FELIPE PAIVA FONSECA

SEMAPHORINS AND NEUROPILINS IN SALIVARY GLAND TUMORS

SEMAFORINAS E NEUROPILINAS EM TUMORES DE GLÂNDULAS SALIVARES

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Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Stomatopathology, in Pathology area.

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Orientador: Prof. Dr. Pablo Agustin Vargas

Este exemplar corresponde à versão final da tese defendida pelo aluno Felipe Paiva Fonseca e orientada pelo Prof. Dr. Pablo Agustin Vargas.

Prof. Dr. Pablo Agustin Vargas

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Abstract

Salivary gland tumors correspond to approximately 3% of all head and neck neoplasms and the malignant neoplasias derived from these anatomic structures still represent a major pitfall in head and neck oncology because of their difficult surgical approach and poor response to other therapies. A better understanding of their molecular basis would significantly aid to an improved future management and the study of salivary gland tumors angiogenic potential represents an interesting target of investigation. It has been shown that semaphorins induce tumor cell apoptosis, modulate tumor cell migration and inhibit angiogenesis in different human neoplasms, competing with vascular endothelial growth factor (VEGF) for biding to their main receptors, the neuropilins-1 and -2, thereby inhibiting mitogenic and pro-angiogenic effects of VEGF. Hence, the objective of this study is to investigate the expression of class 3 Semaphorins A and B, and their receptors neuropilins-1 and -2 in salivary gland tumors, determining their clinical significance. Two hundred and forty eight benign and malignant salivary gland tumors selected from four Brazilian institutions were organized in tissue microarray paraffin blocks and submitted to immunohistochemical reactions against CD34, Sema3A, Sema3B, Np-1 and Np-2. The immunoreactions were quantified using digital algorithms and the results were correlated with clinicopathological parameters and survival rates. Malignant tumors presented an increased vascular density but a lower vascular area than their benign counterparts. In normal salivary glands Np-1 and Np-2 expression was restricted to ductal cells, whereas Sema3A and Sema3B were positive mainly in serous acinar compartment. Benign and malignant tumors revealed a similar expression of all markers and the co-expression of Np-1/Np-2 significantly correlated with the occurrence of paresthesia and higher stages of the tumors. In addition, although not statistically significant, simultaneous overexpression of both receptors also indicated an inferior survival rate. Hence, these results suggest that Sema3A, Sema3B, Np-1 and Np-2 may be involved in the development of normal salivary glands and in the pathogenesis of benign and malignant neoplasms derived from these structures; however, the expression of these proteins did not present a statistically significant prognostic potential in the current study.

Keywords: Salivary gland neoplasms. Semaphorins. Neuropilins.

Resumo

Tumores de glândulas salivares correspondem a aproximadamente 3% de todas as neoplasias de cabeça e pescoço e as neoplasias malignas derivadas destas estruturas anatômicas ainda representam um grande desafio para a oncologia de cabeça e pescoço devido a sua difícil abordagem cirúrgica e pobre resposta às outras abordagens terapêuticas. Um melhor entendimento do seu perfil molecular contribuiria significativamente para um melhor manejo terapêutico futuro e o estudo do potencial angiogênico dos tumores de glândulas salivares representa um interessante alvo de investigação. Tem sido demonstrado que as semaforinas induzem a apoptose de células tumorais, modulam a migração celular neoplásica e inibem a angiogênese em diferentes neoplasias humanas, competindo com o fator de crescimento endotelial vascular (VEGF) pela ligação aos seus principais receptores, as neuropilinas-1 e -2, desta forma inibindo os efeitos mitogênicos e pró-angiogênicos de VEGF. Assim, o objetivo deste estudo é investigar a expressão das semaforinas de classe 3 A e B (Sema3A e Sema3B), e dos seus receptores neuropilinas-1 e -2 (Np-1 e Np-2) em tumores de glândulas salivares, determinando seus significados clínicos. Duzentos e quarenta e oito tumores benignos e malignos de glândulas salivares selecionados de quatro instituições brasileiras foram organizados em blocos de parafina em microarranjo tecidual em matriz e submetidos a reações de imunoistoquímica contra CD34, Sema3A, Sema3B, Np-1 e Np-2. As imunoreações foram quantificadas utilizando algoritmos digitais e os resultados foram correlacionados com parâmetros clinicopatológicos e índices de sobrevida. Tumores malignos apresentaram uma maior densidade vascular, porém uma menor área vascular do que sua contraparte benigna. Em glândulas salivares normais a expressão de Np-1 e -2 esteve restrita às células ductais, enquanto que Sema3A e Sema3B estiveram principalmente no componente acinar. Tumores benignos e malignos revelaram uma expressão similar de todos os marcadores e a co-expressão de Np-1/Np-2 correlacionou-se significativamente com a ocorrência de parestesias e estágios mais avançados dos tumores. Apesar de não ser estatisticamente significativa, a sobre-expressão simultânea de ambos os receptores também indicou uma menor taxa de sobrevida. Desta forma, Sema3A, Sema3B, Np-1 e Np-2 devem estar envolvidas no desenvolvimento das glândulas salivares normais e na patogênese das neoplasias benignas e malignas derivadas destas estruturas; entretanto, a expressão destas proteínas

não apresentou um potencial prognóstico estatisticamente significativo no presente estudo.

Palavras-chave: Neoplasias de glândulas salivares. Semaforinas. Neuropilinas.

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INTRODUÇÃO

Os tumores de glândulas salivares (TGS) representam um grupo de neoplasias altamente heterogêneo do ponto de vista clínico e microscópico, o que torna seu manejo diagnóstico e terapêutico um verdadeiro desafio. As neoplasias benignas e malignas das glândulas salivares correspondem a cerca de 3% dos tumores de cabeça e pescoço, com uma incidência global anual estimada em 0,4 a 13,5 casos/100.000 pessoas (Jones et al., 2008; Fonseca et al., 2012).

Clinicamente, os TGS apresentam-se mais frequentemente como tumefações localizadas comumente assintomáticas que ocasionalmente causam dor e parestesia. As glândulas maiores, em especial as glândulas parótidas são os sítios anatômicos mais afetados; entretanto, uma grande parcela dos casos é diagnosticada na cavidade oral envolvendo principalmente o palato. A evolução clínica dos pacientes afetados por estas neoplasias é bastante heterogênea e está grandemente relacionada ao subtipo histopatológico. Assim, enquanto o carcinoma adenoide cístico apresenta altos índices de metástases a distância, em especial após longos períodos de acompanhamento, o adenocarcinoma polimorfo de baixo grau raramente está associado com estes eventos. Consequentemente, o prognóstico e os índices de sobrevida dos pacientes acometidos são bastante variados atingindo 95% para o adenocarcinoma polimorfo de baixo grau e limitando-se a apenas 40% para aqueles afetados por carcinomas mucoepidermoides de alto grau (Neville et al., 2002; Guzzo et al., 2010).

A abordagem terapêutica dos TGS encontra-se basicamente restrita ao manejo cirúrgico das lesões, seguida ou não de complementação radioterápica adjuvante. O uso de protocolos quimioterápicos atualmente disponíveis não representa uma alternativa satisfatória para os TGS, sendo utilizados apenas em casos onde a cirurgia e a radioterapia de alguma forma não podem ser aplicadas. Muito desta limitação terapêutica se deve à pobre compreensão dos aspectos biológicos destes tumores; por isso, o estudo dos eventos moleculares associados ao surgimento e desenvolvimento dos TGS poderia favorecer a criação de novos regimes quimioterápicos para estas lesões (Koul et al., 2007; Mücke et al., 2009; Lloyd et al., 2010).

Nesta linha de pensamento sabe-se que o crescimento tumoral está intimamente relacionado com o processo de angiogênese uma vez que os vasos capilares neoformados oferecem uma maior disponibilidade de oxigênio e nutrientes para que

haja a expansão tumoral além das fronteiras de uma massa celular avascular de 1 a 2mm^3, levando consequentemente a um maior índice proliferativo e uma diminuição da taxa apoptótica (Folkman, 2003; Cao, 2004; Lequerica-Fernández et al., 2007; Vadasz et al., 2010). Além da importância do processo de angiogênese para a manutenção da massa tumoral e seu desenvolvimento, evidências apontam para uma estreita relação entre neoformação vascular e o desenvolvimento de metástases (Lohela et al., 2003; Cardoso et al., 2009). Assim, o estudo do padrão angiogênico em TGS contribuiria não somente para o entendimento do seu desenvolvimento local, mas também para uma maior compreensão dos fatores possivelmente relacionados à sua disseminação.

A expressão de diferentes fatores e receptores pró- e anti-angiogênicos é necessária para que haja o desenvolvimento de uma nova rede capilar. Portanto, o reconhecimento do perfil de expressão destas proteínas nos TGS pode ser de grande valia para o melhor entendimento da angiogênese destas lesões e a investigação da família das semaphorinas e de seus receptores, as neuropilinas, vem ao encontro deste raciocínio.

Originalmente denominadas collapsinas, as semaforinas são uma grande família de proteínas secretadas ou ligadas à membrana celular que foram originalmente identificadas como fatores envolvidos no crescimento axônico (Blanc et al., 2011). Entretanto, o amplo padrão de expressão das semaforinas sugere a existência de funções adicionais para estas proteínas fora do sistema nervoso. Baseado em sua estrutura, elas podem ser classificadas em sete subclasses, incluindo proteínas transmembrana (classes 1, 4, 5 e 6), secretadas (classes 2 e 3) e proteínas associadas a superfície celular (classe 7) (Staton et al., 2011).

As semaforinas de classe 3 correspondem a sete proteínas secretadas (Sema3A-3G) que atuam através da interação com seus receptores neuropilinas durante o crescimento axônico dos neurônios sensoriais e motores. Existe um certo grau de especificidade na atividade e ligação entre semaphorinas e neuropilinas, com Sema3A ligando-se preferencialmente a Np-1, Sema3F ligando-se a Np-2 e Sema3B ligando-se a ambos os receptores (Potiron et al., 2009).

Estudos *in vitro* sugerem que as semaforinas competem com VEGF165 (Vascular Endothelial Growth Factor 165) nas células endoteliais favorecendo um perfil anti-antiogênico, além de causar apoptose e inibição da migração celular no contexto

neoplásico. Estes efeitos parecem ser principalmente mediados através da ligação Sema3A-Np1 e Sema3F-Np2 (Staton et al., 2007; Yacoub et al., 2009; Potiron et al., 2009). Curiosamente, a região 3p21.3 que está frequentemente deletada em algumas neoplasias humanas, contém os genes para Sema3B e Sema3F, estando ambas sub-expressas em câncer de pulmão e em carcinomas ovarianos, sugerindo um papel supressor tumoral para estes genes (Osada et al., 2006; Staton et al., 2007; Potiron et al., 2009).

Estes estudos sugerem que alterações na relação Sema3:VEGF devem determinar o grau quimiotático entre as células endoteliais tumorais. Em carcinoma de ovário, a expressão de Sema3A diminui com a progressão da lesão, estando inversamente correlacionada com a microdensidade vascular nesta neoplasia, relacionando-se com um pior prognóstico nestes pacientes (Osada et al., 2006). De forma semelhante, em tumores pulmonares primários foi observado que a imunomarcação para Sema3F e VEGF165 está inversamente correlacionada, além de estarem relacionadas com o estadiamento e agressividade tumoral (Lantuéjoul et al., 2003). Observou-se ainda que a expressão de Sema3A, Sema3B e Sema3F está significativamente diminuída em carcinomas de mama se comparadas com tecido de mama normal, estando inversamente correlacionado com a expressão de VEGF também nestas neoplasias (Staton et al., 2011). Estes dados sugerem que a razão entre semaforinas de classe 3 e VEGF165 deve regular o grau de angiogênese e progressão tumoral.

Somado ao padrão de expressão de semaphorinas de classe 3 e do VEGF165 que competem pela ligação aos receptores neuropilinas-1 e -2, o perfil de expressão destes receptores também tem se mostrado de grande valia para o melhor entendimento do processo de angiogênese fisiológica e tumoral, haja vista a significativa correlação estabelecida entre a presença de neuropilinas e a maior agressividade neoplásica em diferentes tumores humanos (Staton et al., 2007; Staton et al., 2011; Staton, 2011).

Neuropilina-1 (Np-1) é uma glicoproteína transmembrana simples (120-130 kDa) inicialmente caracterizada como um receptor neuronal para membros específicos da família das semaforinas. Np-1 desenvolve um papel fundamental no crescimento e direcionamento axônico e sua expressão em embriões diminui conforme ocorre a formação do sistema nervoso central. Entretanto, esta proteína persiste em vários

tecidos adultos não neurais, como na placenta e no coração, além das células endoteliais e em uma variedade de células neoplásicas. Na vasculatura embrionária, Np-1 é expressa por células endoteliais arteriais e, além de ser um receptor para diferentes subtipos de semaforinas de classe 3, incluindo Sema3A, 3B, 3C, 3D e 3F, Np-1 também serve como receptor para alguns membros da família do VEGF, como o VEGF165, VEGF-B, VEGF-E e fator de crescimento placentário (PlGF – Placental Growth Factor) (Staton et al., 2007).

Neuropilina-2 (Np-2) (130 kDa) foi identificada originalmente como um receptor neuronal alternativo para algumas variantes de semaforinas de classe 3, como Sema3B, 3C, 3F e 3G, além de VEGF145, VEGF-C, VEGF-D e PlGF. Os primeiros estudos identificando Np-1 como um receptor para VEGF nas células neoplásicas do câncer de mama, também detectaram a participação de Np-2, que inicialmente era tida como uma proteína não relacionada. Entretanto, Np-2 também foi posteriormente identificada como um receptor funcional para VEGF165 (Staton et al., 2007; Pellet-Many et al., 2008).

Alterações na expressão de Np-1 e Np-2 em ratos transgênicos resultam em morte embrionária, com o surgimento de anormalidades no sistema nervoso central e uma maior densidade vascular e dilatação de vasos sanguíneos. Desta forma, concluiu-se que tanto Np-1 como Np-2, exerceriam um importante papel na formação vascular embrionária e na diferenciação vascular em artérias e veias, além do papel central na orientação da formação de fibras nervosas (Staton et al., 2007).

O número de estudos avaliando o papel de neuropilinas no crescimento e angiogênese tumoral humana vem aumentando rapidamente. A sobre-expressão de Np-1 em tumores de próstata, mama, linfomas, leucemias e outros parece correlacionar-se com um estágio mais avançado e maior agressividade destas neoplasias (Latil et al., 2000; Vanveldhuizen et al., 2003; Staton et al., 2011; Karjalainen et al., 2011). Além disso, uma forte expressão de neuropilinas tem sido observada em cânceres de esôfago, pulmão, bexiga, pâncreas e cólon (Jubb et al., 2012). De fato, células de adenocarcinoma pancreático expressam altos níveis de mRNA de Np-1 e Np-2. Interessantemente, a sub-regulação de Np-1 através da utilização de siRNA aumentou a sensibilidade das células neoplásicas do carcinoma pancreático aos protocolos de

quimioterapia, sugerindo que a combinação da inibição de Np-1 e a quimioterapia teria um significativo potencial terapêutico nesta lesão (Matisushita et al., 2007).

O objetivo deste estudo é analisar a expressão imunoistoquímica das semaforinas de classe 3A e B e de seus receptores neuropilinas-1 e -2 em tumores de glândulas salivares, correlacionando a presença das proteínas com o padrão vascular destas neoplasias e com as variáveis clinicopatológicas dos pacientes afetados.

CAPÍTULO 1

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SEMAPHORINS AND NEUROPILINS EXPRESSION IN SALIVARY GLAND TUMORS

SEMAPHORIN AND NEUROPILIN IN SALIVARY GLAND TUMORS

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Abstract

Aims: To determine the clinical significance of the expression of class 3 semaphorins A (Sema3A) and B (Sema3B) and their receptors, neuropilins-1 (Np-1) and -2 (Np-2), in salivary gland tumors.

Methods and results: Two hundred and forty eight salivary gland tumors were organized in tissue microarray paraffin blocks and expression of CD34, Sema3A, Sema3B, Np-1 and Np-2 was determined through immunohistochemistry. The immunoreactions were quantified using digital algorithms and the results correlated with clinicopathological parameters and specific survival rates. Malignant tumors presented an increased vascular density than their benign counterparts and their increased vascular area significantly correlated with higher rates of recurrence ($p < 0.05$). Patients older than 40 years and presence of recurrences determined an inferior specific survival rate under univariate analysis ($p = 0.0057$ and $p = 0.0303$, respectively). In normal salivary glands Np-1 and Np-2 expression was restricted to ductal cells, whereas Sema3A and Sema3B were positive in the serous acinar compartment. Benign and malignant tumors revealed a similar expression of all markers and the co-expression of Np-1/Np-2 significantly correlated with presence of paresthesia and advanced stages of the tumors ($p = 0.01$ and $p = 0.04$, respectively). Although not statistically significant, simultaneous overexpression of both receptors also indicated an inferior survival rate ($p = 0.09$).

Conclusion: Sema3A, Sema3B, Np-1 and Np-2 may be involved in the pathogenesis of salivary gland tumors, but their expression did not present a statistically significant prognostic potential in the current study.

Keywords: Salivary gland neoplasms; semaphorins; neuropilins; CD34; prognosis.

Introduction

Salivary gland tumors (SGT) are a heterogeneous group of lesions with complex clinicopathological features and biological behavior, corresponding to approximately 3% of all head and neck neoplasms, with an annual incidence of 0.4 to 13.5 cases per 100,000 inhabitants[1,2]. Malignant tumors derived from these structures still represent a major challenge in head and neck oncology because of their difficult surgical approach and poor response to other therapies, which might be attributed to our scarce knowledge concerning their molecular features.

Although the formation of new blood vessels is widely known to represent an essential step in the progression of human cancer[3], the angiogenic properties of SGT has only superficially been investigated and the factors responsible for the development, or inhibition, of new blood vessel formation in SGT requires further exploration. Previous studies have demonstrated the up-regulation of the pro-angiogenic vascular endothelial growth factor (VEGF) in salivary gland malignancies, but its prognostic and therapeutic relevance is debatable and poorly understood[4-6]. Thus, an investigation of VEGF antagonists and related co-receptors, such as semaphorins and neurolipins, could better clarify the role of VEGF in SGT development.

Semaphorins is a large family of secreted or membrane-bound proteins originally identified as axon guidance factors. The family is made up of seven subclasses, including transmembrane proteins (classes 1, 4, 5 and 6), secreted proteins (classes 2 and 3) and proteins associated with the cell surface (class 7). The wide expression pattern of class 3 semaphorins suggests additional functions outside the nervous system and recent studies have shown that class 3 semaphorins induce tumor cell apoptosis, modulate tumor cell migration and inhibit angiogenesis[7-10].

Two receptor families, the neuropilins and plexins, form a complex to mediate the actions of class 3 semaphorins, with the former acting as the ligand-binding moiety and the latter as the signal-transducing component. There is a degree of specificity in semaphorin/neuropilin binding and activity, with Sema3A preferentially binding to Np-1 and Sema3B binding to both receptors. It has recently been shown that neuropilins play an important role as co-receptors for VEGF and that semaphorins interact with the vasculature in an inhibitory manner, competing with VEGF for Np-1/Np-2 binding. In this way, semaphorins inhibit the mitogenic and pro-angiogenic effects of VEGF in endothelial cells, resulting in apoptosis and the inhibition of migration of cancer cells[7-10].

The low expression of class 3 semaphorins and the up-regulation of neuropilins have been shown to favor tumor progression leading to a worse prognosis in a number of human neoplasias[8,11-13]. The aim of the current study therefore was to investigate the expression of Sema3A, Sema3B, Np-1 and Np-2 in a large sample of SGT, in order to determine any possible correlation with angiogenic features and clinicopathological parameters.

Material and methods

Samples

A total of 248 cases of SGT were retrieved from the archives of four Brazilian pathology services (Piracicaba Dental School, Surgical Pathology Laboratory-Cascavel/PR, Federal University of Rio Grande do Sul and A.C. Camargo Cancer Center). Three oral pathologists reviewed the original haematoxylin-eosin stained slides and the diagnoses of all cases were confirmed following the World Health

Organization's 2005 Histological Typing of Salivary Gland Tumors guidelines[1]. Clinical data regarding patient's age, sex, tumor location, presence of pain and paresthesia, tumor stage, treatment employed, tumor recurrence and clinical status and follow-up was retrieved from patient's medical files. The specific-survival time was determined for patients affected by malignant tumors by calculating the time difference between the date of treatment and either the date of death due to the tumor or their last follow-up.

Tissue microarray construction

Tissue microarray (TMA) construction was carried out as previously described[14]. Briefly, tumor areas were selected from the central and most cellular region of the neoplasms and marked on H&E-stained sections using an objective marker (Nikon Corp, Tokyo, Japan). The slides were overlaid on the original paraffin blocks to determine the corresponding areas to be used. TMAs created with cases retrieved from the archives of the Piracicaba Dental School, the Surgical Pathology Laboratory-Cascavel/PR and the Federal University of Rio Grande do Sul were constructed using a manual tissue arrayer (Sakura Co., Tokyo, Japan) and 2 representative cylindrical cores of 2.0mm diameter were taken from each tissue block and arranged sequentially into a recipient ready-to-use paraffin block (Sakura Co., Tokyo, Japan). Two cores of normal parotid gland and one of oral squamous cell carcinoma were inserted in the left upper corner of each recipient block for orientation. Cases retrieved from A.C. Camargo Cancer Center were arrayed as 1.0mm cores, in duplicate, with one core of normal placenta tissue in the left upper corner for orientation. Eleven TMA blocks were

constructed. A map specifying the exact position of each case facilitated the interpretation of the immunohistochemical results.

Immunohistochemistry

Immunohistochemistry was performed using a panel of antibodies to human CD34 (QBEnd10; Dako, Carpentia, USA), Sema3A (*NBP1-51271*; Novus Biologicals, Cambridge, UK), Sema3B (*NB100-2218*; Novus Biologicals, Cambridge, UK), Np-1 (*NBP1-40666*; Novus Biologicals, Cambridge, UK) and Np-2 (*NBP1-86866*; Novus Biologicals, Cambridge, UK). A standard horseradish peroxidase staining procedure was followed using an appropriate biotinylated secondary antibody (Vector Laboratories, Peterborough, UK), and the Elite avidin–biotinylated enzyme complex (ABC) kit (Vector Laboratories). Positive reactions were detected using the chromogen substrates 3,3' diaminobenzidine (DAB) or VectorRed (Vector Laboratories). All sections were counterstained with Harris's haematoxylin (Sigma Aldrich, Gillingham, UK) to visualize cell nuclei. Antigen retrieval for all antibodies was performed by heating the sections in a microwave in 0.01M citrate buffer (pH 6.0) for 8 min on high power. 100% normal goat serum was used to block all sections and for dilution of the primary antibody. Antibody dilutions included 1:50 for CD34, anti-Sema3A 1:150, anti-Sema3B 1:200, anti-Np1 1:200 and anti-Np2 1:200. Sections of mouse brain were used as positive controls for all markers and negative controls were achieved by omission of the primary antibody.

Immunohistochemical digital quantification

Staining for CD34, Sema3A, Sema3B, Np-1 and Np-2 were digitally scored as described previously[15,16]. Briefly, slides were scanned into high-resolution images using the Aperio Scanscope CS® Slide Scanner (Aperio Technologies Inc., Vista, USA) and digital images were analyzed with Pixel Count V9 algorithm (Aperio Technologies Inc., Vista, USA). Sema3A, Sema3B, Np-1 and Np-2 staining was quantified in terms of the percentage of positivity and according to intensity whereby weak staining scored 1, moderate scored 2 and strong scored 3. The final score of each tumor was calculated as the sum of the percentage of each category multiplied by their intensity scores using the following formula: [(%weak x 1) + (%moderate x 2) + (%strong x 3)]. The results always ranged from 100 to 300. The median expression value of each protein was used to divide tumors into 2 groups, below and above the median, exhibiting low and high expression levels, respectively. CD34 staining was analyzed using the Microvessel Analysis V1 algorithm (Aperio Technologies Inc., Vista, USA) and values of vascular density and total vascular area were collected. TMA Lab software (Aperio Technologies Inc., Vista, USA) was used as an auxiliary tool during the analyses.

Statistical analysis

A chi-square (X^2) test was used to compare the immunoexpression of all markers with clinical parameters and a Spearman test was carried out to identify possible correlations among the biomarkers. Wilcoxon, Kruskal-Wallis and Dunn's post-hoc tests allowed differences in expression of the proteins among histological subtypes to be assessed. Log-rank test and Cox regression model were carried out to evaluate the prognostic significance of clinicopathological features and protein expression. An

overall survival curve was acquired using the Kaplan-Meyer method. SAS software was employed for data analyses and a p value < 0.05 was considered statistically significant. The current study was carried out in accordance with the ethical guidelines of Piracicaba Dental School ethical committee (process number CEP/FOP 002/2013 – February 06, 2013).

Results

Sample data

Clinicopathological data is presented in **Table 1**. In the current analysis 248 cases of SGTs were included with an even distribution between benign and malignant tumors (48.4% or 120 cases of benign tumors and 51.6% or 128 cases of malignant neoplasms). Pleomorphic adenoma was the most common benign subtype (39.9% of total sample), whereas mucoepidermoid carcinoma represented the most prevalent malignancy (16.9% of total sample). A slight male predominance was found (M:F ratio of 1.0:0.9) with a mean age of 48.3 years (range of 9 to 91 years). Parotid gland was the most commonly affected site (58.1%), followed by minor glands of the oral cavity (26.2%) and submandibular gland (8.1%). Pain and paresthesia was found only in patients affected by malignant tumors, corresponding to 17.2% and 3.9% of the cases, respectively. Stage I/II malignant neoplasms accounted for 24.2%, and stages III/IV 44.5% of the malignancies. Regarding treatment approaches, all patients affected by benign tumors were treated with surgery only, whereas 30.5% of those affected by malignant tumors received adjuvant radiotherapy following surgical management. No recurrence was observed in benign tumors, whereas 16.4% of the malignant neoplasms

recurred. Follow-up data was available for 70.2% of the patients, with a mean follow-up time of 93 months (range of 1 to 516 months). Considering only malignant tumors, by the time of their last appointment, 46.1% of the patients were alive, whereas 25.8% had died as a result of their tumor and for the remaining 28.1% data was unavailable. No patient affected by benign tumors whose clinical data was available for consultation had died of their neoplasia.

Under univariate analysis, patients younger than 40 and those with recurrences during follow-up time revealed a significant inferior survival rate ($p = 0.0057$ and $p = 0.0303$, respectively); no other clinicopathological variable significantly correlated with survival rate. However, following analysis with the Cox regression model both age and recurrence variables lost their significance ($p > 0.05$). The specific 5-year and 10-year survival rates, calculated by Kaplan-Meyer curve, were 49% and 31%, respectively.

CD34 expression

Vascular network was evaluated by CD34 protein expression in normal salivary gland tissues and in benign and malignant tumors. Although not statistically significant, the number of vessels per unit of area (μm^2) was higher in malignant neoplasms than in their benign counterparts (0.82 ±1.9 *vs* 0.63 ±1.7, respectively) ($p = 0.2215$). Warthin's tumors presented the highest vascular density among histologic subtypes, followed by mucoepidermoid carcinoma, adenocarcinoma NOS, adenoid cystic carcinoma, acinic cell carcinoma, squamous cell carcinoma and pleomorphic adenoma. On the other hand, the total vascular area was significantly higher in benign tumors than in malignancies (267.2 ±166.6 *vs* 184.8 ±97.5, respectively) ($p < 0.0001$). Total vascular area

significantly correlated with an increased number of recurrences (p = 0.04), but there were no further correlations with other clinicopathological parameters and survival rate.

Semaphorins expression

Sema3A and Sema3B were both strongly expressed in the acinar cells of normal serous glands, and only faintly in the ductal system, but they exhibited different staining patterns. Sema3A was strongly and diffusely present throughout the whole serous acinar compartment of the normal gland, whereas Sema3B expression was restricted to the basal area of the acinar cells **(Figure 1)**. Neither protein was expressed by either myoepithelial or mucous cells.

In benign tumors, Sema3A and Sema3B showed cytoplasmic positivity in both pleomorphic adenoma and Warthin's tumors. All malignant neoplasms also demonstrated some cytoplasmic expression, with increased expression of Sema3A in acinic cell carcinomas **(Figures 1)**. Wilcoxon test showed significantly greater expression of Sema3A in benign tumors than in malignancies ($p < 0.001$), whereas no difference was found for Sema3B ($p > 0.05$) **(Figure 2)**. Individual values obtained for Sema3A and Sema3B for each histological subtype is detailed in **Table 2**. In contrast to the positive correlations found with neuropilins, no statistically significant correlations between semaphorins expression, clinicopathological parameters and survival rate of the patients were found.

Spearman correlation test demonstrated a significant correlation between Sema3A and total vascular area of the tumors ($p < 0.0001$) and between Sema3A and Sema3B expression ($p < 0.0001$). Positive correlations were also found between

Sema3A and Np-1 ($p < 0.0001$), Np-2 ($p < 0.0001$) and both Np-1/Np-2 ($p < 0.0001$), and between Sema3B and Np-1 ($p < 0.0001$) and both Np-1/Np-2 ($p < 0.0001$).

Neuropilin-1 and -2 expressions

In normal salivary gland tissue, Np-1 and Np-2 were expressed in striated and intercalated ducts of both mucous and serous glands, with no staining of the acinar or myoepithelial cells **(Figure 3)**. In benign tumors, both proteins revealed an intense cytoplasmic and membrane positivity in pleomorphic adenoma and in the epithelial component of Warthin's tumors. All malignant neoplasms demonstrated intense cytoplasmic and membrane positivity for both Np-1 and Np-2 **(Figure 3)**. Whilst there was no significant statistical difference in the expression of Np-1 between benign and malignant tumors ($p > 0.05$), significantly higher expression of Np-2 was found in malignant tumors compared to benign tumors ($p < 0.0001$) **(Figure 2)**. Individual values obtained for Np-1 and Np-2 for each histological subtype is detailed in **Table 2**. Np-2 over-expression significantly correlated with tumor site, with highest expression in the parotid ($p < 0.0001$). No further significant correlations between either Np-1 or Np-2 with clinicopathological parameters were demonstrated. However, when both receptors were overexpressed simultaneously, a significant correlation was found with a higher stage of tumor ($p = 0.04$), the presence of paresthesia ($p = 0.01$) and location of tumor in the parotid gland ($p = 0.0009$).

Individual expression of neuropilins did not correlate with survival rate under univariate and multivariate analyses; however, using the Log-rank test, there was an unfavorable trend, not statistically significant, of Np-1/Np-2 co-expression on patients' survival rate compared to those expressing low levels of Np-1, Np-2 or both ($p = 0.09$).

Co-expression of Np-1/Np-2 also proved to be significantly increased in malignant tumors ($p < 0.0001$).

Using Spearman correlation test, a significant correlation was found between increased expression of Np-1 and Np-2 and total vascular area measured by CD34 ($p = 0.0005$ and $p < 0.0001$, respectively). When Np-1 and Np-2 were simultaneously overexpressed, a significant correlation with vascular area and vascular density was also seen ($p < 0.0001$). Correlations between neuropilins and semaphorins were described in the previous section.

Discussion

The semaphorin family of proteins and their main receptors the neuropilins-1 and -2 were initially described as important factors for successful neuronal growth. Although expression decreases following embryonic development of the neural system, they are still found in some adult human tissues such as thymus, kidney, placenta, heart and endothelial cells, where they have been implicated in physiologic processes other than neural commitment[9,17,18]. Recent data also suggests that both families play a key role in the onset and development of a number of human neoplasias, where interactions with VEGF result in increased angiogenic potential, which favors neoplastic outgrowth and predicts lower survival rates[9,18]. In the current investigation we analyzed the expression and clinical relevance of Sema3A, Sema3B, Np-1 and Np-2 in benign and malignant SGTs. A site-specific pattern of expression was seen in normal salivary glands and an increased expression in all tumor subtypes studied. Moreover, they expression was significantly associated with the angiogenic profile of the lesions and

simultaneous over-expression of neuropilins significantly correlated with clinical parameters like presence of paresthesia and higher tumor stage.

Np-1 and Np-2 are transmembrane glycoproteins of 923 and 926 amino acids, respectively, with a large extracellular region, a single transmembrane domain and small cytoplasmic domains. Neuropilins have the ability to bind with high affinity to two structurally unrelated classes of ligands with different biological functions, class 3 semaphorins and members of the VEGF family[9,19]. Semaphorins are a large family of secreted and membrane bound proteins divided in different classes. Class 3 semaphorins consist of seven subtypes characterized by a distinct cysteine-rich domain of about 500 amino acids called the "sema domain"[9,17]. Studies suggest that class 3 semaphorins compete with VEGF for Np-1/Np-2 binding, thereby inhibiting the mitogenic effects of VEGF in endothelial cells, and leading to apoptosis and inhibition of cell migration in different human cancers[18]. The expression of semaphorins and neuropilins in normal salivary glands and neoplastic tissues has not been appropriately investigated. Cai et al. (2010)[20] found Np-2 receptor to be only faintly present in the ductal system of normal salivary glands and in agreement with this we have shown that both Np-1 and Np-2 are expressed in the ductal cells of mucous and serous glands, but not in myoepithelial or acinar components. Sema3A and Sema3B, however, were found mainly in the acinar compartment of serous glands, with limited expression in the ductal system and no expression in mucous acinar cells. Chung et al. (2007)[21] investigated the role of Np-1 and class 3 semaphorins during submandibular gland development and suggested that Np-1 and Sema3A/C are involved in the morphogenesis of this organ. Thus, the exact role played by these two family of proteins in the development of normal salivary glands has not yet been fully established, but evidences of previous studies and the

specific distribution pattern observed in our research suggest that neuropilin and semaphorin molecular interactions might occur in salivary gland ductal cells, whilst in serous acinar cells semaphorins would bind preferentially to alternative receptors. Moreover, Sema3B was specifically located in the basal aspect of the acinar cells, which is in contrast to the diffuse staining of Sema3A. Thus, further studies are warranted to confirm and better understand this unusual and unreported distribution of Sema3B in normal glandular cells.

Semaphorins and neuropilins have been extensively investigated in a wide range of human neoplasms[22,23] and various reports have pointed towards an oncogenic role for neuropilins and a tumor suppressor function for semaphorins (some semaphorins occasionally act in a pro-tumorigenic manner)[24]. Neuropilins are highly expressed in breast, ovarian, lung, colonic and prostatic neoplasms, correlating with a lower survival rate and poor prognosis[8,12,13,25-27]. In addition, neuropilin expression increases from pre-invasive to invasive lung tumors[11,26], again correlating with tumor progression in breast cancer[28]. In line with their known biological activities some of the class 3 semaphorins, including Sema3A and Sema3B, have lower expression in human cancers than in normal tissues, allowing increased interaction between the pro-angiogenic factor VEGF and neuropilins[8,12,24]. Because of the site-specific expression pattern of these neuropilins and semaphorins in normal salivary glands, we did not quantitatively compare their expression with that seen in the tumors, but we did subjectively note that neuropilins had a stronger and more diffuse staining pattern in the neoplasms than in normal glands, whereas Sema3A and 3B had a similar staining profile in both neoplastic and normal tissues.

Considering previous studies by both our group and others[4-6,29] reporting VEGF overexpression in malignant SGTs, the expression of the proteins investigated in this study is in line with the rationale proposed for VEGF/class 3 semaphorins/neuropilins molecular interactions in a neoplastic environment, with a higher expression of neuropilins (especially Np-2) and their co-expression in malignant tumors than in benign lesions and normal tissues, and an inverted distribution for semaphorins, especially Sema3A whose expression is significantly decreased in malignancies, favoring, therefore, the pro-angiogenic and oncogenic action of VEGF in malignant SGTs through its possible interaction with neuropilin co-receptors.

Although we did not find a statistically significant association between neuropilins/semaphorins and prognosis, we did find an inferior survival rate for those patients that co-expressed high levels of Np-1/Np-2; this has been previously documented in other human cancers[8,12,26] confirming the importance of the simultaneous expression of both receptors to promote an increased oncogenic potential, possibly through VEGF function.

In the current study CD34–measured vascular area proved to be significantly correlated with an increased rate of recurrences, which together with age represented the only clinicopathologic parameters determining an inferior survival for patients affected by malignant tumors in this sample. These results illustrate the clinical importance of the vascular pattern of the tumors, but because vascular density and vascular area did not directly correlate with patients' prognosis, these results are also in contrast with previous reports that found CD34 vascular density as an independent determinant of tumor aggressiveness and prognostic biomarker for patients affected by SGTs[30-33].

Some limitations of the current investigation must be highlighted to better understand the results obtained. Although we used a large number of cases, the sample size of specific malignant microscopic subtypes remained relatively small, possibly precluding statistically significant results. Moreover, some of the conclusions were obtained analyzing all malignant tumors as a single group, not considering their biological heterogeneity. Finally, although a multicenter collaborative study allows the inclusion of a higher number of cases for investigation, it also includes patients who have been subjected to different therapeutic protocols, which could influence the overall survival rate of the studied sample, which in the current research was proved to be lower than previously described in the literature[34].

In conclusion, Sema3A, Sema3B, Np-1 and Np-2 are expressed in normal salivary gland tissue in a site-specific manner, suggesting involvement in the development of these structures. They are also strongly expressed in benign and malignant tumors, indicating that they might play important roles in salivary gland tumorigenesis; however, despite the significant correlation between neuropilins with advanced tumor stage and increased rate of paresthesia, the expression of all proteins did not present a statistically significant prognostic potential in the current study.

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Authors' contributions: **FPF** – designed the study, performed the research, analyzed the data and wrote the paper; **LB** – designed the study, performed the research and analyzed the data; **ARSS** – Analyzed the data and provided cases for the study sample; **MAL** – Analyzed the data and provided cases for the study sample; **OPA –** Analyzed the data and provided cases for the study sample; **BABA** – performed some of the immunohistochemical reactions and analyzed the data; **FVM –** Constructed some of the TMA blocks and analyzed the data; **LP**K **–** Analyzed the data and provided cases for the study sample; **ALCAR –** Analyzed the data and provided cases for the study sample; **MDM –** Analyzed the data and provided cases for the study sample; **LM –** Analyzed the data and provided cases for the study sample; **PMS –** designed the study and analyzed the data; **PAV** – designed the study and analyzed the data.

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Table 1. Clinicopathological features of the studied sample.

Parameter	*n*	*(%)*
Age		
< 40	78/248	*31.5*
≥ 40	156/248	*62.9*
NS	14/248	*5.6*
Sex		
Male	124/248	*50.0*
Female	110/248	*44.4*
NS	14/248	*5.6*
Site		
Parotid	144/248	*58.1*
Submandibular	20/248	*8.1*
Sublingual	4/248	*1.6*
Minor glands	65/248	*26.2*
NS	15/248	*6.0*
Pain*		
Yes	22/128	*17.2*
No	68/128	*27.4*
NS	38/128	*29.7*
Paresthesia*		
Yes	5/128	*3.9*
No	84/128	*65.6*
NS	39/128	*30.5*
Histologic subtype		
Pleomorphic adenoma	99/248	*39.9*
Warthin tumor	21/248	*8.5*
MEC	42/248	*16.9*
AdCC	35/248	*14.1*
Adenocarcinoma, NOS	31/248	*12.5*
SCC	11/248	*4.4*
AcCC	9/248	*3.6*
Stage*		
I/II	31/128	*24.2*
III/IV	57/128	*44.5*
NS	40/128	*31.3*
Treatment*		
Surgery	38/128	*29.7*
Surgery + Radiotherapy	39/128	*30.5*
Other	8/128	*6.3*
No treatment	7/128	*5.5*
NS	36/128	*28.1*
Recurrence*		
Yes	21/128	*16.4*
No	68/128	*53.1*
NS	39/128	*30.5*
Follow-up*		
Alive	59/128	*46.1*
Dead	33/128	*25.8*
NS	36/128	*28.1*

* In relation to total number of malignant tumors (128 cases). NS: Not specified. MEC: Mucoepidermoid carcinoma. AdCC: Adenoid cystic carcinoma. SCC: Squamous cell carcinoma. AcCC: Acinic cell carcinoma.

Table 2. Medians and standard deviations obtained for Sema3A, Sema3B, Np-1 and Np-2 in each tumor subtype.

Tumors	CD34 (Dens.)	CD34 (area)	Np-1	Np-2	Sema3A	Sema3B
PA	0.44 (±1.3)	254.8 (±75.2)	158.1 (±25.7)	124.8 (±21.3)	126.6 (±16.2)	157.4 (±26.7)
Warthin Tumor	3.83 (±1.4)	631.9 (±143.6)	220.1 (±24.6)	197.1 (±46.6)	161.5 (±26.6)	178.6 (±21.1)
AdCC	0.66 (±1.7)	221.9 (±115.3)	187.1 (±40.9)	164.1 (±41.1)	124.4 (±27.7)	175.5 (±38.5)
AcCC	0.54 (±1.5)	208.4 (±97.7)	157.75 (±33.8)	115.5 (±29.8)	186.4 (±50.6)	167.8 (±37.6)
MEC	1.83 (±1.9)	175.7 (±95.4)	146.1 (±23.1)	172.5 (±43.7)	114.0 (±22.1)	149.9 (±26.6)
SCC	0.48 (±2.2)	138.6 (±79.9)	142.7 (±34.7)	223.5 (±30.7)	111.9 (±8.3)	148.9 (±23.9)
Adenoc. NOS	0.70 (±1.8)	159.1 (±58.6)	167.9 (±27.9)	199.7 (±40.5)	112.8 (±18.5)	158.6 (±26.9)

PA: Pleomorphic adenoma. AdCC: Adenoid cystic carcinoma. AcCC: Acinic cell carcinoma. MEC: Mucoepidermoid carcinoma. SCC: Squamous cell carcinoma. Adenoc. NOS: Adenocarcinoma not otherwise specified. CD34 density measured as number of vessels/unit of area (μm^2) and CD34 area as μm^2.

Figures legends

Figure 1. Immunoexpression of Sema3A and Sema3B in normal salivary gland tissue and in benign and malignant tumors (DAB and VectorRed; 200x). **A)** Sema3A was strongly and diffusely present throughout the whole serous acinar compartment of normal parotid gland. **B)** Cytoplasmic staining of Sema3A in pleomorphic adenoma, **C)** mucoepidermoid carcinoma and **D)** acinic cell carcinoma. **E)** Sema3B was observed in the basal portion of normal acinic cells of parotid glands, exhibiting only a faint staining in the ductal system. **F)** Cytoplasmic expression of Sema3B in pleomorphic adenoma, **G)** adenoid cystic carcinoma and **H)** acinic cell carcinoma.

Figure 2. Distribution of Sema3A, Sema3B, Np-1 and Np-2 in benign and malignant salivary gland tumors.

Figure 3. Immunoexpression of Np-1 and Np-2 in normal salivary gland tissue and in benign and malignant tumors (VectorRed; 200x). **A)** Expression of Np-1 in normal salivary gland tissue was found to be restricted to intercalated and striated ducts, with no staining of myoepithelial or acinous cells. **B)** Cytoplasmic and membrane staining of Np-1 was found in pleomorphic adenoma, **C)** adenoid cystic carcinoma and **D)** mucoepidermoid carcinoma. **E)** Np-2 in normal salivary gland tissue was also found in the intercalated and striated ducts only. **F)** Cytoplasmic and membrane staining was found in cases of pleomorphic adenoma, **G)** adenoid cystic carcinoma and **H)** mucoepidermoid carcinoma.

FIGURES

Figure 1.

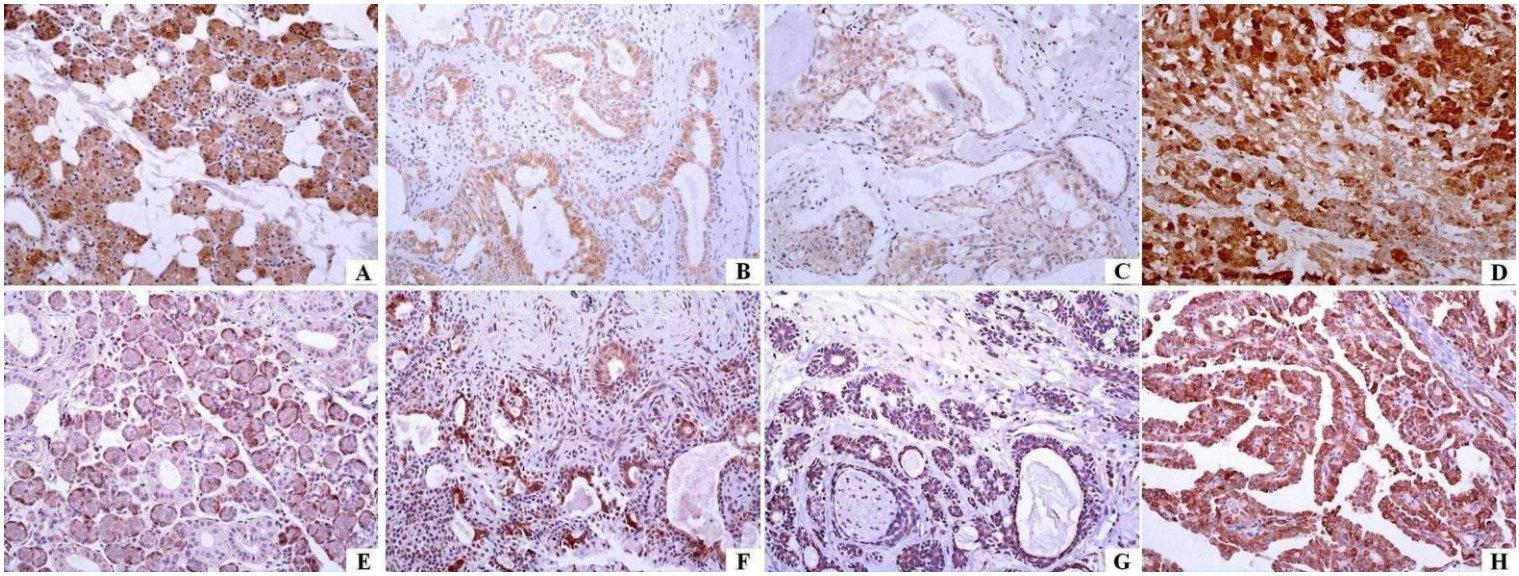


Figure 2.

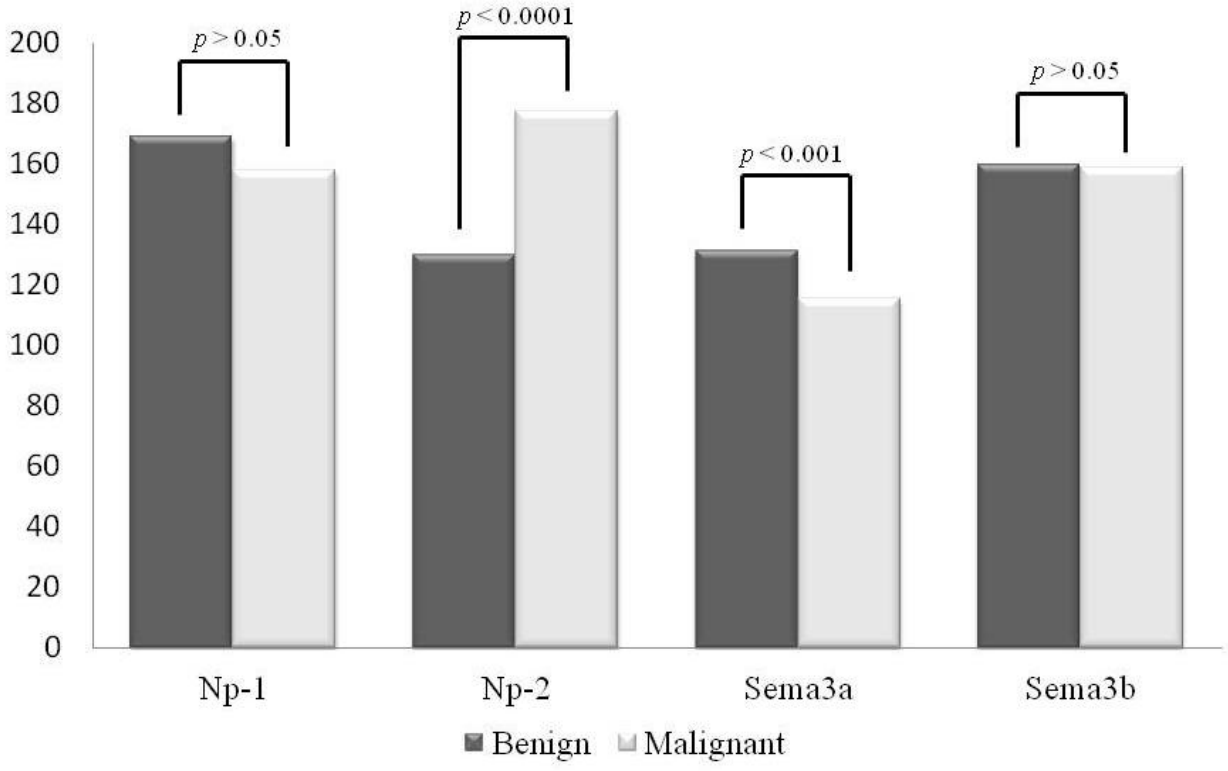
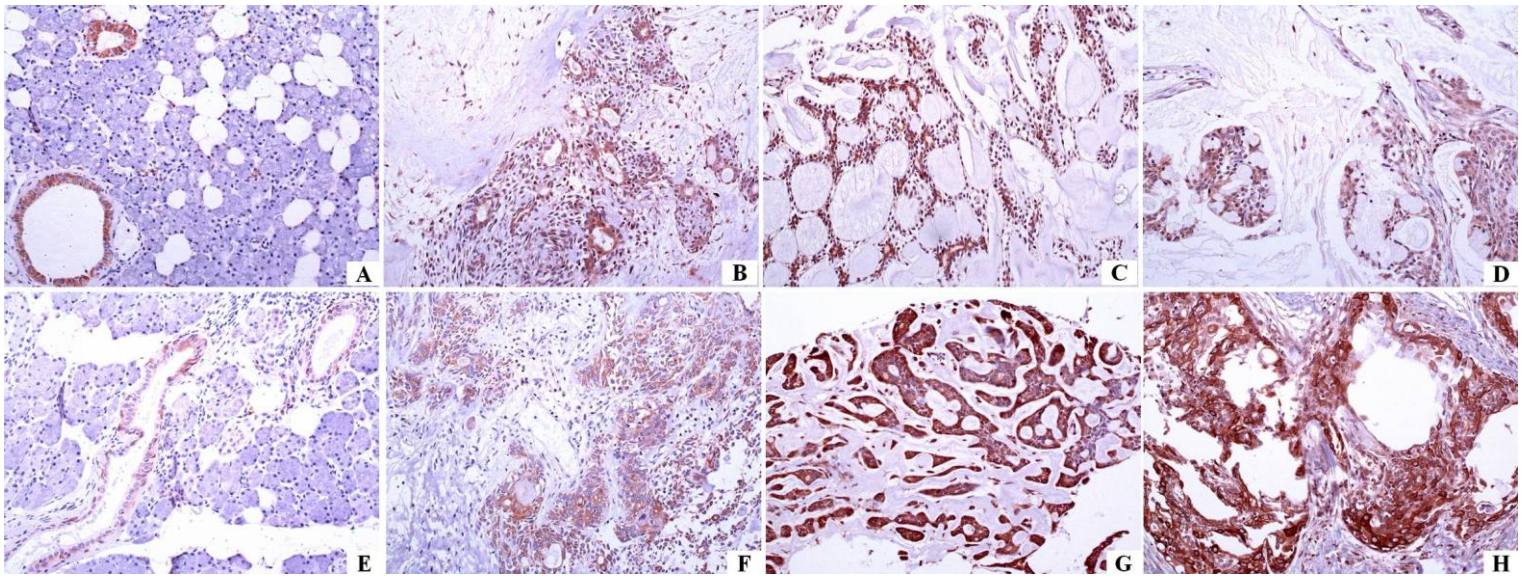


Figure 3.



CONCLUSÃO

- Semaforinas de classe 3A e B, e seus receptores neuropilinas-1 e -2 apresentam um padrão de expressão sítio-específico em glândulas salivares normais, o que associado com dados prévios da literatura, sugere um possível papel destas proteínas no desenvolvimento glandular normal.
- Semaforinas de classe 3A e B, e os receptores neuropilinas-1 e -2 também estão frequentemente expressos em tumores benignos e malignos de glândulas salivares, possivelmente participando do processo de tumorigênese glandular salivar.
- Apesar da expressão simultânea das neuropilinas ter se correlacionado com neoplasias malignas em estádios mais avançados e maior índice de recidivas, a expressão das semaforinas de classe 3A e B e das neuropilinas-1 e -2 não representaram determinantes prognósticos significativos na amostra estudada.

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[*] De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

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Anexo 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise da expressão de citoqueratinas, do padrão angiogênico, proliferativo e de conteúdo de DNA celular de tumores de glândulas salivares "**, protocolo nº 002/2013, dos pesquisadores Felipe Paiva Fonseca e Pablo Agustin Vargas, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 06/02/2013.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Analysis of the expression of cytokeratins, of the angiogenic, proliferative and cellular DNA content patterns of salivary gland tumors"**, register number 002/2013, of Felipe Paiva Fonseca and Pablo Agustin Vargas, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Feb 06, 2013.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

Anexo 2

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15-Dec-2014

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Anexo 3

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