and their receptors, as well as circulation patterns and structural features of capillaries in the secondary organ should be important (reviewed in [28]). Differential tropism occurs in breast cancers, which frequently spread to lung, bone, brain, and liver, whereas in head and neck carcinomas, regional lymph nodes are the preferential target sites and distant metastases are a late and rare finding [29]. Why do HNSCCs have this behavior whereas small cell carcinomas of the head and neck [30] and several tumors of salivary gland [31, 32], located in the same anatomical site, typically have distant metastases? The answer probably lies in the tumor and stromal cell features but the lymphatic network may be also important. In addition to their permissive structure as previously outlined, the hydrostatic pressure in the lymphatic system is lower compared to blood circulation, decreasing the mechanical challenge. Otherwise, lymph is richer in immune response factors which, although insufficient to destroy tumor cells [33], may play an important role in selecting immune resistance phenotypes.

Considering the atypical characteristic of HNSCC to remain a locoregional disease and the limitations of clinical and histological criteria for the diagnosis of lymph node metastasis [34], still the most powerful prognostic factor for these cancers [35], it is urgently necessary to define appropriate biomarkers of the metastatic phenotype for this group of diseases. In the present study, we aimed to characterize the proteomic profile of lymph node metastasis from 32 squamous cell carcinomas of the tongue, floor of the mouth and larynx using two-dimensional electrophoresis and mass spectrometry analysis.

Material and Methods

Tissue samples

Thirty-two samples of lymph nodes were obtained from patients with surgically resected head and neck squamous cell carcinomas before radio- or chemotherapy. This set included 20 metastatic (N+ or positive) and 12 non-metastatic (N0 or negative) lymph nodes from 9 tongue, 14 floor of the mouth and 9 larynx carcinomas**.** These sites were classified by The International Statistical Classification of Diseases and Related Health Problems 10th Revision ICD-10 as C02, C04, C32.8, respectively (http://www.who.int/classifications/icd/en/). An overlapping set of 22 samples (8 tongue, 8 floor of the mouth and 6 larynx carcinomas, 11 N+ and 11 N0) was analyzed by Western blot.

The samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from 9 institutions in São Paulo State, Brazil, whose aim is to develop clinical, genetic and epidemiological analysis of head and neck squamous cell carcinomas.

Tissue samples were obtained immediately after the removal of the surgical specimen, snap-frozen and stored in liquid nitrogen. Analysis of hematoxylin and eosin-stained sections detected the presence or absence of tumor cells in N+ and N0 lymph nodes, respectively, confirming the results of routine pathological analysis. 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.

Proteomic analysis

Proteomic analysis was performed according to the protocol described by de Marqui *et al.* [36]. All chemicals used were of high quality (Merck, Calbiochem, GE Healthcare, Sigma and Bio-Rad).

Sample preparation. In brief, lymph node samples were cut into small pieces and washed with 500 μl of lysis buffer containing 7M Urea, 2M Thiourea, 4% CHAPS, 65 mM DTT, and 0.2% carrier ampholytes. The specimens were disrupted by sonication twice for 60 s at 0°C and vortexed vigorously for approximately 2 min at 0^{o}C. The lysates were centrifuged at 10,000 g for 3 min at 4°C. Protein concentration of the resulting supernatant was determined by the Bradford method [37]. The protein samples were stored in aliquots at -80°C.

To minimize individual differences and to enable duplicate analysisof samples with limited amount of proteins, one- and two-dimensional electrophoresis experiments were performed using 3 pools (C02, C04, C32.8, respectively) of metastatic and 3 pools of non-metastatic lymph node samples from the same anatomical sites. The pools combined equal amounts of protein from each sample, resulting in a total of 1800 ug per pool.

One-dimensional gel electrophoresis. The pools of lymph nodes (N+ and N0) from larynx tumors were separated by one-dimensional 12% resolving/5% stacking sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE) according to Laemmli [38]. Under reducing conditions, the proteins were denatured at 96^{o} C for 5 min in 5X loading buffer with β-mercaptoetanol, and 100 ug of each pool were loaded into the wells. SDS-PAGE was carried out on a vertical polyacrylamide gel system (SE 400 Vertical Unit, GE Healthcare, Uppsala, Sweden) at a voltage of 120 V. Proteins were detected by Coomassie Blue staining, and the molecular mass was estimated using low molecular weight standard proteins of 14.4–97 kDa (LMW Calibration Kit for SDS Electrophoresis, GE Healthcare). Bands in the range of 10-20 kDa and 20-30 kDa were excised manually from gels and subjected to in-gel protein digestion and mass spectrometry protocols.

Two-dimensional gel electrophoresis (2-DE). The pooled samples were analyzed by 2-DE according to the protocol previously described by de Marqui et al.[36]. Proteins were cleaned using ice-cold acetone 100%, and centrifuged at 13,000g for 5 min at 4^{o} C. Aliquots containing approximately 1500 ug of protein were diluted with rehydration buffer (8 M urea, 2% CHAPS, 0.6% DTT, 0.5% IPG buffer pH 3-10, trace of bromophenol blue) to a total volume of 250 µL before loaded onto an immobilized linear pH gradient (IPG) strip (13 cm, pH 3-10 L, GE Healthcare).

After isoelectric focusing (IEF) on an IPGphor apparatus (GE Healthcare), the IPG strips were equilibrated for 15 min in equilibration solution (6 M urea, 50 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS, trace of bromophenol blue) containing 1% DTT, followed by incubation for 15 min in the same solution containing 2.5% iodoacetamide instead of DTT. IPG strips were sealed on top of a 12.5% SDS-polyacrylamide gel using 0.5% low-melting agarose in SDS running buffer with bromophenol blue.

Electrophoresis was performed using a SE 600 Ruby vertical electrophoresis unit (GE Healthcare) under conditions of 15 mA/gel for 30 min and 30 mA/gel for 7 h at 10^{o} C. The samples were run in triplicate and the LMW Calibration Kit (GE Healthcare) was used as a protein standard. After Coomassie Blue staining, the gels were scanned using an

ImageScanner (GE Healthcare) and the images were analyzed using the ImageMaster 2D Elite software (GE Healthcare) for spot detection, quantification, and comparative analysis. Only spots showing at least a two-fold change in their relative volumes were considered for mass spectrometry analysis. One protein-free gel piece and a gel piece from a protein ladder band were processed in parallel and used as negative and positive controls, respectively.

In-gel protein digestion and mass spectrometry (MS).

Slices from 1-DE gel and differentially expressed protein spots from 2-DE gels were manually cut out from the gels and digested with trypsin, according to the protocol previously described by de Marqui et al.[36], with modifications. The samples were destained in 250 μL of 50% acetonitrile (ACN)/25 mM ammonium bicarbonate and dehydrated with 50 μL of ACN for 15 min. Acetonitrile was discarded and the gel pieces were dried in Speed Vac for 30 min. A trypsin solution was added to each gel piece, and the sample was incubated for 24 h at 37^{o}C. Peptides were extracted with 50 μL 1% trifluoroacetic acid (TFA) for 12 h and 50 μL 1% TFA/50% ACN for 2 h. The supernatants were mixed and concentrated in a vacuum centrifuge to 5-10 μL.

Digested samples from one-dimensional gel were separated by C18 RP-HPLC column coupled with nanoUPLC (nanoAcquity)-electrospray tandem mass spectrometry on a Q-TOF Ultima mass spectrometer (Waters Corporation, Milford, MA, USA) at a flow rate of 0,6 μL/min. The gradient was 0-50% acetonitrile in 0.1% formic acid over 60 min. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected.

Peptide digests from two-dimensional gels were mixed with matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid, 0.1% TFA in 50% ACN) in a 1:1 (v:v) ratio, spotted on a stainless steel sample plate and air dried. Mass determinations were performed on a MALDI-Q-TOF (Matrix Assisted Laser Desorption Ionization - Quadrupole Ion Trap - Time of Flight) Premier (Waters Corporation). Each sample was run in duplicate.

For protein identification, the MS/MS data were searched against MSDB (Mass Spectrometry Protein Sequence Database) using Mascot Distiller version 2.2.1.0 and Mascot Daemon version 2.2.0 (Matrix Science Ltd., London, UK). The parameters for spectra acquisition were set up as follows: *Homo sapiens* taxonomy; trypsin enzyme; one missed cleavage site; carbamidomethylation of cysteine and oxidation of methionine as modifications; peptide tolerance of 0.1 Da; MS/MS tolerance of 0.1 Da; monoisotopic

masses. The criteria for positive identification of proteins were: (1) individual ion scores at $p<0.05$, and (2) molecular weights and isoelectric points matched to values obtained from image analysis.

Gene ontology (GO) annotation (http://www.geneontology.org/) was used to assign biological process terms for differentially expressed proteins.

Immunodetection

For Western blot analysis, the antibodies used were polyclonal primary anti-FABP5 and monoclonal primary anti-profilin 1 (Abcam, Cambridge, MA, USA) diluted 1:500, and monoclonal primary anti-β-Actin antibody (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:5000. In brief, protein samples (10 µg) were loaded onto 12% resolving gel with 5% stacking gel (SDS-PAGE) in denaturing conditions, at 120 V for 120 min. The molecular weight ladder was the PageRuler™ Prestained Protein Ladder (Fermentas Life Sciences, Vilnius, Lithuania).

The proteins were then transferred electrophoretically (90 V for 90 min, in Mini Protean 4 Cell, BioRad, Hercules, CA, USA) to PVDF membrane (Immobilon-P Membrane, Millipore, Bedford, MA, USA) using transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol). The PVDF membranes were submitted to chromogenic staining using the Western Breeze kit (Invitrogen, Carlsbad, CA, USA). The blots were then scanned and analyzed using a Kodak Gel Logic 2200 Digital Imaging System, (Carestream Health, Rochester, NY, USA).

Results

A total of 32 lymph nodes (20 N+ and 12 N0) from patients with head and neck squamous cell carcinoma were combined in 6 pools (A-F) and analyzed using 2-DE and mass spectrometry. The mean age of the patients was 57.4 years (range, 45-79 years), and the male/female sex ratio was 9.7:1. Most patients were smokers or former smokers (28/32) and had a history of chronic alcohol abuse (30/32). Median follow-up of the patients after primary surgery was 30 months. The clinicopathological features of these patients, including tumor stage, are presented in Supplementary Table 1.

Pooled samples were run on 2-DE, and gel triplicates exhibited a nearly identical protein spot map. After analysis by the imaging software and detailed manual checking, comparison of the protein profiles between metastatic and the corresponding non-

metastatic lymph-nodes from tongue, floor of the mouth and larynx carcinomas revealed 128 spots showing consistent differences in expression. Two-dimensional gel images of paired protein samples are shown in Figure 1 and Supplementary Figure 1.

Thirty-four proteins were identified by mass spectrometry and database searches. Observed and calculated molecular weight and pI showed a high correlation, reinforcing the validity of the results. Some proteins were present in trains of spots, suggesting distinctive post-translational modifications and isoforms derived from alternative splicing.

Nineteen proteins were overexpressed and 15 underexpressed in metastatic lymph nodes compared with non-metastatic ones (Supplementary Table 2, gel areas I-XI). These proteins are involved in cytoskeleton organization, cell adhesion and migration, signal transduction, transcription, translation, transport, cell proliferation, apoptosis, homeostasis, metabolic processes, response to stimulus and developmental process, as summarized in Table 1. It should be mentioned that several up- (albumin, galectin-7, glutathione S-transferase, heat shock 27 kDa protein, S100-A9 and stratifin) and downregulated (γ-actin, carbonic anhydrase 1 and tropomyosin alpha-3 chain) proteins in N+ lymph nodes were also detected in various primary tumors analyzed by our group (data not shown).

Some proteins exhibited a diverse profile in metastasis of tongue, floor of the mouth and larynx carcinomas. For example, A-FABP and apolipoprotein A-I only showed up-regulation in positive lymph nodes of floor of the mouth tumors, whereas differential expression of ARL-1, PDI, S100-A7, GAPDH, GRP94 and PKM was not observed in lymph nodes of larynx tumors, and CPI-B in floor of mouth tumor metastasis.

Because many differentially expressed proteins were observed in the range of 10 to 30 kDa in 2-DE gels, we also analyzed bands in this size range of one-dimensional gels, to evaluate the differences between both profiles of lymph node samples from larynx carcinoma. In addition to having many proteins in common with the pattern obtained by 2-DE (Supplementary Tables 3 and 4), one-dimensional electrophoresis analysis revealed several other interesting proteins involved in apoptosis **(**elongation factor 1-alpha 2**),** cell cycle **(**eukaryotic peptide chain release factor GTP-binding subunit ERF3B), adhesion (periostin, collagen alpha-2 (VI) chain), signaling **(**protein DJ-1**,** Ras-related C3 botulinum toxin substrate 2**,** Rho GDP-dissociation inhibitor 2 and several members of Rab family), protein folding (T-complex protein 1 subunit zeta**,** peptidyl-prolyl cis-trans isomerase A), cytoskeleton (coactosin-like protein**),** differentiation (transgelin-2), inflammation (annexin 1) and, not surprisingly, in T cell receptor signaling pathways **(**ubiquitin-conjugating enzyme E2 N), transport (hemoglobin chains), immune **(**Rho GDP-dissociation inhibitor 2 and immunoglobulin chains) and stress response **(**superoxide dismutase [Mn],

mitochondrial). These results, although nonquantitative, provided additional information on the protein profile of head and neck cancer metastasis.

Two proteins displaying up- (E-FABP) or down-regulation (PFN1) in lymph nodes metastasis were selected for validation by Western blotting experiments in 22 samples. The selection was carried out after a literature analysis on the potential involvement of the proteins in cancer development and progression. The results for E-FABP paralleled those observed in 2-DE gels. For PFN1, besides the expected 14-kDa band, which was present in all samples, a band of 26 kDa was observed in N+ samples, again confirming the data obtained by 2-DE (Figure 2).

Discussion

HNSCC is an anatomically heterogeneous malignancy related to alcohol and tobacco consumption, which arises from the mucosa of distinct anatomical sites within the head and neck, including oral cavity, pharynx, and larynx. Although this group of carcinomas is considered one disease, clinical and molecular data indicate that it comprises different entities [33]. In fact, gene expression analysis shows that even oral subsites have different molecular profiles [39]. Divergences in tumor behavior are also well known; for instance, carcinomas of the base of tongue, hypopharynx and supraglottis are typically aggressive whereas glottic carcinomas exhibit better prognostic [33, 40]. Such differences may be in part explained by the influences of micro-environmental factors including the lymphatic network in head and neck region.

Lymph node metastasis remains one of the most important prognostic factors in HNSCCs and critical for delineating their treatment [41]. However, there is a high incidence of occult metastases even in N0 patients and no suitable method with high sensitivity to detect them [42], making the understanding of the molecular basis of lymphatic spreading a very important issue. Proteomic tools provide a powerful approach to identifying many novel proteins or groups of proteins involved in this process, especially those showing post-translational modification, such as phosphorylation, glycosylation or proteolytic cleavage [43].

In the present study, we investigated the proteomic profile of lymph node metastasis from squamous cell carcinomas of tongue, floor of the mouth and larynx, by using uni and two-dimensional electrophoresis and mass spectrometry analysis. Nineteen proteins were up-regulated and 15 down-regulated in metastatic lymph nodes. Western blot analysis

confirmed the results for two representative proteins of under and over expressed groups (E-FABP and PFN1, respectively)

Some of the over expressed proteins may play an important role in the head and neck tumorigenesis and metastatization processes. For example, ARL-1 (aldo-keto reductase family 1 member B10), an enzyme induced by tobacco carcinogens [44] and probably responsible for inactivating toxic aldehydes [45], is a potential biomarker for non-small cell lung cancer [46]. ARL-1 exhibits a very high retinal reductase activity [47], thus depleting the pool of retinal available to form retinoic acid, and consequently inducing cell proliferation and loss of differentiation [48].

ARL-1 [49] as well as several other upregulated proteins have already been associated with HNSCC, such as cystatin B [50], E-FABP [51, 52] , heat shock 27 [53, 54], galectin-1 [53, 55], galectin-7 [49, 51, 56], glutathione S-transferase P [49, 51, 54, 57], S100-A9 or calgranulin B [53], S100-A7 or psoriasin [58], and stratifin [49, 54, 58-60]. These proteins have also been associated with other cancers, such as A-FABP [61], apolipoprotein A-I or APO-A1 [62], cytosol aminopeptidase [63], E-FABP [64], glutathione S-transferase P heat shock 27 [65, 66], protein disulfide-isomerase [67], S100-A9 [64, 68]) and S100-A11 or calgizzarin [69, 70] .

The increased expression of these proteins in the primary tumor may explain deregulation of cell adhesion, cell growth, apoptosis and migratory capabilities (heat shock 27, galectin-1 and -7, GSTP1-1, stratifin), epidermis development (E-FABP), xenobiotic metabolism (GSTP1-1), as well as tumor immune escape (galectin-7) and defense or inflammatory response (S100-A9, S100-A7). However, in regard to the metastasis environment, many questions remain. What proteins are predictive biomarkers for regional metastasis in HNSCC? Likewise, what features were previously selected and manifest in cells leaving the primary tumor? After arriving in the lymph nodes, what would be the new challenge for the tumor cells? Tentative answers to these questions may be exemplified by the findings we obtained for S100-A7, A-FABP, galectin-1 and protein disulfide-isomerase. These proteins have been shown to be associated with hypoxia [71-74], a common adverse condition faced by metastatic, as well as primary tumor cells. The recent findings of Chaudary and Hill [75] reinforce the idea that ‘hypoxia-related’ factors regulate lymph node metastasis under intermittent hypoxic conditions. According to these authors, lymphatic vessels occur more often only in the periphery of tumors, which are regions of acute hypoxia and may stimulate cell spreading by lymphatics, leading to increased lymph node metastasis [76].

Concerning E-FABP, our proteomic approach detected that this member of the fatty acid-binding protein family is over expressed in lymph node metastasis, a result also supported by the Western blotting experiments. This finding is contrary to the data of Uma and collaborators in India [77] and the difference may be caused by distinctive patterns of tobacco exposure and by RNA versus protein analysis, since the expression of both does not necessarily correspond.

Ruse et al [78] observed that E-FABP and S100-A7, both over expressed in our samples, stabilize the level of each other, and colocalize in focal adhesion-like structures in response to calcium, possibly as part of a proteic complex with an important role in the metastatic process. Abnormal expression of S100 proteins has already been detected in metastasis of lung and colorectal cancers [79, 80] and associated with lymph node positive tumors [69, 81-83] and invasive/migratory phenotype [84]. Similar results have been observed for stratifin and apolipoprotein A-I, which show high expression in lymph node metastasis of primary colonic adenocarcinomas [62] and human lung squamous carcinoma [85] and even in serum of patients with metastatic lung cancer [86].

Among the down-regulated proteins in metastatic lymph nodes were proteins involved in cell motility (γ-actin, tropomyosin alpha-3 chain), cell differentiation, proliferation and communication (calreticulin), apoptosis and response to stress (peroxiredoxins-2, calreticulin, heat shock proteins), cytoskeleton organization (profilin-1), transcription, translation (calreticulin) and metabolic processes (carbonic anhydrase 1, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase isozym, triosephosphate isomerase). For several proteins, the observed expression profile confirmed the results of previous studies on primary tumor cells. For example, carbonic anhydrase 1, an enzyme involved in maintaining the pH homeostasis by catalyzing hydration of carbon dioxide, is lost in colorectal tumors [87].

Profilin 1 [88] and tropomyosin alpha-3 chain [89, 90] have also been observed downregulated in cancer cells. Profilin 1 regulates signal-dependent actin polymerization during cell motility [91]. The fact that this protein binds to many ligands involved in cytoskeletal control and other functions [92] may explain an unexpected band of 26 kDa in our Western blots of N0 lymph nodes, in addition to the 14-kDa band typical of profilin. Tropomyosin is another regulator of the actin cytoskeleton [93] that has been described forming oncogenic fusion proteins derived from chromosomal rearrangements [94-96] and related to new blood vessel formation [97]. These data, added to the observation of γ-actin loss, suggest the involvement of systemic dysregulation of cellular cytoskeletal proteins that probably occurs before the tumor cells have left the primary site.

For several of the identified proteins, divergent results have been described in the literature with respect to their expression in cancer cells. One example is calreticulin, which has been observed upregulated [98-101] or downregulated [102-104] in tumors. These findings may be explained by changes in calcium content and fluxes across the endoplasmic reticulum (ER) in different tissues. As Bergner and Huber [105] clearly explain in their review, increased proliferation, decreased differentiation and decreased apoptosis are all regulated by calcium. Therefore, cells showing low levels of calreticulin, a chaperone in the ER involved in regulation of calcium homeostasis, probably have a low level of ER Ca^{2+} and are more resistant to apoptotic stimuli [106]. Peroxiredoxin-2 is another protein showing divergent results in cancer, both down [65, 107, 108] and upregulation [109]. This protein has antioxidant activities and is also implicated in cell proliferation and differentiation. As Memon et al [107] concluded, the differences in expression may be related to cancer type and stage of the tumor.

Unexpected results were observed for heat shock proteins GRP75 and GRP94, and enzymes of the glycolytic pathway (GAPDH, triosephosphate isomerase and pyruvate kinase), which showed downregulation in our samples. The explanation for these findings may be related to the effect of the lymph node immune environment in controlling tumor growth or in metabolic reprogramming of the metastatic cell considering the blood flow, oxygen and nutrient supplies in the secondary site. In addition, some proteins may show enhanced expression in normal cells of lymph nodes, as previously observed for pyruvate kinase [110], making difficult the differentiation between N+ and N0 samples.

Conclusion

To our knowledge, this is the first study of lymph node metastasis of head and neck carcinomas using proteomic approaches. Most results were in accordance with the expected protein profile of the metastatic cell behavior. New potential markers such as profilin-1 and FABPs were identified and may prove useful for defining the metastatic phenotype of head and neck carcinomas.

The increased expression of the proteins associated with hypoxia S100-A7, A-FABP, galectin-1 and protein disulfide-isomerase is in accordance with the idea that hypoxia stimulates cell spreading by lymphatics, leading to increased lymph node metastasis. In turn, the down-regulation of γ-actin, tropomyosin alpha-3 chain and profilin-1 evoke a systemic actin cytoskeletal dysregulation, initiated probably before the tumor cells have left the primary site. In addition, our data on the expression of heat shock proteins and

enzymes of the glycolytic pathway suggest an effect of the lymph node environment in controlling tumor growth or in metabolic reprogramming of the metastatic cell.

The observation of several proteins with differential expression between lymph node metastasis from tongue, floor of the mouth and larynx carcinomas reinforces the idea that head and neck sites and subsites are dissimilar entities and their behavior may be influenced by micro-environmental factors including the lymphatic network.

Authors' contributions

AV participated in the design of the study, carried out proteomic and Western blot experiments, data analysis and drafted the manuscript. AML helped with Western blot experiments. PMC carried out histophatological analysis for sample selection. GMP helped with proteomic experiments. MBC and EEF carried out clinical data analysis for sample selection. GENCAPO team members were responsible for sample collection and initial on-site sample processing provided the pathological analysis of the cases obtained the informed consent and discussed the findings. EHT participated in the design and coordination of the study, data analysis and drafted the manuscript. All authors revised and approved the final manuscript.

Appendix

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

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FIGURE LEGENDS

Figure 1. Enlarged 2-DE gels of human lymph node proteins from HNSCC patients. Partial images showing proteins with different levels in patients with tongue (C02), floor of the mouth (C04) and larynx cancer (C32.8). **Up-regulated proteins.** A-FABP - fatty acid-binding protein, adipocyte; APO-AI - apolipoprotein A-I; ARL-1 - aldo-keto reductase family 1 member B10; CPI-B - cystatin-B; E-FABP - fatty acid-binding protein, epidermal; Gal-7 - galectin-7; PDI - protein disulfide-isomerase*;* S100-A7 – psoriasin; S100-A9 – calgranulin-B; S100-A11 – calgizzarin. **Down-regulated proteins**. GRP75 - heat shock 70 kDa protein 9; GRP94 - heat shock protein 90 kDa beta member 1; PFN1 – profilin-1; PRP - peroxiredoxin-2; TIM - triosephosphate isomerase.

Figure 2. **Analysis of E-FABP and PFN 1 proteins.** Representative Western blots illustrating the (A) E-FABP and (B) PFN1 expression in tumor-free (N0) and positive (N+) lymph nodes. β-actin was used as an internal control. MW=PageRuler Prestained Protein Ladder.

ADDITIONAL FILES

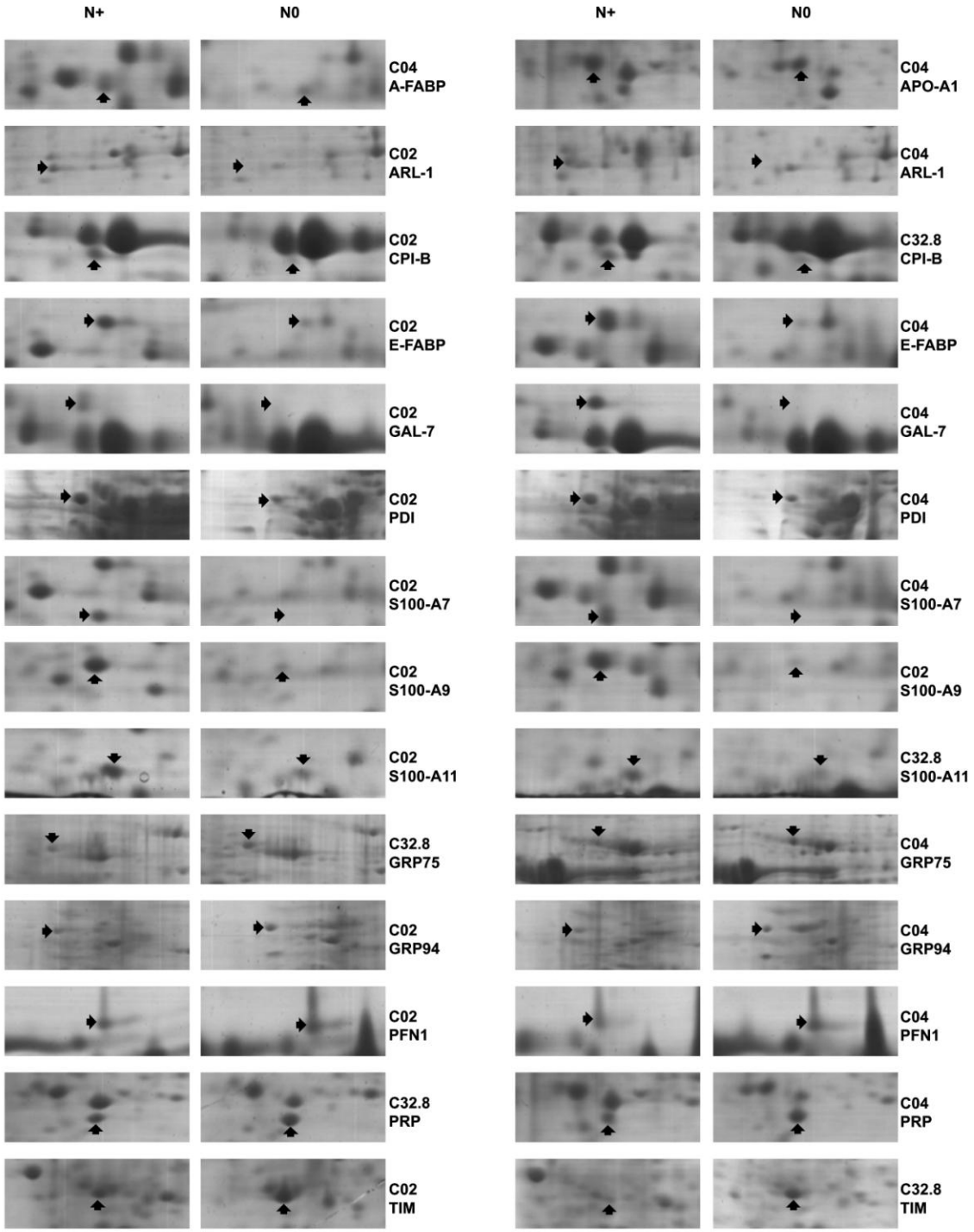
Supplementary Figure 1. Two-dimensional electrophoresis maps of human lymph node pools from HNSCC patients. (A) Negative lymph nodes (N0) from patients with (A) tongue – C02, (C) floor of the mouth – C04 and (E) larynx cancer –C32.8; positive lymph nodes (N+) from patients with (B) tongue, (D) floor of the mouth and (F) larynx cancer.

Supplementary Table 1. Clinicopathological features of patients.

Supplementary Table 2. Proteins expressed in lymph node pools from HNSCC patients. Proteins separated by two-dimensional electrophoresis and identified by MALDI-Q-TOF MS/MS.

Supplementary Table 3. Proteins expressed in negative lymph nodes (N0). Proteins separated by one-dimensional gel electrophoresis and identified by ESI-Q-TOF MS/MS.

Supplementary Table 4. Proteins expressed in positive lymph nodes (N+). Proteins separated by one-dimensional gel electrophoresis and identified by ESI-Q-TOF MS/MS.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N0
N+
N0
C04
A-FABP
C04
APO-A1
C02
ARL-1
C04
ARL-1
C02
CPI-B
C32.8
CPI-B
C02
E-FABP
C04
E-FABP
C02
GAL-7
C04
GAL-7
C02
PDI
C04
PDI
C02
S100-A7
C04
S100-A7
C02
S100-A9
C02
S100-A9
C02
S100-A11
C32.8
S100-A11
C32.8
GRP75
C04
GRP75
C02
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C04
GRP94
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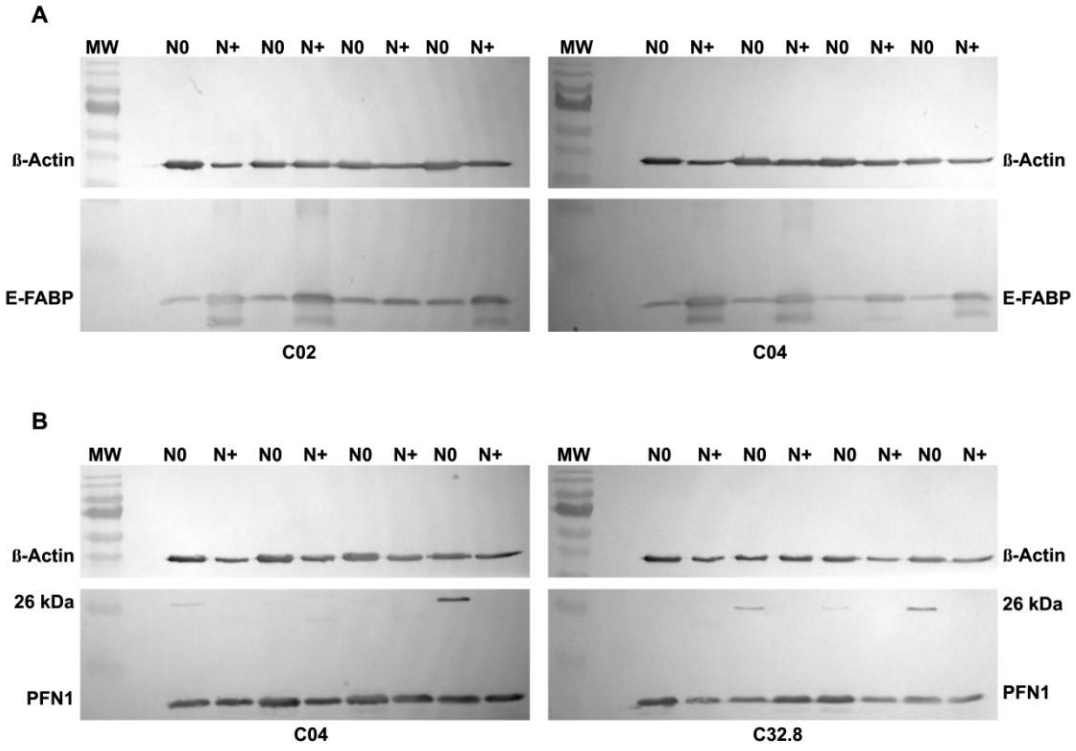
PFN1
C32.8
PRP
C04
PRP
C02
TIM
C32.8
TIM

Figure 1


A
MW N0 N+ N0 N+ N0 N+ N0 N+
MW N0 N+ N0 N+ N0 N+ N0 N+
ß-Actin
E-FABP
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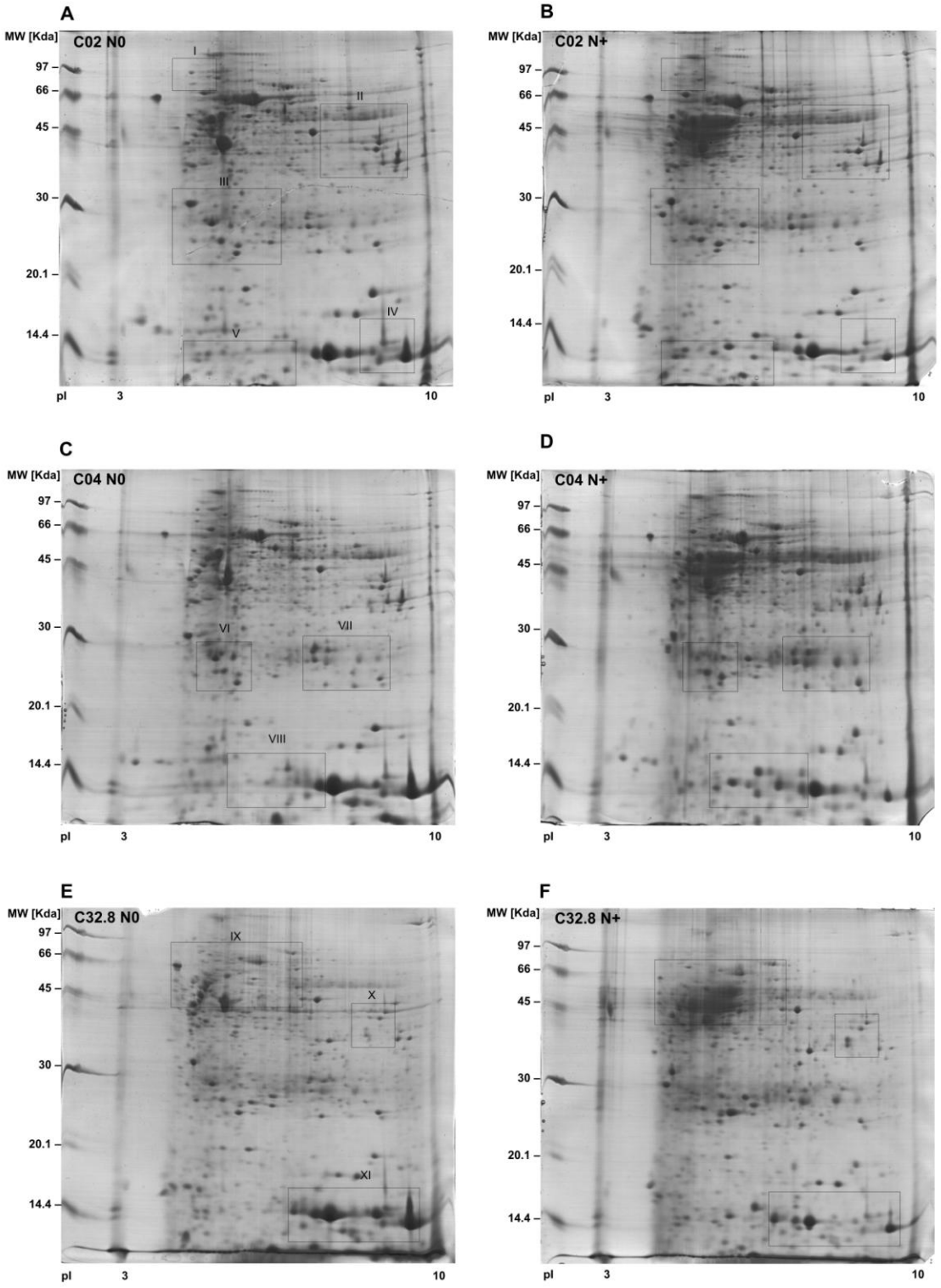
E-FABP
C02
C04
B
MW N0 N+ N0 N+ N0 N+ N0 N+
MW N0 N+ N0 N+ N0 N+ N0 N+
ß-Actin
26 kDa
PFN1
ß-Actin
26 kDa
PFN1
C04
C32.8

Figure 2



Supplementary Figure 1

Table 1. Information on biological processes based on Gene ontology. Up- and down-regulated proteins selected from proteomic analysis of positive lymph node samples.

Biological process	Up-regulated proteins
Apoptosis	Gal-1
induction	14-3-3σ
anti-apoptosis	ALB, GSTP1-1, HspB1
Cell adhesion	
cell-cell adhesion	Gal-7
Cell communication	
signal transduction	Apo-AI, ARL-1, Gal-1, S100-A11, 14-3-3σ
cell-cell signaling	S100-A9
Cell migration	
cell motility	HspB1
Cell proliferation	
negative regulation	S100-A11
Developmental process	
system development	GSTP1-1
cell differentiation	A-FABP
epidermis development	CK-1, E-FABP, S100-A7
Metabolic process	ARL-1
protein metabolic process	Apo-AI, CPI-B, LAP
lipid metabolic process	Apo-AI, ARL-1, A-FABP, E-FABP
protein modification process	PDI
aldehyde metabolic process	ARL-1
Response to stimulus	A-FABP, HspB1, S100-A7
defense response	S100-A7
inflammatory response	A-FABP, S100-A9
immune response	Apo-AI, Igγ1, IgκC
response to stress	ALB
response do DNA damage	14-3-3σ
Replication	
negative regulation of DNA replication	S100-A11
Transcription	A-FABP
Translation	HspB1
Transport	ALB, A-FABP, Apo-AI
Biological process	**Down-regulated proteins**
Apoptosis	
regulation of apoptosis	CRP55,
anti-apoptosis	GRP94, GRP75, GRP78, PRP
Cell communication	
signaling	CRP55
Cell migration	
cell motility	γ-actin, TMα3
Cell proliferation	
positive regulation	CRP55
Cytoskeleton organization	PFN1
Developmental process	
cell differentiation	CRP55
Metabolic process	CA-I
protein modification process	CRP55
lipid metabolic process	TIM
protein metabolic process	GRP75
monosaccharide metabolic process	GAPDH, PKM, TIM
Response to stimulus	
response to stress	GRP78, GRP94, PRP
Replication	
DNA replication	CRP55
Transcription	CRP55
Translation	CRP55
Transport	α1 globin; β globin, δ globin, CRP55

Up-regulated proteins. A-FABP - Fatty acid-binding protein, adipocyte; ALB - albumin; Apo-AI - Apolipoprotein A-I; ARL-1 - Aldo-keto reductase family 1 member B10; CK-1 - Keratin, type II cytoskeletal 1; CPI-B - Cystatin-B; E-FABP - Fatty acid-binding protein, epidermal; Gal-1 - Galectin-1; Gal-7 - Galectin-7; GSTP1-1 - Glutathione S-transferase P; HspB1 - Heat shock 27 kDa protein; Igγ1 - Ig gamma-1 chain C region; IgκC - Ig kappa chain C region; LAP - Cytosol aminopeptidase; PDI - Protein disulfide-isomerase; S100-A11 - Calgizzarin; S100-A7 - Psoriasin; S100-A9 - Calgranulin-B; 14-3-3σ – Stratifin

Down-regulated proteins. γ-actin - Actin, cytoplasmic 2; CA-I - Carbonic anhydrase 1; CRP55 - Calreticulin; GAPDH - Glyceraldehyde-3-phosphate dehydrogenase; α1 globin - Hemoglobin subunit alpha; β globin - Hemoglobin subunit beta; δ globin - Hemoglobin subunit delta; GRP94 - Heat shock protein 90 kDa beta member 1; GRP78 - Heat shock 70 kDa protein 5; GRP75 - Heat shock 70 kDa protein 9; PFN1 - Profilin-1; PKM - Pyruvate kinase isozym; PRP - Peroxiredoxin-2; TIM - Triosephosphate isomerase; TMα3 - Tropomyosin alpha-3 chain

Supplementary Table 1. Clinicopathological features of patients.

Case[a]	Primary site*	Sex	Age (yrs)	Pathologic stage	Lifetime smoking (yrs)	Lifetime alcohol consumption (yrs)	Patient status	Survival months
CP1/0017	C02	M	55	T2N0M0	45	29	alive	61
CP1/0151	C02	M	47	T3N0M0	33	33	dd	18
CP1/0212	C02	M	69	T4N0M0	54	49	alive	50
CP1/0232	C02	F	79	T3N0M0	-	-	alive	41
CP1/0057	C02	M	57	T4N2BM0	49	49	dc	02
CP1/0273	C02	M	56	T4N2BM0	-	-	alive	24
CP1/0280	C02	M	45	T3N2BM0	30	30	dd	14
CP1/0281	C02	M	57	T4N2BM0	49	39	dd	17
CP3/0113	C02	M	67	T4N1M0	-	49	alive	64
CP3/0332	C02	M	56	T3N2bM0	38	43	dd	09
CP1/0053	C04	M	52	T2N0M0	24	24	alive	50
CP1/0086	C04	M	61	T1N0M0	40	40	alive	52
CP1/0240	C04	M	75	T2N0M0	63	63	alive	29
CP1/0248	C04	M	50	T4N0M0	36	36	alive	24
CP3/0094	C04	M	68	T4N0M0	50	33	dd	13
CP1/0055	C04	M	56	T4N2BM0	36	31	dd	18
CP1/0070	C04	F	48	T4N1M0	33	31	dd	19
CP1/0095	C04	M	63	T3N2CM0	46	37	dd	11
CP1/0154	C04	M	59	T4N2bM0	45	40	dc	04
CP1/0171	C04	M	70	T2N1M0	-	59	alive	26
CP1/0183	C04	M	72	T4N2BM0	63	59	dd	13
CP1/0225	C04	F	48	T3N2CM0	32	30	alive	50
CP1/0230	C04	M	63	T4N2CM0	46	43	dd	04
CP1/0175	C32.8	M	61	T4N0M0	47	40	alive	47
CP1/0180	C32.8	M	74	T4N0M0	38	37	alive	47
CP3/0301	C32.8	M	54	T4aN0M0	29	19	alive	38
CP1/0042	C32.8	M	70	T4N2CM0	55	48	dd	23
CP1/0058	C32.8	M	50	T4N2CM0	40	26	alive	65
CP1/0066	C32.8	M	72	T4N2BM0	5	52	dd	04
CP3/0041	C32.8	M	52	T4N2cM0	32	-	alive	70
CP3/0105	C32.8	M	67	T4N2cM0	37	53	dd	<1
CP3/0290	C32.8	M	50	T4N2cM0	18	18	alive	41

dd = dead by disease; dc = dead by commorbidity.
*Sites classified by ICD-10 in C02 (tongue), C04 (floor of mouth), C32.8 (larynx).

Supplementary Table 2. Proteins expressed in lymph nodes pools from HNSCC patients. Proteins separated by two-dimensional electrophoresis and identified by MALDI-Q-TOF MS/MS.

Protein name	Mascot Accession	Swiss-Prot accession	pI	Mass	Sequence coverage (%)	Score	Queries matched	Area	Tumor
Actin, cytoplasmic 2	ATHUG	P63261	5.31	42108	12	230	3	VI	Down
Albumin	ABHUS	P02768	5.92	71317	4	78	3	VIII	Up
Aldo-keto reductase family 1 member B10	Q6FHF3_HUMAN	O60218	7.12	36226	12	149	3	II	Up
Apolipoprotein A-I	LPHUA1	P02647	5.56	30759	11	104	3	VI	Up
Calgizzarin	I37080	P31949	6.56	11847	23	76	2	V	Up
Calgranulin-B	B31848	P06702	5.71	13291	42	100	3	VIII	Up
Calreticulin	A37047	P27797	4.29	48283	9	167	3	IX	Down
Carbonic anhydrase 1	1AZM	P00915	6.65	28620	16	116	3	VII	Down
Cystatin-B	UDHUB	P04080	7.90	11224	24	64	2	XI	Up
Cytosol aminopeptidase	AMPL_HUMAN	P28838	6.29	53006	4	71	3	IX	Up
Fatty acid-binding protein, adipocyte	FABPA_HUMAN	P15090	6.81	14692	22	83	2	VIII	Up
Fatty acid-binding protein, epidermal	FABPE_HUMAN	Q01469	6.84	15366	18	94	2	VIII	Up
Galectin-1	LEG1_HUMAN	P09382	5.34	14917	8	54	1	V	Up
Galectin-7	LEG7_HUMAN	P47929	7.00	14992	19	73	2	VIII	Up
Glutathione S-transferase P	GSTP1_HUMAN	P09211	5.44	23438	12	78	2	VI	Up
Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	P04406	8.58	36070	4	71	1	II	Down
Heat shock 27 kDa protein	HHHU27	P04792	5.98	22826	20	105	3	III	Up
Heat shock 70 kDa protein 9	Q6GU03_HUMAN	P38646	6.03	73967	3	78	2	IX	Down
Heat shock protein 90 kDa beta member 1	A35954	P14625	4.76	92696	4	69	3	I	Down
Hemoglobin subunit alpha	Q53F97_HUMAN	P69905	8.72	10703	42	263	3	XI	Down
Hemoglobin subunit beta	Q6R7N2_HUMAN	P68871	6.75	16112	23	127	3	XI	Down

Hemoglobin subunit delta	HDHU	P02042	7.97	16028	19	64	2	IV	Down
Ig gamma-1 chain C region	GHHU	P01857	8.46	36596	9	71	2	X	Up
Ig kappa chain C region	Q6GMW1_HUMAN	P01834	7.55	26077	14	48	2	VII	Up
Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	8.16	66018	4	82	2	VII	Up
Peroxiredoxin-2	PRDX2_HUMAN	P32119	5.67	21918	19	135	4	VI	Down
Profilin-1	PROF1_HUMAN	P07737	8.48	15085	10	50	2	IV	Down
Protein disulfide-isomerase	ISHUSS	P07237	4.76	57480	8	201	4	IX	Up
Psoriasin	S10A7_HUMAN	P31151	6.26	11433	34	103	3	VIII	Up
Pyruvate kinase isozyme	KPYM_HUMAN	P14618	7.95	58339	8	152	3	II	Down
Triosephosphate isomerase	TPIS_HUMAN	P60174	6.51	26807	8	51	1	VI	Down
Tropomyosin alpha-3 chain	AAF87083	P06753	4.79	29019	14	177	4	III	Down
14-3-3 protein sigma	Q6FH51_HUMAN	P31947	4.64	27874	15	98	3	III	Up

Supplementary Table 3. Proteins expressed in negative lymph nodes (N0). Proteins separated by one-dimensional gel electrophoresis and identified by ESI-Q-TOF MS/MS.

Mascot Acession	Swiss-Prot accession	Protein name	Sequence coverage (%)	Score	Queries matched
1BJ5	P02768	Albumin	23	754	15
AAN84548	P68871	Hemoglobin subunit beta	78	729	19
AAZ83699	P02042	Hemoglobin subunit delta	78	656	14
TPIS_HUMAN	P60174	Triosephosphate isomerase	45	550	10
PSHUAM	P63104	14-3-3 protein zeta/delta	34	548	13
Q1HDT5_HUMAN	P69905	Hemoglobin subunit alpha	64	524	32
LPHUA1	P02647	Apolipoprotein A-I	33	436	10
PROF1_HUMAN	P07737	Profilin-1	52	355	6
CAI13096	Q06830	Peroxiredoxin-1	47	347	9
PRDX2_HUMAN	P32119	Peroxiredoxin-2	27	346	5
ATHUG	P63261	Actin, cytoplasmic 2	18	331	7
GDIS_HUMAN	P52566	Rho GDP-dissociation inhibitor 1	33	304	6
1433B_HUMAN	P31946	14-3-3 protein beta/alpha	34	302	9
CSHUA	P62937	Peptidyl-prolyl cis-trans isomerase A	28	278	6
AAG41947	P04264	Keratin, type II cytoskeletal 1	8	256	4
BLVRB_HUMAN	P30043	Flavin reductase	19	205	3
PRDX6_HUMAN	P30041	Peroxiredoxin-6	14	184	4
HHHU27	P04792	Heat shock 27 kDa protein	14	178	3
CAA82315	P35527	Keratin, type I cytoskeletal 9	5	177	3
HSHUB1	P06899	Histone H2B type 1-J	28	176	3
CAB66585	P61026	Ras-related protein Rab-10	16	172	3
Q6FIG4_HUMAN	Q6FIG4	RAB1B protein	18	160	3
CAH1_HUMAN	P00915	Carbonic anhydrase 1	14	158	3

JC2488	Q15286	Ras-related protein Rab-35	10	143	2
AAH40679	P59190	Ras-related protein Rab-15	10	142	2
Q6GMX4_HUMAN	Q6GMX4	IGL@ protein	16	141	3
S15076	P27348	14-3-3 protein theta	16	136	6
Q5U0C3_HUMAN	Q5U0C3	RAP1A, member of RAS oncogene family	12	132	2
JC5394	Q99497	Protein DJ-1	16	124	3
G3P_HUMAN	P04406	Glyceraldehyde-3-phosphate dehydrogenase	8	120	2
H3T_HUMAN	Q16695	Histone H3.1t	17	117	4
Q7Z7M7_HUMAN	Q7Z7M7	Superoxide dismutase	15	115	4
B49002	Q9UL80	Myosin-reactive immunoglobulin light chain variable region	35	114	2
TAGL2_HUMAN	P37802	Transgelin-2	13	109	2
MOHU6M	P60660	Myosin light polypeptide 6	27	107	3
GSTP1_HUMAN	P09211	Glutathione S-transferase P	14	106	2
Q3T1C1_HUMAN	P28066	Proteasome subunit alpha type-5	12	104	3
CAG46469	Q13162	Peroxiredoxin-4	7	97	2
H2A1A_HUMAN	Q96QV6	Histone H2A type 1-A	12	90	2
COTL1_HUMAN	Q14019	Coactosin-like protein	17	81	3
THIO_HUMAN	P10599	Thioredoxin	12	78	1
Q5JXB2_HUMAN	Q5JXB2	Putative ubiquitin-conjugating enzyme E2 N-like	12	77	2
Q6FI44_HUMAN	Q6FI44	RAB5A, member RAS oncogene family, isoform CRA_a	10	74	2
Q4W5B0_HUMAN	Q9H082	Ras-related protein Rab-33B	4	67	1
VIME_HUMAN	P08670	Vimentin	2	62	1
EFHU1	P68104	Elongation factor 1-alpha 1	4	57	2
MLE1_HUMAN	P05976	Myosin light chain 1, skeletal muscle isoform	6	56	1
FABPE_HUMAN	Q01469	Fatty acid-binding protein, epidermal	6	55	1
Q8IYD1_HUMAN	Q8IYD1	Eukaryotic peptide chain release factor GTP-binding subunit ERF3B	1	52	1
B34386	P15153	Ras-related C3 botulinum toxin substrate 2	7	51	1

Supplementary Table 4. Proteins expressed in positive lymph nodes (N+). Proteins separated by one-dimensional gel electrophoresis and identified by ESI-Q-TOF MS/MS.

Mascot acession	Swiss-Prot accession	Protein name	Sequence coverage (%)	Score	Queries matched
CAA23759	P68871	Hemoglobin subunit beta	68	495	11
Q6P5S8_HUMAN	Q6P5S8	IGK@ protein	39	473	34
1AIN	P04083	Annexin A1	25	374	8
K2C6A_HUMAN	P02538	Keratin, type II cytoskeletal 6A	14	314	8
Q53F97_HUMAN	P69905	Hemoglobin subunit alpha	50	309	29
B31848	P06702	Calgranulin-B	68	283	25
1BJ5	P02768	Albumin	15	245	9
TPIS_HUMAN	P60174	Triosephosphate isomerase	31	235	6
K1C13_HUMAN	P13646	Keratin, type I cytoskeletal 13	11	229	4
HHHU27	P04792	Heat shock 27 kDa protein	25	224	5
Q29XZ0_HUMAN	Q15063	Periostin	4	223	2
PSHUAM	P63104	14-3-3 protein zeta/delta	22	198	5
KRHU9	P08727	Keratin, type I cytoskeletal 19	8	173	4
ATHUG	P63261	Actin, cytoplasmic 2	10	135	4
CSHUA	P62937	Peptidyl-prolyl cis-trans isomerase A	23	117	4
CAI13096	Q06830	Peroxiredoxin-1	22	111	4
PRDX6_HUMAN	P30041	Peroxiredoxin-6	8	87	2
G3P_HUMAN	P04406	Glyceraldehyde-3-phosphate dehydrogenase	4	83	1
PGAM1_HUMAN	P18669	Phosphoglycerate mutase 1	7	83	1
TAGL2_HUMAN	P37802	Transgelin-2	30	83	4
AAF17709	P30085	UMP-CMP kinase	6	76	1
SAHUP	P07602	Proactivator polypeptide	2	75	1
AAH03018	Q7Z4W1	L-xylulose reductase	11	72	1

TCPZ_HUMAN	P40227	T-complex protein 1 subunit zeta	3	68	1
FRIL_HUMAN	P02792	Ferritin light chain	16	63	2
TAGL_HUMAN	Q01995	Transgelin	10	62	2
K3HUNG	P01621	Ig kappa chain V-III region NG9	9	61	1
S10A7_HUMAN	P31151	Psoriasin	19	58	1
1433B_HUMAN	P31946	14-3-3 protein beta/alpha	14	55	3
Q6FHW5_HUMAN	Q6FHW5	COL6A2 protein	11	53	1
CAB66585	P61026	Ras-related protein Rab-10	5	52	1
Q5JXB2_HUMAN	Q5JXB2	Putative ubiquitin-conjugating enzyme E2 N-like	7	48	1
TFHUP	P02787	Serotransferrin	2	46	1

ARTIGO CIENTÍFICO V

Salivary and serum proteomics in head and neck carcinomas – before and after surgery and radiotherapy

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Running title:

Key words: head and neck carcinoma, saliva, serum, proteomics, two-dimensional electrophoresis, mass spectrometry

Competing interests:
Authors declare that they have no competing interests.

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ABSTRACT

In the present study, we performed bidimensional electrophoresis (2-DE) and mass spectrometry to evaluate saliva and serum proteins from patients presenting head and neck squamous cell carcinoma (HNSCC) and compared the results before and after therapy and also with those of healthy donors. Thirteen proteins showed an altered pattern of expression in saliva of cancer patients, including over-expression of keratins, immunoglobulins, alpha-amylase, PLUNC and zinc-alpha-2-glycoprotein and down-regulation of myosin. In serum samples, six proteins were over-expressed (serum albumin, alpha-1-microglobulin/bikunin precursor, apolipoprotein A-I, haptoglobin, serotransferrin, transthyretin) and two were under-expressed (hemoglobin subunit alpha, hemoglobin subunit beta) compared to the control group. This is the first report showing over-expression of protein PLUNC and zinc-alpha-2-glycoprotein in saliva from HNSCC patients. Both proteins may contribute to control tumor growth and, therefore, represent targets for new analysis. Similarly, a modified transthyretin form was detected with altered levels in serum from patients and may be involved in head and neck tumorigenesis, as suggested for other tumors. Although the number of patients analyzed and proteins detected with differential expression between healthy and HNSCC patients was low, the present study adds information on the complex group of secreted proteins and their role in cancer process and emphasizes the potential of saliva and serum analysis for diagnosis and monitoring of HNSCC patients.

INTRODUCTION

The ability to monitor health and disease status and the treatment outcome through noninvasive means is a desirable aim in the health promotion. In this context, human body fluids, such as saliva, plasma, serum, urine, tears and breast nipple aspirate, provide several key advantages in comparison with tissue biopsy and other body fluids, including low invasiveness and cost, easy sample collection and processing, as well as reduced anxiety and discomfort for patients, especially when longitudinal monitoring over time is necessary (reviewed by [1, 2])

Composed of a mix of oral fluids from major and minor salivary glands and blood derivatives [3], saliva probably is the most easily accessible fluid of our body. It plays important roles in maintaining oral health and homeostasis, participating of teeth enamel remineralization, defense against microorganisms, lubrification, preliminary digestion of foods, pH modulation and taste perception process [3-6](These attributes are derived from different components, which include proteins, hormones, electrolytes as calcium, bicarbonate, phosphate and fluoride, and small molecules as urea [3]. Salivary proteins have been studied by traditional biochemical or high-throughput proteomics approaches and more than one thousand of them have already been identified both in whole saliva and in secretions from individual glands, although those expressed in low levels have certainly not yet been detected [7-30] (http://www.biosino.org/bodyfluid/fluid.jsp?bf=Saliva; http://hspp.dent.ucla.edu/cgi-bin/hspmscgi-bin/search_pro_c.cgi). The reported proteins and peptides are mucins, immunoglobulins, agglutinins, proline-rich proteins, cystatins, histatins, defensins, peroxidases, amylase, albumin, lipase, lysozyme, lactoferrin, statherin and several others. Many of them form complexes and show posttranslational modifications like glycosylation, acetylation, deamidization, sulfatation and phosphorylation. Regarding the distribution in functional categories, salivary proteins mainly belong to immune response, cell communication, cytoskeleton, metabolic process and cell proliferation categories, and also to protein pathways involved in neurodegenerative diseases, diabetes and cancer [29].

The increasing interest in saliva as an attractive fluid for diagnosis has led to standardization of collection and storage processes [31] mainly because several factors

may affect salivary flux and composition. Different collecting methods for unstimulated or stimulated whole oral fluid, single glandular secretion, crevicular fluid and mucosal transudate are available, each one designed to address distinctive objectives [32]. In addition to blood and gland contributions as well as arrest and stimulation of salivation, other factors such as circadian rhythm, physiological status, medication, food intake, gender and age also affect the flow rates and composition of saliva [30] (also reviewed by [33]). These features have been exploited in the monitoring of hormone [34] and drug levels [35], exposition to environmental pollutants [36] and infections [37]. The analysis of flow rates and saliva constituents also has potential to be used as a valuable tool for monitoring several pathological conditions since substantial changes in both parameters have been associated with local and systemic diseases including periodontitis [38], diabetes mellitus [39], cystic fibrosis [40], rheumatoid arthritis, Sjögren's syndrome [41], salivary gland disorders [42], breast [43, 44], ovarian [45] and oral cancer [46-49].

With respect to oral cancer, the first reports on the use of saliva for screening or diagnostic purpose are relatively recent [50, 51]. The proximity to head and neck tumors offers to saliva an important advantage in relation to other body specimens, besides the noninvasive characteristic and the compatibility with proteomic approaches. Really, salivary proteomics can be explored for early detection of tumors, predicting aggressiveness and prognosis, and surveillance for cancer recurrence [29]. A comparison between samples from healthy and patients may reveal unique or increased levels of specific proteins as well as the presence of isoforms due to post-translational modifications that may be used as diagnostic biomarkers [17].

Similarly to saliva, serum and plasma also are very important sources of biological markers and, although with different compositions, both may provide rich information on physiological and pathological processes. However, differently of saliva, the analysis of serum and plasma for diagnostic purpose is largely known [52-54]. These fluids have intracellular and extracellular origin and reflect cell metabolism or death. Most proteins are synthesized by the liver, endothelium, central nervous system and blood cells and their main functions include salt-water balance, transport and defense, as do vasopressin, transferrin and immunoglobulins (reviewed by [1]). Because protein levels in plasma/serum show inter and intra-individual variations and also vary in time and

space, standardization of collection and storage process is a critical point for consistent results [2].

Recently, high-throughput proteomics approaches have been used to identify specific and sensitive biomarkers in serum/plasma [55, 56]. A huge amount of different proteins have been identified by collaborative studies [57], although approximately 20 of the most abundant ones in plasma correspond to 99% of the total mass of protein. Among these are apolipoproteins, transthyretin (prealbumin) and retinol-binding protein [58].

Many diseases have been the focus of serum proteomics profiling such as diabetes [59], as well as autoimmune [60], heart [61] and infection diseases [62]. Promising data have been reported for a variety of cancers, including colon [63, 64], ovarian [65], breast [66], head and neck [67, 68] and other cancers [69].

In the present study, we performed bidimensional electrophoresis (2-DE) and mass spectrometry to evaluate saliva and serum proteins from patients presenting head and neck squamous cell carcinoma (HNSCC) and compared the results before and after therapy and also with those of healthy donors. Our data add more information to the biology of HNSCC and confirm the potential application of saliva and serum proteome analysis to the identification of biomarkers associated with this group of diseases.

MATERIAL AND METHODS

Case selection. Unstimulated whole saliva was obtained from 7 male patients with head and neck squamous cell carcinoma at Hospital de Base de São José do Rio Preto, SP (6 smokers or former smokers, mean age=65±11.4 years) and 10 unmatched healthy donors (1 female and 9 male, nonsmokers, mean age=53±11.0 years). Serum samples were obtained from 15 HNSCC patients (1 female and 14 male, 13 smokers or former smokers, mean age=69.1±10.0 years) treated with surgery and radiotherapy at the same Hospital. Unmatched healthy controls for serum analysis consisted of 20 smokers or former smokers and 16 nonsmokers (mean age=41.2±11.3 years), of which 8 of them were also farm workers exposed to smoke from sugar cane burning.

The patient set included four metastatic (N+ or positive) and 11 non-metastatic (N0 or negative) carcinomas from the base (n=1) and border of tongue (n=1), floor of

mouth (n=1) and other parts of the mouth (n=2), piriform sinus (n=1), palate (n=2), and larynx (n=7), classified by The International Statistical Classification of Diseases and Related Health Problems 10th Revision ICD-10 (http://www.who.int/classifications/icd/en/) and TNM system (Ed Sobin LH WC. TNM Classification of Malignant Tumours. 6th Edition ed. New Jersey: John Wiley & Sons, Hoboken; 2002).

The study protocol was approved by the Committee of Ethics in Research of at Faculdade de Medicina de São José do Rio Preto - FAMERP (CEP 266/2005) and informed consent was obtained from the individuals enrolled.

Saliva samples. Samples from patients and controls were collected after awakening and after midday. The donors were asked to abstain from eating, drinking, smoking, or brushing their teeth for at least 2 hours prior to collection. Samples from patients were also collected before and <1-60 months after surgery and radiotherapy. Radiotherapy was administered in 180 cGy fractions daily to a total dose of 5040-7020 cGy. To evaluate the effect of circadian rhytm on proteomic profile, samples from one control were obtained after awakening, just before lunch and dinner and just after lunch.

Approximately 2 mL of whole human saliva was collected by spitting directly into a clean 15 mL conical tube, without protease inhibitors. To minimize degradation, the samples were kept on ice during the collection procedure. Immediately after the collection, samples were centrifuged at 13,000g for 5 min at 4^{o}C to remove debris. For proteolysis evaluation, a sample from a female control was collected in the same way but kept on ice for 3 hours before processing. The protein concentration of the resulting supernatants was determined by the Bradford method [(70)] and the samples were stored at -80°C until use.

The experiments were performed using three pools: one pool for patients before (n=2) and one pool for patients after treatment (n=6) and one for controls (n=8). The pools combined equal amounts of protein from each sample, resulting in a total of 1200 ug per pool.

Serum samples. Blood samples were obtained by venipuncture and were allowed to clot at 5°C for 60-120 min. The tubes were centrifuged at 3500*g* for 10 min; the serum was aspirated and the protein concentration was determined by the Bradford method [(70)] . The samples were aliquoted and stored at –80 °C.

The experiments were performed using individual samples from patients, one collected before and one after treatment. In addition, one experiment was performed using two pools of samples from patients with metastatic (n=3) and non-metastatic (n=9) carcinomas, respectively. Samples from controls were pooled in four batches of 8, 4, 8 and 8 samples, respectively. The pools combined equal amounts of protein from each sample, resulting in a total of 1800 ug per pool.

Two-dimensional gel electrophoresis (2-DE) and in-gel digestion. Two-dimensional gel electrophoresis and in-gel digestion were performed essentially as described previously by de Marqui AB et al [71]. Briefly, the saliva supernatant or serum sample was mixed with 3 volumes of ice-cold acetone, and the protein precipitate was isolated by centrifugation at 13,000g for 5 min at 4° C. Aliquots containing 1000 ug and 1500 ug of protein were diluted with rehydration buffer (8 M urea, 2% CHAPS, 0.6% DTT, 0.5% IPG buffer, bromophenol blue trace) to a total volume of 250 μL and applied onto Immobiline DryStrips (13 cm, pH 3-10 L, GE Healthcare). After isoelectric focusing (IEF) on an IPGphor apparatus (GE Healthcare), the IPG strips were placed on top of a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Electrophoresis was performed using a SE 600 Ruby vertical electrophoresis unit (GE Healthcare) under conditions of 15 mA/gel for 30 min and 30 mA/gel for 5 h at room temperature. The samples were run in duplicate.

Proteins were detected by Coomassie Blue staining protocol. The gels were scanned using an ImageScanner (GE Healthcare) and the resulting images were analyzed using the ImageMaster 2D Elite software (GE Healthcare) for spot detection, quantification, and comparative and statistical analysis. Only spots showing at least a two-fold change in their relative volumes were considered for mass spectrometry analysis.

Protein spots of interest were cut manually from the gel, distained, dehydrated, rehydrated and digested with proteomics-grade modified trypsin (Promega-USA) for 16 h at room temperature. Negative and positive control digests were performed on gel slices that did not contain any protein and on slices cut from an eletrophoretic band of the protein molecular weight marker, respectively.

Mass spectrometry. The peptide digest and the matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid, 0.1% v/v TFA in 50% v/v ACN) were mixed at a ratio

of 1:1. A volume of 1 µL of the mixture was spotted onto a stainless steel sample plate and left to dry at room temperature. Samples were analyzed using MALDI-Q-TOF (matrix-assisted laser desorption ionisation-quadrupolo-time of light) PREMIER (Waters-USA), mass spectrometry (MS/MS) operating in reflectron mode. Triplicate runs of each sample were made to ensure an accurate analysis. For protein identification, the resulting MS/MS data were interpreted by the MASCOT software (MS/MS Ions Search) and searched against the Mass Spectrometry Protein Sequence Database (MSDB). Only proteins with a score higher than $p<0.05$ were considered.

Gene ontology (GO) annotation (http://www.geneontology.org/) and KEGG Pathways (Kyoto Encyclopedia of Genes and Genomes) (http://cgap.nci.nih.gov/Pathways/) were used for the functional classification of differentially expressed proteins.

RESULTS

Clinical and/or demographic information for the HNSCC and control groups are presented in Tables 1 and 2.

Saliva proteins from 7 HNSCC patients and 10 healthy donors were analyzed by combined 2-DE and MALDI-TOF-TOF mass spectrometry. In the control group, several changes were observed between samples from a male and a female subject, but not for samples from the same individuals collected over the day. The delay in processing specimens also showed no differences relative to samples immediately processed. Image analysis of the gels showed good matching between aliquots, suggesting that a massive degradation of proteins did not take place for up to 3 hours after collection, at least under the conditions the sample was taken.

With respect to HNSCC patients, significant differences were observed in comparison to the healthy subjects, considering saliva samples collected at the same time point. Radiotherapy affected saliva flux and composition: approximately 1ml/5min could be collected from the patients after radiation therapy, which represents about 2 times less than that of the control subject. Thirteen proteins showed an altered pattern of expression in cancer patients, including over-expression of keratins, immunoglobulins, alpha-amylase, PLUNC (palate lung and nasal epithelium clone protein) and zinc-alpha-

2-glycoprotein and down-regulation of myosin (Figure 1, Supplementary Table 1). These proteins are involved in cell adhesion, cell differentiation and epidermis development, metabolic processes, transport and immune response (Table 3).

In serum samples of HNSCC patients, six proteins were over-expressed (serum albumin, alpha-1-microglobulin/bikunin precursor, apolipoprotein A-I, haptoglobin, serotransferrin, transthyretin) and two were under-expressed (hemoglobin subunit alpha, hemoglobin subunit beta) compared to the control group. They are involved in apoptosis, cell killing, signaling, homeostasis, metabolic processes, response to stimulus and transport (Figure 2, Table 4, Supplementary Table 2). Farm worker protein profiles were similar to those of controls, except for apolipoprotein A-1 that exhibited over-expression, similarly to the patients.

The data of saliva and serum analysis of patients after treatment were compared with those obtained in the preoperative period. The results showed that the protein profile after treatment reverted to a pattern closer to those observed for controls, except for haptoglobin, which exhibited higher level of expression after radiotherapy.

DISCUSSION

HNSCCs constitute a group of aggressive diseases affecting different subsites of the head and neck. An early diagnosis is critical for the successful treatment of the patients and the prognosis depends on the lymph node status at the time of diagnosis. Still nowadays, even with remarkable advances in molecular biology extending our understanding of cancer, no sensitive and specific marker is available to help the management of HNSCC patients and for monitoring the disease course or to evaluate individuals at risk.

Most HNSCC research, as for other cancers, has focused on identifying molecular alterations in tumor cells. More recently, body fluids have been evaluated as new sources for biomarkers discovery. There is no doubt that the analysis of several body fluids has enormous advantages over those of surgical specimens, including low invasiveness, easy sample collection, storage and processing. In this context, saliva and serum seem promising diagnostic or predictive tools for head and neck diseases, especially because they may contain cell components released by the tumor, therefore

may reflect the complex processes taking place in the tumor microenvironment. Otherwise, many variables challenge the optimization of protocols for each step of both saliva and serum analysis.

In the present study, we used two-dimensional gel electrophoresis coupled with mass spectrometry (MS/MS) to evaluated serum and saliva from HNSCC patients and controls. Some differences were noted between the 2-DE protein profiles from saliva of a male and a female healthy subject. Albeit limited to two samples, the results indicated that patients and controls should be matched for sex, in accordance of the data published by Papale et al. [30] . In addition, samples that remain in the surgical center beyond a time window of 3 hours, even if kept on ice, may result in a distinctive protein profile in relation to those processed immediately or less than three hours after collection. Although no differences have been observed between samples of the same donor over the day, patient and control samples were collected only at two points (after awakening and after midday). As discussed by Papale et al. [30] , endogenous proteases may have an increased release during the post-prandial period. Therefore, collecting the saliva at different time points may account for some of the changes we observed in protein expression levels.

As expected, flow rates were reduced after radiotherapy as well as the total protein concentration, which can be explained by radiation effect on the salivary gland activity [72]. In fact, Marzi et al [73] demonstrated that the recovery of salivary gland function takes place within approximately 1 year after radiotherapy. Compared to non-irradiated patients or healthy subjects, flow rates changed in irradiated patients.

Significant differences in saliva profile were detected between patients and healthy subjects. Over-expression of immunoglobulins, keratins, PLUNC and zinc-alpha-2-glycoprotein should reflect immune responses to tumor antigens as well as mucosa degradation due to proteolytic events. Up-regulation of PLUNC has recently been detected in gastric carcinomas and their metastasis [74], salivary gland tumors [75] and even in peripheral blood and pleural fluid of lung patients [76]. Interestingly, Zhou et al [77] observed a lower frequency of PLUNC transcript expression in nasopharyngeal carcinomas than in chronic inflammation of nasopharyngeal mucosa, reinforcing the role of this protein in the immune response. Zinc-alpha-2-glycoprotein is another

interesting protein that inhibits the proliferation of oral squamous cell carcinoma cells [78] and may be part of a mechanism to control tumor growth.

Similarly to saliva, changes in the serum protein profile of HNSCC patients were detected. Albumin, protein AMBP, apolipoprotein A-1, haptoglobin, transferrin and transthyretin are common circulating or carrier proteins. Of them, serum apolipoprotein A-1, haptoglobin and transthyretin have already been described as putative cancer markers [69, 79] and were observed up-regulated in most of our samples. Transthyretin is a 55-kDa tretamer protein which transports thyroid hormones and retinol (vitamin A) and is associated with the commonest form of systemic amyloid disease [80]. In our study, an increased level of a transthyretin fragment at ~37 kDa and pI 5,52 was observed in 2-DE gels from patients. The studies of Olofsson et al [81] and Matsubara et al [82] demonstrated that variants of transthyretin exist as dimers and are prone to aggregate formation. One of this variant (Ser112Ile) promotes cytotoxicity in a human neuroblastoma cell line [82]. Our findings suggested that conformational changes of this protein may participate of the neoplastic process although its role is yet unclear. Other authors have also noticed altered levels of truncated or modified transthyretin forms in different cancer types [83-85] and explained the results by the presence of inflammatory processes or an abnormal activity of proteases in the tumor microenvironment.

This is the first report showing over-expression of protein PLUNC and zinc-alpha-2-glycoprotein in saliva from HNSCC patients. Both proteins may contribute to control tumor growth and, therefore, represent targets for new analysis. Similarly, a modified transthyretin form was detected with altered levels in serum from patients and may be involved in head and neck tumorigenesis, as suggested for other tumors. Although the number of patients analyzed and proteins detected with differential expression between healthy and HNSCC patients was low, the present study adds information on the complex group of secreted proteins and their role in cancer process and emphasizes the potential of saliva and serum analysis for diagnosis and monitoring of HNSCC patients.

AUTHORS' CONTRIBUTIONS

AV participated in the design of the study, obtained informed consent from patients, carried out proteomic and Western blot experiments, data analysis and drafted the manuscript. JVM coordinated sample collection and carried out clinical data analysis. EHT participated in the design and coordination of the study, data analysis and drafted the manuscript. All authors revised and approved the final manuscript.

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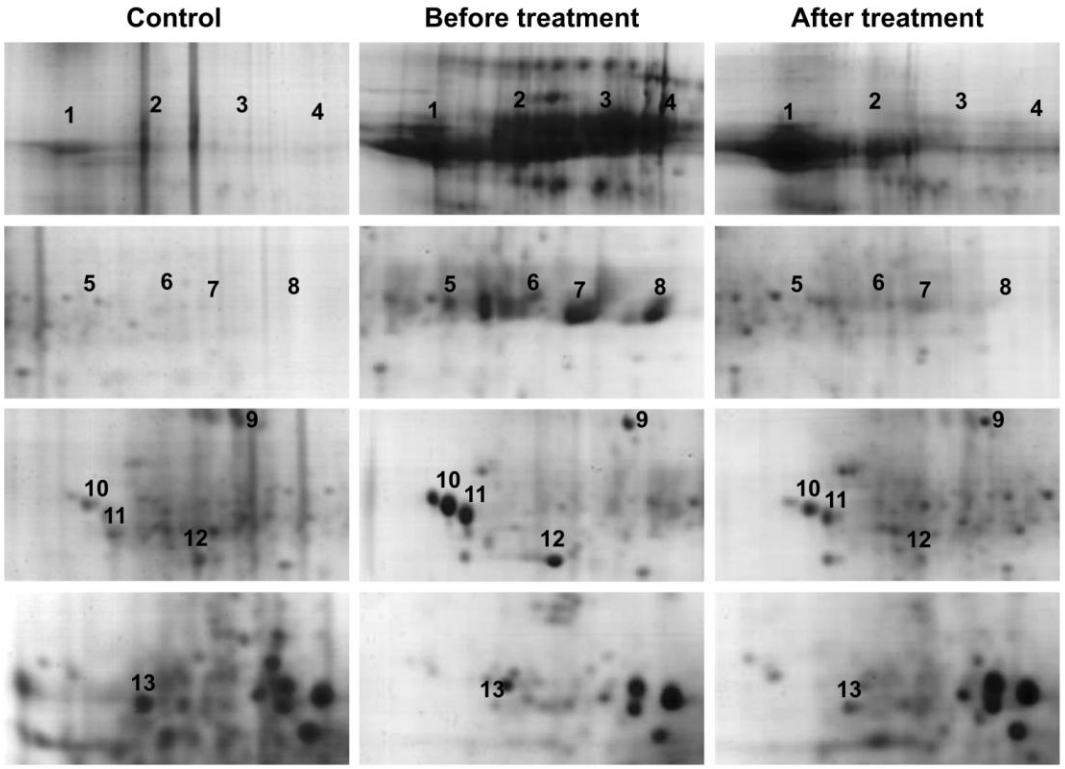
Control
Before treatment
After treatment
1
2
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Figure 1

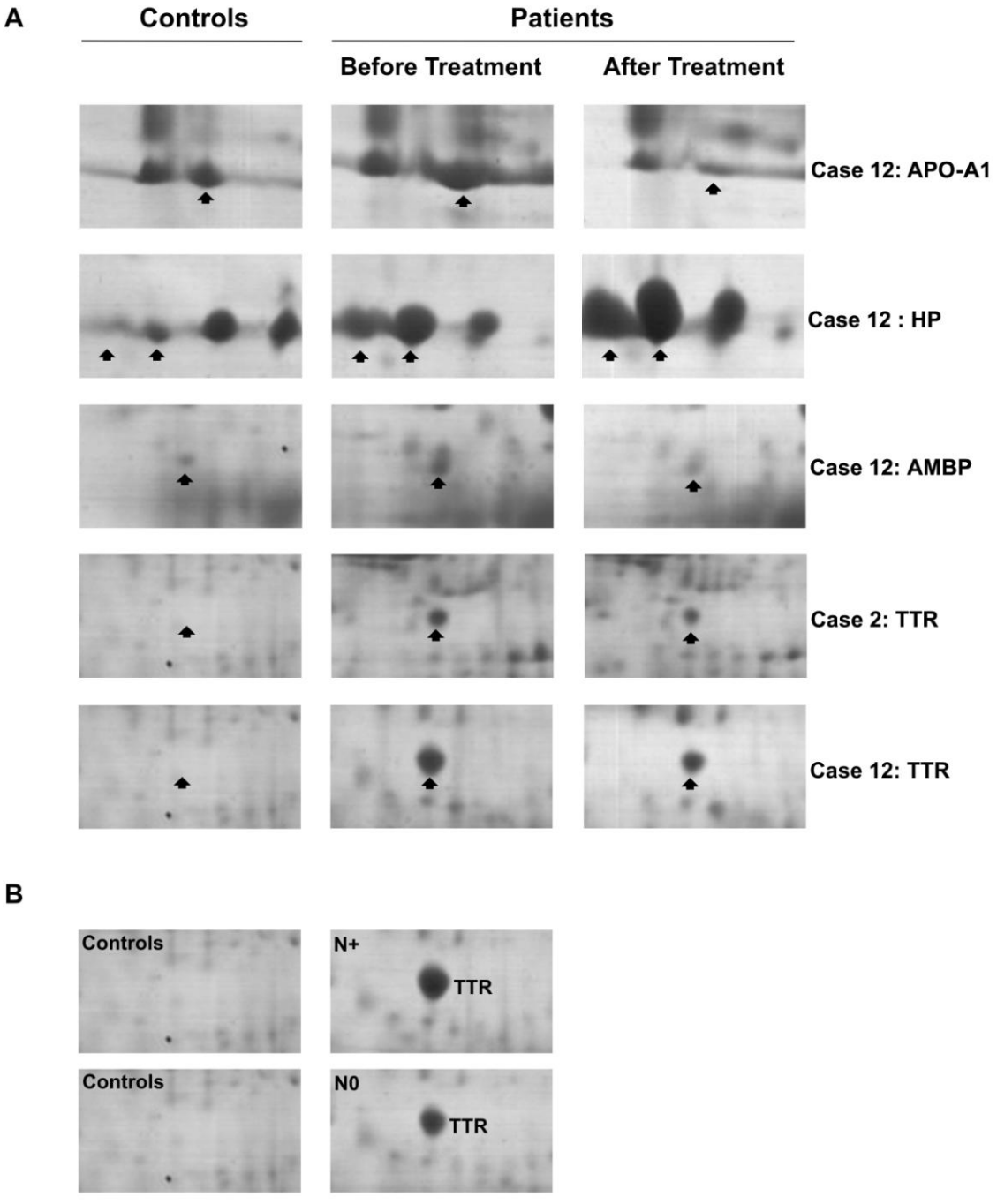
A
Controls
Patients
Before Treatment
After Treatment
Case 12: APO-A1
Case 12 : HP
Case 12: AMBP
Case 2: TTR
Case 12: TTR
B
Controls
N+
TTR
Controls
N0
TTR

Figure 2

Table1. Clinical and demographic data of the HNSCC patients. S= Surgery; RxT= Radiation therapy; Sa= Saliva; Se= Serum

Case	Age (yrs)	Gender	Ethnic group	Smoking status	Alchool use	Pathological TNM	Treatment	Site ICD-10	Samples
01	69	M	White	Current	Current	T2N0M0	S	C32.9	Se
02	50	M	White	Current	Former	T2N0Mx	S / RxT	C06.2	Se
03	58	M	White	Current	Former	T1N0M0	S	C32.9	Sa / Se
04	56	F	White	Never	Never	T1N0M0	S	C06.0	Se
05	61	M	White	Current	Former	T4N2bM0	Rxt	C32.9	Se
06	57	M	White	Current	Current	T2N2bMx	S / RxT	C12.9	Se
07	75	M	White	Current	Current	T4N0M0	S / RxT	C32.9	Se
08	54	M	White	Current	Current	T2N2cM0	RxT	C01.9	Se
09	54	M	White	Current	No data	T1N0M0	S	C04.9	Se
10	58	M	White	Former	Current	T1bN0M0	RxT	C32.0	Sa / Se
11	49	M	White	Current	Current	T2N0M0	RxT	C05.1	Sa / Se
12	66	M	White	Current	Current	T3N2cM0	RxT	C05.0	Sa / Se
13	65	M	White	Former	Never	T1bN0M0	RxT	C32.9	Sa / Se
14	81	M	White	Current	Current	T2N0M0	RxT	C32.0	Sa / Se
15	78	M	Black	Never	No data	T2N0M0	S / RxT	C02.1	Sa / Se

Table 2. Demographic data of the control group.

Age (yrs)	Gender	Ethnic group	Smoking status	Alchool use	Samples
29	Male	White	Never	Never	Serum
47	Male	Intermediate	Never	Current	Serum
50	Male	White	Never	Current	Serum
32	Male	White	Former	Current	Serum
23	Male	White	Current	Current	Serum
27	Male	White	Current	Current	Serum
36	Male	Intermediate	Current	Current	Serum
46	Female	Black	Former	Former	Serum
44	Male	White	Never	Current	Serum
57	Male	Black	Never	Never	Serum
44	Male	White	Former	Former	Serum
45	Male	White	Former	Former	Serum
47	Male	Intermediate	Former	Current	Serum
48	Male	White	Former	Current	Serum
48	Male	White	Former	Current	Serum
55	Male	Intermediate	Current	Current	Serum
60	Male	Intermediate	Current	Former	Serum
52	Male	White	Current	Former	Serum
22*	Male	Pardo	Never	Current	Serum
48*	Male	White	Never	Never	Serum
31*	Male	White	Current	Current	Serum
45*	Male	Intermediate	Current	Never	Serum
23*	Male	Intermediate	Current	Current	Serum

31*	Male	Intermediate	Current	Current	Serum
50*	Male	Intermediate	Former	Current	Serum
30*	Male	Black	Current	Current	Serum
35	Male	White	Never	Current	Saliva
32	Female	White	Never	Current	Saliva
59	Male	White	Never	Current	Saliva
60	Male	White	Never	Current	Saliva
56	Male	White	Never	Current	Saliva
49	Male	White	Never	Current	Saliva
60	Male	Intermediate	Never	Current	Saliva
56	Male	White	Never	Current	Saliva
59	Male	White	Never	Current	Saliva
64	Male	White	Former	Current	Saliva

* Farm workers

Table 3. Information on biological processes based on Gene ontology. Up- and down-regulated proteins selected from proteomic analysis of saliva samples from HNSCC patients.

Biological process	Up-regulated proteins
Cell adhesion	Zn-alpha-2-GP
Developmental process	
epithelial cell differentiation	
negative regulation of epithelial cell proliferation	CK-4
epidermis development	CK-13
Metabolic process	
carbohydrate metabolic process	AMY1A
Response to stimulus	
immune response	IGJ, IGKC, PLUNC, Zn-alpha-2-GP
Biological process	**Down-regulated protein**
Transport	Myosin Va variant

Up-regulated proteins. AMY1A: alpha-amylase 1; CK-4: cytokeratin 4; CK-13: cytokeratin 13; IGJ: immunoglobulin J chain; IGKC: immunoglobulin kappa chain C region; PLUNC: protein Plunc, Zn-alpha-2-GP: zinc-alpha-2-glycoprotein. **Down-regulated proteins.** myosin Va variant.

Table 4. Information on biological processes based on Gene ontology. Up- and down-regulated proteins selected from proteomic analysis of serum samples from HNSCC patients.

Biological process	Up-regulated proteins
Apoptosis	
Negative regulation of apoptosis	ALB
Biologic Process	
Cell killing	ALB
Cell communication	
Signal transduction	AMBP, APO-A1
Cellular homeostasis	TF
Metabolic process	
Protein metabolic process	APO-A1
Lipid metabolic process	APO-A1
Hormone metabolic process	TTR
Response to stimulus	
Inflammatory response	
Defense response	HP
Immune response	APO-A1
Response to external stimulus	ALB
Transport	ALB, APO-A1, TF, TTR
Biological process	**Down-regulated proteins**
Transport	HBA1, HBB

Up-regulated proteins. ALB: serum albumin; AMBP: protein AMBP or alpha-1-microglobulin/bikunin precursor; APO-A1: apolipoprotein A-I; HP: haptoglobin; TF: serotransferrin; TTR: transthyretin. **Down-regulated proteins.** HBA1: hemoglobin subunit alpha; HBB: hemoglobin subunit beta.

Supplementary Table 1. Proteins expressed in saliva samples from HNSCC patients. Proteins separated by two-dimensional electrophoresis and identified by MALDI-QUAD-TOF MS/MS.

Spot	Mascot acession	Swiss-Prot accession	Protein name	pI	Mass	Sequence coverage (%)	Score	Queries matched
1	K1C13_HUMAN	P13646	Cytokeratin-13	4.91	49898	10	190	4
2	I37942	P19013	Cytokeratin-4	6.25	57602	10	264	5
3, 4	ALHUS	P04745	Alpha-amylase 1	6.47	58443	4	76	2
5, 6, 7, 8	K3HU	P01834	Ig kappa chain C region	5.58	11773	32	104	2
9	Q5XKQ4_HUMAN	P25311	Zinc-alpha-2-glycoprotein	5.71	34451	14	150	3
10, 11	AAA58902	P01591	Immunoglobulin J chain	4.62	16041	16	103	3
12	AAF70860	Q9NP55	Protein Plunc	5.65	26810	23	198	4
13	Q59FF5_HUMAN	Q59FF5	Myosin Va variant	9.14	166071	0	53	1

Supplementary Table 2. Proteins expressed in saliva samples from HNSCC patients. Proteins separated by two-dimensional electrophoresis and identified by MALDI-QUAD-TOF MS/MS.

Mascot acession	Swiss-Prot accession	Protein name	pI	Mass	Sequence coverage (%)	Score	Queries matched
HCHU	P02760	Protein AMBP	5.95	39886	19	240	4
LPHUA1	P02647	Apolipoprotein A-I	5.56	30759	12	133	3
HPHU2	P00738	Haptoglobin	6.13	45861	6	68	1
Q9BX83_HUMAN	Q9BX83	Hemoglobin alpha 1 globin chain	7.07	10703	31	159	2
Q549N7_HUMAN	P68871	Hemoglobin subunit beta	7.14	16098	30	153	3
ABHUS	P02768	Serum albumin	5.92	71317	7	82	3
TFHUP	P02787	Serotransferrin	6.81	79280	5	139	3
VBHU	P02766	Transthyretin	5.52	15991	24	159	2

CONCLUSÕES

3. CONCLUSÕES

Para o nosso conhecimento, esse é o primeiro estudo que avalia metástases em linfonodos de carcinoma epidermóide de cabeça e pescoço utilizando abordagens proteômicas. A maioria dos resultados está de acordo com o perfil protéico esperado para células metastáticas.

As principais conclusões deste estudo são:

1. A solubilização de proteínas é uma etapa fundamental para a obtenção de resultados consistentes pela técnica de eletroforese bidimensional com posterior análise por espectrometria de massas;

2. As proteínas com expressão alterada em metástases regionais procedentes de pacientes com carcinoma epidermóide de cabeça e pescoço possuem um papel importante na tumorigênese e no processo de metastatização e atuam em migração, adesão e proliferação celular, desenvolvimento da epiderme, metabolismo de xenobióticos, escape imune e resposta inflamatória;

3. Duas proteínas, E-FABP e profilina-1, são marcadores potenciais de metástases regionais em carcinoma epidermóide de cabeça e pescoço;

4. A hipóxia possui um papel importante na disseminação linfática de células tumorais;

5. A desregulação sistêmica do citoesqueleto de actina ocorre no processo tumorigênico e de metastatização;

6. O microambiente linfonodal atua no controle do crescimento do tumor ou na reprogramação metabólica da célula metastática;

7. Os carcinomas epidermóides de língua, soalho de boca e laringe são entidades diferentes, embora estejam em sítios anatômicos próximos;

8. A resposta inflamatória é um evento importante na interação do tumor com seu estroma;

9. O perfil protéico de saliva é mais influenciado pelo sexo do doador que pelo ritmo circadiano;

10. O perfil protéico da saliva e do soro é distinto entre pacientes com carcinoma epidermóide de cabeça e pescoço e indivíduos saudáveis. As proteínas diferencialmente expressas representam marcadores potenciais desses tumores;

11. A composição de proteínas da saliva e do soro é influenciada pela radioterapia.

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