RESEARCH ARTICLE

Overexpression of Antiangiogenic Vascular Endothelial Growth Factor Isoform and Splicing Regulatory Factors in Oral, Laryngeal and Pharyngeal Squamous Cell Carcinomas

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Abstract

Background: Overexpression of proangiogenic vascular endothelial growth factor A family *VEGFA$_{xxx}$* is associated with tumor growth and metastasis. The role of the alternatively spliced antiangiogenic family *VEGFA$_{xxx}$b* is poorly investigated in head and neck squamous cell carcinomas (*HNSCCs*). The antiangiogenic isoform binds to bevacizumab and its expression level could influence the treatment response and progression-free survival. In this study, the relative expression of *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* isoforms and splicing regulatory factors genes was investigated in a series of *HNSCCs*. **Methods:** *VEGFA$_{xxx}$*, *VEGFA$_{165}$b*, *SRSF6*, *SRSF5*, *SRSF1* and *SRPK1* gene expression was quantified by quantitative real time PCR in 53 tissue samples obtained by surgery from *HNSCC* patients. Protein expression was evaluated by immunohistochemistry. **Results:** *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* were overexpressed in *HNSCCs*. Elevated protein expression was also confirmed. However, *VEGFA* isoforms demonstrated differential expression according to anatomical sites. *VEGFA$_{xxx}$* was overexpressed in pharyngeal tumors while the *VEGFA$_{165}$b* isoform was up-regulated in oral tumors. The *VEGFA$_{165}$b* isoform was also positively correlated with expression of the splicing regulatory genes *SRSF1*, *SRSF6* and *SRSF5*. **Conclusions:** We concluded that *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* isoforms are overexpressed in *HNSCCs* and the splicing regulatory factors *SRSF1*, *SRSF6*, *SRSF5* and *SRPK1* may contribute to alternative splicing of the *VEGFA* gene. The findings for the differential expression of the antiangiogenic isoform in *HNSCCs* could facilitate effective therapeutic strategies for the management of these tumors.

Keywords: Head and neck cancer - *VEGFA* - alternative splicing - pathologic angiogenesis - gene expression

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Introduction

Angiogenesis is an essential mechanism for tumor growth and results from genetic and/or environmental alterations. The formation of new vessels is a complex process that involves receptors, cytokines, enzymes and growth factors (Carmeliet et al., 2002), such as the vascular endothelial growth factor A (*VEGFA*). *In vitro* and *in vivo* experiments have shown that increased expression of *VEGFA* is associated with tumor growth and metastasis (Harper et al., 2008).

VEGFA gene consists of eight exons and seven introns with approximately 14 kilobases in length (Houck et al., 1991, Arcondéguy et al., 2013). Alternative splicing of exon 8 from pre-mRNA originates two families of protein: *VEGFA$_{xxx}$* and *VEGFA$_{xxx}$b*, where *xxx* is the number of amino acids encoded. *VEGFA* isoforms are generally co-expressed in all tissues and *VEGF$_{165}$* and *VEGFA$_{165}$b* are the main isoforms. *VEGFA$_{165}$b* protein has 96% homology with *VEGFA$_{165}$*, but it presents different C-terminus amino acid sequence (Eswarappa and Fox, 2015). Studies have shown that *VEGFA$_{165}$b* binds to *VEGFR2* with the same affinity as *VEGF$_{165}$*, but does not activate it completely resulting in alteration of the downstream signaling (Cébe-Suarez et al., 2006, Biselli-Chicote et al., 2012). This alternative isoform was associated with inhibition of endothelial proliferation, migration and vasodilation (Bates et al., 2002), and can reduce the physiological angiogenesis and tumor growth (Qiu et al., 2007), because the isoforms compete for the receptor ligation (Woolard et

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al., 2004). Tumors expressing $VEGF_{165}b$ grow significantly slowly than tumors expressing $VEGF_{165}$ (Woolard et al., 2004), suggesting that a switch in splicing from $VEGF_{165}$ to $VEGF_{165}b$ can inhibit the tumor growth.

The regulation of $VEGFA_{xxx}$ and $VEGFA_{xxx}b$ splicing remains unclear. However, it is known that exon splicing depends on the balance of the activity of serine-rich (*SR*) proteins, such as *SRSF1*, *SRSF5* and *SRSF6*, in determining the C-terminus region. The splicing factors *SRSF1* and *SRSF5* support the proximal splicing site selection of the *VEGFA* pre-mRNA, leading to $VEGFA_{xxx}$ expression, while *SRSF6* supports the distal splicing site selection promoting $VEGFA_{xxx}b$ expression (Nowak et al., 2008). Other important regulators of splicing include some SR protein kinases, such as *SRPK1*, that phosphorylate serine-arginine domains present in splicing factors (Manley et al., 1996). SR proteins can be regulated both directly by *SR* kinase proteins, such as *Clk1* and *SRPKs*, and indirectly by mitogen-activated protein kinase (*MAPK*) and protein kinase C (*PKC*). *SRPKs* phosphorylate *SRSF1* favoring the proximal splicing and *Clk1* results in phosphorylation of *SRSF1*, *SRSF6* and *SRSF5* (Prasad et al., 1999, Lai et al., 2003).

Since most previous studies on *HNSCC* have not distinguished between proangiogenic and antiangiogenic isoforms and there are insufficient data for understanding the role of $VEGF_{165}b$ isoform in *HNSCC*, mainly in oral tumor, we aimed to evaluate the expression of $VEGFA_{xxx}$ and $VEGFA_{165}b$ isoforms in *HNSCC*, and investigate alternative splicing of *VEGFA* in this tumor type.

Materials and Methods

Patients and tissue samples

The study protocol was approved by Institutional Ethics Committee. Informed consent was obtained from the participants of the study. Fresh tissue from 52 *HNSCC* and 26 adjacent non-tumor tissues were collected from 1998 to 2000 at the Head and Neck Surgery Service, Arnaldo Vieira de Carvalho Cancer Institute, Sao Paulo, Brazil, and from 2007 to 2012 at the Otolaryngology and Head and Neck Surgery Service, Hospital de Base / FAMERP, Sao Jose do Rio Preto, Brazil. Samples were immediately frozen in liquid nitrogen and stored at -80ºC until processing. Microdissection of the samples was performed in Pathology Laboratory at Hospital de Base. Representative formalin-fixed block from 26 tumor samples and 15 adjacent non-tumor tissues were selected for immunohistochemical staining.

Median age of the patients was 58±11.56 years. Eighty three percent of the patients were smokers, and 68% were alcohol consumers. The study cohort consisted mostly of male patients (72.1%) and the most frequent primary tumor site was the oral cavity (51%) followed by larynx (26%) and pharynx (23%). Twenty-two percent of the patients performed radio and/or chemotherapy. Despite the relationship of Human Papillomavirus (HPV) infection with *HNSCC* development in some populations, HPV status was not evaluated in our study because the low prevalence of HPV infection in *HNSCC* in Brazilian population (Ribeiro et al., 2011). Tumor staging was performed according to the 7th edition of the *TNM* staging system (Sobin et al., 2010). *T1N0* tumors were classified as stage I; *T1N1* and *T2N0-1* tumors as stage II; *T3N0-1* and *T1-3N2* tumors as stage III; and *T4N0-3*, *T1-3N3* and *T1-4N0-3M1* as stage IV (Fleming et al., 1997). For statistical analyses stage I and II tumors were grouped and classified as non-advanced tumors and stage III and IV tumors were classified as advanced tumors. Based on histopathological examination of the surgical specimen, 33% presented nodal metastasis and 56% of the patients had advanced primary tumors.

Quantitative real time PCR

Total RNA was extracted with TRIzol Reagent (Ambion, TX) following manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed using RT-PCR kit (Applied Biosystems, CA). $VEGFA_{xxx}$ and $VEGFA_{165}b$ expression in *HNSCC* and adjacent non-tumor tissues was evaluated using primers that distinguish both families of *VEGFA* isoform. Primers and probe were designed using Primer Express v.3.0 software (Applied Biosystems, CA) using the *VEGFA* complementary DNA (cDNA) sequence (GenBank: NM_001171623.1). Primer set and probe used for detection of $VEGFA_{xxx}$ family amplify $VEGFA_{148}$, $VEGFA_{165}$, $VEGFA_{183}$, $VEGFA_{189}$ and $VEGFA_{206}$ isoforms: forward primer 5' AACACAGACTCGCGTTGCAA 3', reverse primer 5' CGCCTCGGCTTGTCACAT 3' and TaqMan MGB 6-FAM probe 5' AGCTTGAGTTAAACGAAC 3'. Reactions were performed in triplicate in 96 wells plate using 100ng of cDNA, 100 nM of forward primer, 300 nM of reverse primer and 250 nM of probe (Applied Biosystems, CA). The reactions were performed on StepOne Plus Real-Time PCR System (Applied Biosystems, CA) and cycled following manufacturer's instructions.

Foward primer and probe used for $VEGFA_{xxx}$ were also used for $VEGFA_{165}b$ (GenBank:NM_001171629.1); however a specific reverse primer to the end of the exon 7 and beginning of the exon 8b was used to detect only the $VEGFA_{165}b$ isoform (5' TTCCTGGTGAGAGATCTGCAAGTA 3').

The reactions for $VEGFA_{165}b$ quantification were performed using 100 ng of cDNA, 900 nM of forward and reverse primers and 250 nM of probe (Applied Biosystems, CA). Primers and probes sequences for *SRSF6*, *SRSF5*, *SRSF1* and *SRPK1* genes were analyzed using TaqMan Gene Expression Assay (Applied Biosystems, CA).

Raw qPCR data were calculated by StepOne software version 2.0 (Applied Biosystems, CA) after manual adjustment of the basal fluorescence signal and the threshold. Relative gene expression from tumor samples was analyzed using non-tumor samples as calibrator group and *TBP* and *RPLPO* as reference genes (Applied Biosystems, CA). Fold change (FC) was calculated by ddCt algorithm.

Immunohistochemistry

Three-micrometer thick sections of formalin-fixed paraffin-embedded tissues were cut and mounted onto silanized glass slides. Sections were dewaxed, rehydrated,

Table 1. Association Between Gene Expression and Clinicopathologic Features of the *HNSCC* Patients

Variables		$VEGFA_{xxx}$		$VEGFA_{165}b$		SRSF1		SRSF6		SRSF5		SRPK1	
	N	FC*	p Value	N	FC	p Value	N	FC	p Value	N	FC	p Value	N	FC	p Value
Total Tumors	51	1.50	<0.0001	49	3.06	<0.0001	52	0.86	0.293	52	0.68	0.305	52	0.75	0.545	52	1.02	0.3171
Primary site																		
Oral	25	1.30	0.038	24	2.9	0.02	26	0.31	**	26	0.61	0.169	27	0.79	0.033	27	0.65	<0.0001
Pharynx	12	4.93		12	1.55		-	-		11	0.93		11	1.45		13	12.05	
Larynx	14	2.30		14	1.39		14	0.20		13	0.44		13	2.0		14	2.96	
TNM stage																		
I-II (non-advanced)	14	1.47	0.214	15	1.45	0.153	16	0.97	0.412	16	0.75	0.034	16	0.86	0.439	16	0.81	0.745
III-IV (advanced)	31	2.40		30	4.50		30	0.82		30	0.62		30	0.55		30	1.12	
Tumor extent																		
T1-T2	15	1.48	0.800	16	1.55	0.188	17	0.82	0.601	17	0.75	0.108	17	0.58	0.543	17	0.81	0.406
T3-T4	29	1.49		29	4.50		29	0.91		29	0.62		29	0.85		29	1.13	
Nodal metastasis																		
N0	26	1.49	0.765	27	1.76	0.492	28	0.90	0.220	18	0.73	0.442	28	1.0	0.229	28	0.95	0.721
N+	19	1.50		18	3.88		18	0.5		28	0.57		18	0.51		18	1.12	

FC , fold change. *Median; ** No amplification of the calibrator sample; Significant p Value in bold.

washed in distilled water and the antigenic retrieval was performed in microwave with sodium citrate (pH 6.0) heated at 97 ºC for 20 minutes. Reactions were performed with REVEAL Biotin-Free Detection System (Spring Bioscience, CA) following manufacturer's instructions. Incubation with pan-VEGF antibody (*MAB293*, R and D Systems, MN) diluted 1:50 or *VEGFA$_{165}$b* antibody (*MAB3045*, R and D Systems, MN) diluted 1:50 was performed overnight at 4ºC. Subsequently, the slides were washed with phosphate-buffered saline for 5 minutes and incubated with HRP conjugate and diaminobenzidine (DAB) as chromogen. The development time in DAB solution was 10 minutes. The slides were counterstained with Harris's hematoxylin for 40 seconds. Sections from mammary tumor tissue were used as positive control, and slides without primary antibody treatment were used as negative control. Images were obtained with a Camera Retiga 4000R (QImaging, CA) attached to an Olympus microscope (Model BX53, NY), and captured, averaged, and digitized using Image-Pro Plus 7.01 software (Media Cybernetics, USA). Illumination exposure was uniformly maintained and regularly checked in order to prevent any distortion of measurements (immunopositive area, gray level) among the samples. After capture, the image was analyzed using the software ImageJ, version 1.43m (Bethesda, USA) (Jensen et al., 2013). After selection of the squamous cell region, the software calculated the immunopositive area by counting all pixels with gray intensity equal or superior to the threshold of staining. The threshold was defined for each protein evaluated, based on the mean immunopositivity of all control cases and taking into account the nuclear hematoxylin staining. Results were shown as a percentage of immunopositive area/total area. Three areas from each image were evaluated and the mean of percentage of immunopositivity was obtained for statistical analyses.

Statistical analyses

Statistical analyses were performed using the GraphPad Prism software version 5.01 and StatsDirect software version 3.0.171. Wilcoxon Signed Rank Test was performed to analyze gene expression in tumor samples. Analyses of gene expression according the tumor progression was performed by Mann-Whitney Test. Comparison of gene expression among the primary tumor sites was evaluated by Kruskal-Wallis Test followed by Dunn's Multiple Comparison Post Test. Spearman correlation was used to evaluate the correlation between *VEGFA* isoforms and splicing factor expression in tumors. Immunohistochemistry data were evaluated by Mann-Whitney Test or Kruskal-Wallis Test. Survival analysis was performed using the Kaplan-Meier curve and Log-rank test. Results with a p value < 0.05 were considered statistically significant.

Results

Overexpression of VEGFA$_{xxx}$ and VEGF$_{165}$b in HNSCC

VEGF$_{xxx}$ transcripts was detected in 51 samples and VEGF$_{165}$b transcripts was detected in 49 samples. Overexpression of *VEGFA$_{xxx}$* ($p < 0.0001$) and *VEGFA$_{165}$b* ($p < 0.0001$) was observed in tumor samples as compared to adjacent non-tumor tissues (Table 1 and Figure 1). Radio and/or chemotherapy did not change *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* expression in the analyzed casuistic (data not shown). Corroborating gene expression data, immunohistochemistry analyses showed that *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* proteins are significantly ($p < 0.0001$) overexpressed in tumor samples (75.11% and 63.89% of the stained area, respectively) as compared to non-tumor

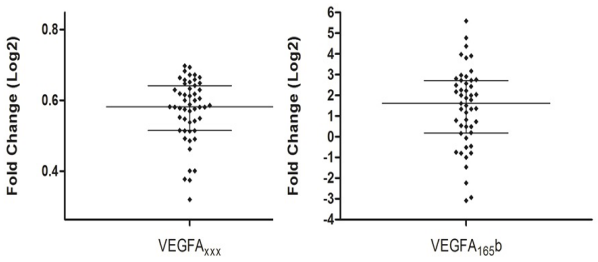

Figure 1. *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* Expression in *HNSCC* as Compared to Non-Tumor Tissues. Fold change were Log2 transformed (y-axis). *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* were overexpressed in tumors (Wilcoxon Signed Rank Test: $p < 0.0001$). The bars represent median with interquartile variation (25th percentile and 75th percentile). Calibrator (non-tumor tissues) log RQ = 0.

tissues (33.64% and 18.16%) (Figure 2).

Differential expression of VEGFA isoforms according to the anatomical sites

Analysis of expression in tumors compared to non-tumor tissues showed that *VEGFA$_{xxx}$* was significantly overexpressed in pharynx tumors ($p = 0.001$). Overexpression of *VEGFA$_{xxx}$* was also observed in

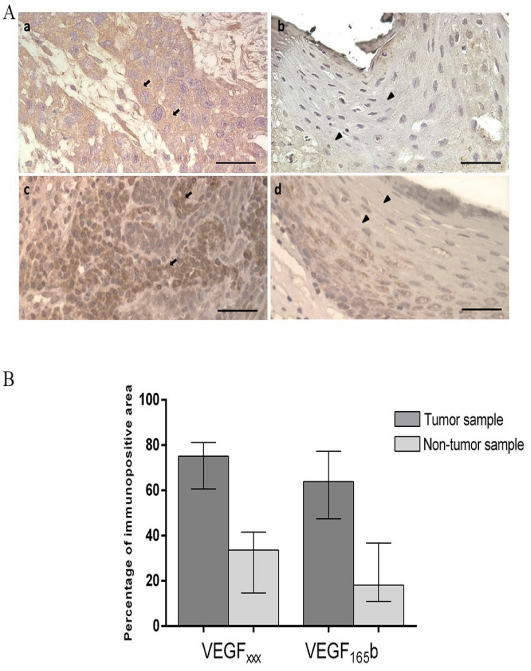

Figure 2. A. Representative Graphic of Immunolocalization of *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* proteins in *HNSCC* and non-tumor tissue. A. Cytoplasmic immunostaining for *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* in formalin-fixed paraffin-embedded sections of oral tumors (a and c, respectively) and non-tumor tissues (b and d, respectively). Arrows indicate strong staining in the cytoplasm of tumor samples and arrows head indicate weak staining in the cytoplasm of non-tumor samples. Bar = 1000µM. B. Comparison of *VEGFA$_{165}$b* and *VEGFA$_{xxx}$* immunopositive area in *HNSSC* and non-tumor tissue. Tumors presented increased *VEGFA$_{xxx}$* and *VEGFA$_{165}$b* immunoreactivity compared to non-tumor tissue (Mann-Whitney test, $p < 0.0001$). The bars represent median with interquartile variation (25th percentile and 75th percentile).

Table 2. Correlation among Splicing Factors and *VEGFA* Isoforms in *HNSCC*.

Splicing factor	*VEGFA$_{xxx}$*		*VEGFA$_{165}$b*	
	r[a]	p Value	r[a]	*p value*
SRSF1	0.081	0.566	0.387	0.005
SRSF6	-0.056	0.695	0.337	0.017
SRSF5	0.193	0.17	0.444	0.001
SRPK1	-0.06	0.673	0.224	0.118

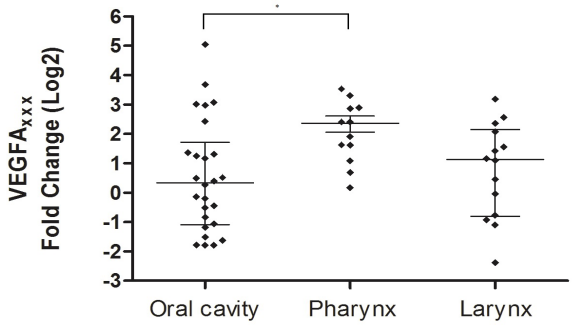

Figure 3. *VEGFA$_{xxx}$* Expression According to the Anatomical Sites. Fold change were Log2 transformed (y-axis). The bars represent median with interquartile variation (25th percentile and 75th percentile). Calibrator (non-tumor tissues) log RQ = 0. *Statistically significant (Kruskal-Wallis test, $p=0.038$).

larynx tumors ($p = .035$). *VEGFA$_{xxx}$* expression differed significantly among the anatomical sites ($p = 0.038$), and the Post Test showed higher expression in the pharynx than oral tumors (Table 1 and Figure 3). *VEGFA$_{165}$b* showed significantly higher expression only in oral tumors as compared to non-tumor tissues ($p = 0.0005$) and presented differential expression among the anatomical sites ($p = 0.02$); the Post Test showed higher expression in oral tumor than in larynx tumor (Table 1 and Figure 4). Regarding protein expression, there was no significant difference in expression of VEGFA$_{xxx}$ and VEGFA$_{165}$b among the anatomical sites ($p = 0.8473$).

Expression of VEGFA isoforms was not associated with tumor progression

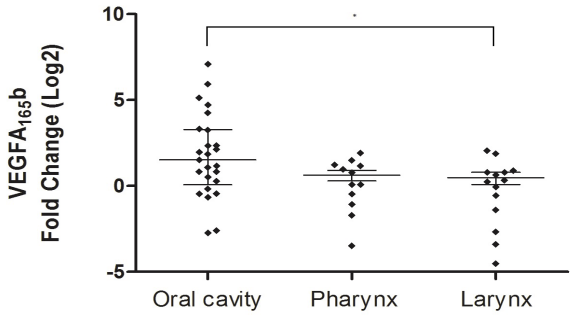

Figure 4. *VEGFA$_{165}$b* expression according to the anatomical sites. Fold change were Log2 transformed (y-axis). The bars represent median with interquartile variation (25th percentile and 75th percentile). Calibrator (non-tumor tissues) log RQ = 0. *Statistically significant (Kruskal-Wallis test, $p=0.02$).

Table 3. Analysis of the Prognostic Role of $VEGFA_{xxx}$ and $VEGFA_{165}b$ Expression in HNSCC

	12-month disease-free survival (%)	*p value*	12-month overall survival (%)	*p value*
$VEGFA_{xxx}$				
Overexpression	75	0.963	100	0.902
Down expression	81		90	
$VEGFA_{165}b$				
Overexpression	75	0.687	100	0.91
Down expression	79		100	

Information about TNM was possible for 47 patients. Comparison between tumors T1/T2 and T3/T4 showed no difference in $VEGFA_{xxx}$ and $VEGFA_{165}b$ expression (p = 0.800 and p = 0.188, respectively) (Table 1). $VEGFA_{xxx}$ and $VEGFA_{165}b$ (p = 0.765 and p = 0.492, respectively) did not differ between tumors grouped according to the presence of nodal metastasis (Table 1). Thirty one patients presented advanced tumors (stage III and IV) and 16 presented non-advanced tumors (stage I and II). There was no difference in gene (p = 0.214 for $VEGFA_{xxx}$ and p = 0.153 for $VEGFA_{165}b$) and protein (p = 0.557 for VEGFAxxx and p = 0.103 for $VEGFA_{165}b$) expression between advanced and non-advanced tumors.

Expression of SR splicing factors in HNSCC

SRSF1, *SRSF5*, *SRSF6* and *SRPK1* transcripts were detected in 52 tumor samples. *SRSF5* presented higher expression in pharynx tumor as compared to non-tumor tissue (p = 0.003) and to the other anatomical sites (p = 0.033) (Table 1). *SRPK1* was overexpressed in pharynx (p = 0.001) and larynx (p = 0.0009) tumors as compared to non-tumor tissue. *SRPK1* presented differential expression among the anatomical sites (p < 0.0001), and the Post Test showed higher expression in pharynx and larynx tumors than in oral tumor (Table 1). *SRSF6* expression was lower in advanced tumors as compared to non-advanced tumors (p = 0.0339) (Table 1).

Correlation between the expression of VEGFA isoforms and SR proteins

VEGF165b presented positive correlation with *SRSF1*, *SRSF5* and *SRSF6* expression in tumors (Table 2). No correlation was observed among SR factors and $VEGFA_{xxx}$. Analysis of correlation between SR kinase *SRPK1* and SR factors showed positive correlation with *SRSF1* (r = 0.533, p < 0.0001), *SRSF5* (r = 0.509, p = 0.002) and *SRSF6* (r = 0.552, p < 0.0001) in tumor tissues.

Survival Analysis

The prognostic role of $VEGFA_{xxx}$ and $VEGFA_{165}b$ was evaluated by Kaplan-Meier survival analysis. Disease-free survival was defined as the time from surgical resection of the primary tumor to the tumor recurrence. Overall survival was defined as the time from surgical resection to the death or last follow-up. Patients were followed for a period of 48 months (median) and a maximum of 101 months. Fold change (FC) above the median was considered high expression of $VEGFA_{xxx}$ (FC = 1.5) and $VEGFA_{165}b$ (FC = 3.06). The results showed that high expression of $VEGFA_{xxx}$ and $VEGFA_{165}b$ have no significant effect on disease-free survival or overall survival time (Table 3).

Discussion

In the present study, *VEGFA* isoforms were overexpressed in *HNSCC* tumor samples as compared to non-tumor tissue. Overexpression of *VEGFA* is associated with tumor growth and results in increased angiogenesis (Das et al., 2007). Anti-VEGFA drugs, as bevacizumab and sunitinib, are often used in the treatment of patients with cancer (Prager et al., 2010). *VEGFA* overexpression have been found in solid tumors, such as breast cancer (Schneider et al., 2005), colorectal tumor (Ferroni et al., 2006) and head and neck squamous cell carcinoma (Uehara et al., 2004, Jaiswal et al., 2011). For oral tumors, *VEGFA* expression was significantly associated with a poor prognosis (Uehara et al., 2004). In bladder cancer, *VEGFA* protein was positively correlated with the tumor progression (Yang et al., 2015).

In 2002, Bates and colleagues identified the $VEGFA_{165}b$ isoform in renal tissue and proposed an antiangiogenic role for this variant. Analyses in vitro have shown that the recombinant $VEGFA_{165}b$ and $VEGFA_{121}b$ proteins induced human umbilical vein endothelial cell (*HUVEC*) proliferation and *VEGFR-2* phosphorylation (Catena et al., 2010). However, *HUVEC* proliferation was approximately 50% less stimulated by $VEGFA_{121/165}b$ in comparison to $VEGFA_{165}$. According to the authors, both $VEGFA_{xxx}$ and $VEGFA_{xxx}b$ isoforms equally compete for binding to the receptor, although $VEGFA_{xxx}b$ isoform induces less effectively the angiogenesis. Our findings show overexpression of the antiangiogenic isoform $VEGF_{165}b$ in *HNSCC*. Individuals with high relative levels of $VEGFA_{165}b$ could not benefit with antiangiogenic treatment, once this isoform also binds bevacizumab, preventing the binding with the proangiogenic isoforms and inhibition of angiogenesis.

The expression of *VEGFA* isoforms was not associated with head and neck tumor progression in this study. Overexpression of total *VEGFA* in oral and pharynx advanced tumors (T3 and T4) has been observed in the presence of nodal metastasis (Boonkitticharoen et al., 2008). High expression of total *VEGFA* was associated with larger tumor size, tumor progression and metastasis in larynx (Sullu et al., 2010). However, regarding the antiangiogenic isoform, recent data have shown that $VEGF_{xxx}b$ was overexpressed in 97.3% of the pharynx and larynx tumor, independent of HPV status, but the expression was not associated with lymph node metastasis and survival (Wilkie et al., 2016). These findings showed overexpression of $VEGF_{xxx}$b in *HNSCC*, although it does not seem a reliable prognostic biomarker for tumors with presence of nodal metastasis, corroborating our results.

Analyzing the *VEGFA* isoforms expression data according to the tumor primary sites, we observed that pharynx tumors presented higher $VEGFA_{xxx}$ expression, and oral tumors presented higher $VEGFA_{165}b$ expression, reflecting the differential expression of these isoforms

among head and neck tumor sites. In this study, the overexpression of *SRSF5* in the pharynx tumor is in accordance with the high expression of $VEGFA_{xxx}$ isoforms in this anatomical site. *SRSF5* and *SRSF1* are associated with the selection of proximal splicing site favoring the synthesis of $VEGFA_{xxx}$. Although the *SRSF1* overexpression was not observed in tumor samples in the present study, it is possible that the high availability of *SRPK1* results in more efficient activation of *SRSF1* protein. *SRPK1* is associated with the proximal splicing site selection, and presented high expression in pharynx and larynx tumors, as well as $VEGFA_{xxx}$. *SRSF6* was down expressed in advanced tumors as compared to non-advanced tumors. *SRSF6* promotes $VEGF_{165}b$ expression; however, other factors, such as *TGFβ1* and *IGF-1*, could also play a role in the regulation of the *VEGFA* alternative splicing (Nowak et al., 2008; Slomiany et al., 2004).

Concerning the splicing regulation of *VEGFA* isoforms by *SR* proteins, positive correlation was observed between gene expression of $VEGFA_{165}b$ isoform and all splicing factors, *SRSF1*, *SRSF6* and *SRSF5*, in the present study. On the other hand, no correlation was found between the expression of *SR* proteins and $VEGFA_{xxx}$. Although the effect of these splicing factors has been proposed by in vivo studies (Nowak et al., 2008; Nowak et al., 2010) the interaction between *SR* proteins and their target RNA at a specific site is influenced by several and multiple determining factors, such as competition with other SR proteins (Pandit et al., 2013) or other RNA binding proteins, including heterogeneous ribonucleoprotein proteins, and RNA secondary structure (Long et al., 2009).

The expression of the *SR* kinase *SRPK1* presented positive correlation with *SRSF1*, *SRSF6* and *SRSF5* in tumor tissues. These results suggest the co-expression of these splicing factors and the *SR* kinase *SRPK1* and their involvement in *VEGFA* splicing. To date, it is known that *SRPK1* phosphorylates *SRSF1*, but it can also phosphorylate other factors, including *SRSF6*. It is also known that other protein kinases like Clk1 are associated with the phosphorylation of *SRSF1*, *SRSF6* and *SRSF5*. Further, *SR* proteins can be indirectly regulated by PKC and MAPK (Prasad et al., 1999; Lai et al. 2003). Thus, other factors may contribute to the expression of these proteins in tumor samples.

In our point of view, the main finding was the overexpression of $VEGFA_{165}b$ in *HNSCC* as compared to non-tumor tissue. Importantly, there are no published studies investigating the gene expression pattern of $VEGFA_{165}b$ isoform in *HNSCC*. These results are important for the knowledge of the biology of head and neck cancer and may contribute to directing more effective therapeutic strategies in the treatment of these tumors. In addition, it is important to note that anti-*VEGFA* therapies currently used in cancer treatment target both the pro- and antiangiogenic isoforms, which could prevent the antiangiogenic activity of $VEGFA_{xxx}b$ isoforms in head and neck cancer, since this isoform is up regulated in this tumor type. Considering these issues, the use of therapy targeting only $VEGFA_{xxx}$ could improve the treatment outcomes in *HNSCC* (Carter et al., 2015).

In summary, the results showed that $VEGFA_{xxx}$ and $VEGFA_{165}b$ are overexpressed in *HNSCC* development with possible contribution of *SRSF1*, *SRSF6*, *SRSF5* and *SRPK1* regulatory factors in alternative splicing of *VEGFA* gene. The findings contribute to the understanding of the role of $VEGFA_{165}b$ in cancer angiogenesis and increase the knowledge about the mechanism related to carcinogenesis development in head and neck.

Conflict of interest

The authors declare that they have no conflict of interest.

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