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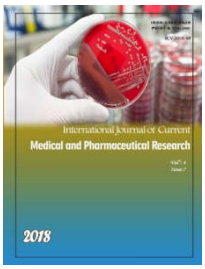

IMMUNOHISTOCHEMICAL EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR-C (VEGF-C) IN SALIVARY GLAND TUMOURS

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ABSTRACT

Context/Background: Vascular endothelial growth factor C expression has been focus of research for variety of neoplasms owing to its potential role played in lymphangiogenesis and metastasis through interactions with various molecules.
Objective: This study was designed to determine the immunohistochemical expression of VEGF-C in benign and malignant salivary glands tumours in local Pakistani population.
Materials & Methods: This descriptive study was conducted at the Department of Morbid Anatomy and Histopathology/ Oral Pathology, University of Health Sciences Lahore, Pakistan. Biopsies and detailed clinical data of 85 cases of salivary gland neoplasms (31 benign and 54 malignant) were obtained from different local tertiary care hospitals in Lahore from Jan. 2015 to Sep 2016. After confirming the histologic diagnosis,immunohistochemical expression of VEGF-C was determined in the salivary gland tumours. SPSS version 21.0 Chi-square and Fischer Exact tests were applied for statistical analysis and $p<0.05$ was considered to be statistically significant.
Results: Expression of VEGF-C and staining patternwas found to be significantly associated with benign and malignant salivary gland tumours ($p=0.002$ and $p<0.0001$).
Conclusion
Higher expression of VEGF-C in malignant salivary gland tumours may predict its potential advantage as a biologic marker and promising therapeutic target to restrict the metastatic spread of malignant salivary gland neoplasms to distant organs.

INTRODUCTION

Lymphangiogenesis is a crucial player for metastasis in various cancers and this role is verified by the expression of various lymphangiogenic markers. These lymphangiogenic markers enable the pathologists to predict behaviour and prognosis for various malignant neoplasms. Vascular endothelial growth factor-C (VEGF-C) is one such marker[(1)].

Vascular endothelial growth factor-C (VEGF-C), a 21-kD non-disulfide-linked homodimeric protein, is a member of vascular endothelial growth factor (VEGF) family and platelet-derived growth factor (PDGF) superfamily [(2)]. It has high affinity for both vascular endothelial growth factor receptor 2 & 3 (VEGFR-2 & VEGFR-3). Through its interaction with VEGFR3 and VEGFR2, VEGF-C promotes lymphangiogenesis (both peritumoral and stromal) and angiogenesis [(2)]. Molecular interaction of VEGF-C with chemokines facilitates entry of tumour cells into the lymphatic vessels [(2)], stimulate dilation of lymphatic vessels and hyperplasia of sentinel lymph nodes, thus ultimately leading to metastasis and tumour progression [(3)].

Salivary gland tumours exhibit tremendous morphological variability in their histologic profile including features like hybrid tumours, anaplasia, lack of proper grading systems and tendency for benign tumours to transform into malignant ones,

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so necessitating the use of specialized techniques for proper diagnosis and prediction of their biological behavior[4].

It is a common notion that carcinoma spreads through lymphatics and sarcomas spread through blood vessels[5], the fact that lymphangiogenesis can be a mechanism during malignant transformation of benign salivary gland tumours[6] lead us to study the expression of VEGF-C in benign and malignant salivary gland tumours.

MATERIALS AND METHODS

This study was conducted at the Department of Morbid Anatomy and Histopathology/Oral Pathology, University of Health Sciences, Lahore. A total of 85 biopsies, 25 each of pleomorphic adenoma (PA), adenoid cystic carcinoma (AdCC)& mucoepidermoid carcinoma (MEC), 6 of Warthin tumour (WT) and 2 each of carcinoma ex pleomorphic adenoma (CEPA) and basal cell adenocarcinoma (BCA) of salivary glands reported at Histopathology Departments of University of Health Sciences, King Edward Medical College/Mayo hospital, Sheikh Zaid hospital and Fatima Jinnah Medical College /Ganga Ram Hospital, Lahore from January, 2015 to September, 2016 were included in the study. Detailed clinical data including clinical stage (wherever possible) was retrieved from the respective departmental records. After approval from the ethical committee of University of Health Sciences, Lahore, we proceeded towards the laboratory procedures.

Hematoxylin &eosin staining

Paraffin embedded tissue sections were made from biopsy specimens. Tissue sections of 4µm were cut using rotary microtome and were stained with hematoxylin and eosin stain. Diagnosis was confirmed by 2 oral pathologists/ Histopathologists. Subtype determination of PA was done according to the criteria provided by Seifert [7].

Histologic grading of AdCC was done according to the grading criteria provided by Spiro[8] where mostly tubular or cribriform pattern (no stipulations or minor solid components) was given grade I, 50% solid pattern was grade IIand mostly solid pattern was assigned grade III.

Grading of MECs was done on the basis of less than 20% cystic component (+2), presence of neural invasion (+2), necrosis (+3), ≥ 4 mitoses per 10 high power fields (+3) and anaplasia (+4). Sum of the point values was used to determine low (0-4), intermediate (5-6) or high (7-14) grade MEC[9].

Immunohistochemistry

About 4 µm thick tissue sections were cut with the help of rotary microtome and taken on Poly-L-lysine coated slides for immunohistochemical staining with anti-VEGF-C antibody. Two sections were taken from each block, dried at 60^0 C for 50 minutes followed by de-waxing in xylene and rehydration in alcohol. Next, the slides were placed in Coplin jars containing citrate buffer (pH 6.0) solution and then in hot water bath (95^0C) for 40 minutes in order to retrieve antigens (Heat Induced Epitope Retrieval). After removing the slides from hot water bath, they were allowed to cool at room temperature and hydrogen peroxide was added to block endogenous peroxidase activity followed by thorough washing with PBS (phosphate buffered saline).Sections were then incubated with 1-2 drops of protein blocker for 10 minutes to block endogenous enzymatic activity and then again washed with PBS. This was followed by incubation with primary antibody, rabbit anti-human VEGFC polyclonal antibody (Abcam ab135506, Cambridge, UK) diluted to 1:25µg/ml (suggested dilution by the manufacturer) for 1 hour. Then, sections were incubated successively with Biotinylated Secondary Antibody for 10 minutes and Streptavidin Peroxidase Reagent for 10 minutes before application of DAB (di-amino-benzidine) (2 minutes) to avoid false positive staining. All incubation steps were separated by thorough washing with PBS. Counter staining with hematoxylin was done followed by dehydration and mounting of sections with coverslips using DPX. Positive (oral mucosa and skin) and negative (omission of primary antibody) controls were run with each batch of 20 histological sections of salivary gland tumours. VEGF-C staining was evaluated on the basis of extent and intensity immunolabeling of tumor cells [10].
The intensity (qualitative variable) of staining was scored:
0 (absent), 1 (weak), 2(moderate) and 3(strong)

The proportion (quantitative variable) of tumor cells staining was semi-quantitatively evaluated as:

0 (no positive tumor cells);
1 (1 - 10% positive tumor cells);
2 (11% to 49% positive tumor cells);
3 (>50% positive tumor cells)

Total/Final Score*:* The sum of the intensity and extent scores was the final score (0-6).
Negative: 0-1
Weak positive (1+): 2
Moderate positive (2+): 3-4
Strong positive (3+): 5-6

Statistical Analysis

The clinical, histological and immunohistochemical data was analyzed statistically using SPSS 21.0. Chi-square and Fischer Exact tests were applied and p-value <0.05 was considered to be statistically significant.

RESULTS

The clinical parameters of the salivary gland tumours studied are summarized in Table 1.

The mean age for benign tumours was found to be 32.52± 16.326 years with an age range of 12-70 years. Most patients were seen in 2nd& 3rd (25.8% each) decades of life. Almost equal gender predisposition was noted (F:M, 1.06:1). Parotid gland (61.3%) was the most frequent site affected followed by minor salivary glands (22.6%) with palate being the commonest site (71.4%).
Mean age for the malignant cases was calculated to be 35.65±14.130 years with an age range of 9-70 years. Most patients were seen in 5th decade (37%) of life. A slight male predilection of 1:1.25 was noted in malignant salivary gland tumours. Most of these tumours arose in minor salivary glands (48%) followed by parotid gland (42.6%). Of the minor salivary gland sites, palate (34.6%) was the most frequent site involved. Regarding the clinical stage (TNM staging system) of AdCC and MEC, most cases were in stage I & II (Table 1).
Statistically significant association was noted among tumour type and age of patients (p=0.028), gland involved (p<0.0001) and laterality (p= 0.041). Clinical stage and histological grades in both AdCC and MEC were significantly associated (p=0.003 and p<0.001 respectively) (Table 1).

Table 1 Frequencies, Percentages and p-value Regarding Clinical Data of the Salivary Gland Tumours (n=85)

Parameter	PA		WT		AdCC		MEC		BCA		CEPA		Total		p-value
	F	%	F	%	F	%	F	%	F	%	F	%	F	%	
Age															
Mean Age	30.36 ± 14.838		41.50 ± 20.550		41.32 ± 11.022		31.44 ±2.999		34.00 ± 19.799		19.00 ± 8.485		34.51 ± 14.949		
Minimum (years)	12		18		22		9		20		13		09		0.028
Maximum (years)	70		70		70		70		48		25		70		
Commonest decade	2nd& 3rd		6th		5th		3rd		-		-		5th		
Gender															
Female	14	56	2	33	9	36	13	52	1	50	1	50	40	47	0.724
Male	11	44	4	67	16	64	12	48	1	50	1	50	45	53	
F:M	1.3 : 1		1 : 2		1 : 1.8		1.1 : 1		1 : 1		1 : 1		1:1.1		
Gland															
Parotid	15	60	04	66.7	4	16	17	68	01	50	1	50	42	49.4	<0.001
Submandibular	04	16	01	16.7	0	0	02	08	0	0	1	50	08	9.4	
Sublingual	0	0	0	0	0	0	02	08	0	0	0	0	02	2.4	
Minor	06	24	01	16.7	21	84	04	16	01	50	0	0	33	38.8	
Laterality															
Right	10	40	01	16.7	09	36	13	52	1	50	2	100	36	42	0.041
Left	10	20	05	83.3	09	36	1	4	1	50	0	0	33	39	
Not mentioned	05	20	0	0	07	28	11	44	0	0	0	0	16	19	
Clinical stage															
Tumour size															
T1-2	-	-	-	-	16	64	17	68	-	-	-	-	-	-	0.001
T3-4	-	-	-	-	09	36	08	32	-	-	-	-	-	-	
Nodes															
Positive	-	-	-	-	02	08	12	48	0	0	02	100	23	42.6	
Negative	-	-	-	-	16	64	11	44	0	0	0	0	16	29.6	
Not reported	-	-	-	-	07	28	02	8	02	100	0	0	15	27.8	
Metastasis															
M_0	-	-	-	-	25	100	24	96	-	-	-	-	-	-	
M_1	-	-	-	-	0	0	01	04	-	-	-	-	-	-	
Final stage															
I/II	-	-	-	-	15	60	13	52	-	-	-	-	-	-	
III/IV	-	-	-	-	07	28	08	40	-	-	-	-	-	-	
Unknown	-	-	-	-	03	12	04	16	-	-	-	-	-	-	

Cell-rich or cellular subtype of PA (n=12; 48%) was the commonest subtype noted in PA, closely followed by classic (n=11; 44%). Only 2(8%) cases of stroma rich/hypo-cellular subtype were seen in the current study.

Regarding tumour morphology of malignant tumours, 17 (68%) cases of AdCC were of grade I and 8(32%) were grade III. Cribriform pattern (n=15; 60%) was the predominant pattern noted in AdCC followed by tubular (n=6; 24%) and solid (n=4; 16%). As for MEC, 9(36%) were grade I, 7(28%) were grade II and 9(36%) were grade III. Both cases of basal cell adenocarcinoma were of solid subtype characterized by solid nests of cells delineated by basement membrane like material.

Positive nodes were noted in 2(8%) of AdCC, 12(48%) cases of MEC and 2(100%) cases of carcinoma ex PA.

Perineural invasion (PNI) was noted in 9(36%) and 10(40%) cases of MEC and AdCC respectively. One (50%) case of basal cell adenocarcinoma showed PNI.

Vascular invasion (VI) was noted in 18(72%) cases in of AdCC and 1(50%) case of basal cell adenocarcinoma.

In the normal peritumoral salivary gland tissue, strong nuclear and cytoplasmic expression of VEGF-C was noted in ductal structure while strong nuclear reaction was noted in acinar cells (Figure 1A &B).

The total scores for VEGF-C in benign and malignant salivary gland tumours are summarized in Table 2.

The VEGF-C positive scores were significantly higher in malignant neoplasms *(p*=0.002). Significant association was noted among the total score of VEGF-C staining in the benign tumours (*p*=0.004), however, no significant association was noted within the malignant group (*p*=1.000).In contrast, the type of staining pattern was statistically significant (differed significantly) not only in benign and malignant tumours but also within the groups (*p*<0.0001) (Table 3).

Table 2 VEGF-C Total Score in Benign and Malignant Salivary Gland Tumours (n=85)

Tumour	Total Score VEGF-C	Negative		Weak positive		Moderate positive		Strong positive		p-value
		F	%	F	%	F	%	F	%	
Pleomorphic adenoma	5.36±0.490	0	0	0	0	0	0	25	100	
Warthin tumour	4.33±0.516	0	0	0	0	03	50	03	50	
Adenoid cystic carcinoma	5.48±0.510	0	0	0	0	0	0	25	100	
Mucoepidermoid carcinoma	5.40±0.577	0	0	0	0	01	04	24	96	**0.002**
Basal cell adenocarcinoma	5.50±0.707	0	0	0	0	0	0	02	100	
Carcinoma ex pleomorphic adenoma	6.00±0.00	0	0	0	0	0	0	02	100	

Table 3 VEGF-C Staining Pattern in Benign and Malignant Salivary Gland Tumours (n=85)

Tumour	Staining Pattern						p-value
	Cytoplasmic alone		Membranous & Cytoplasmic		Nuclear & Cytoplasmic		
	F	%	F	%	F	%	
Pleomorphic adenoma	-	-	-	-	25	100	<0.0001
Wartin tumour	6	100	-	-	-	-	
Adenoid cystic carcinoma	4	16	-	-	21	84	
Mucoepidermoid carcinoma	-	-	18	72	7	28	
Basal cell adenocarcinoma	-	-	2	100	-	-	
Carcinoma ex pleomorphic adenoma	1	50	-	-	1	50	

The VEGF-C positivity was strong in 28(90.3%) of benign tumours followed by moderate in 3(9.7%) (Table 2). In PA, both the epithelial component and mesenchymal/myoepithelial cells showed strong nuclear and cytoplasmic staining reaction (Table 3 &Figure 1C-1E). Predominant staining pattern was significantly associated with the type of tumour (p<0.0001). It was nuclear and cytoplasmic in all cases of PA while cytoplasmic alone in WT (Table 3 and Figure 1F). Nuclear reaction was noted in abluminal cells around the ductal structures. Stroma showed weak staining in most cases most cases of PA while WT showed strong reaction in stroma of all cases (Figure1C-F).

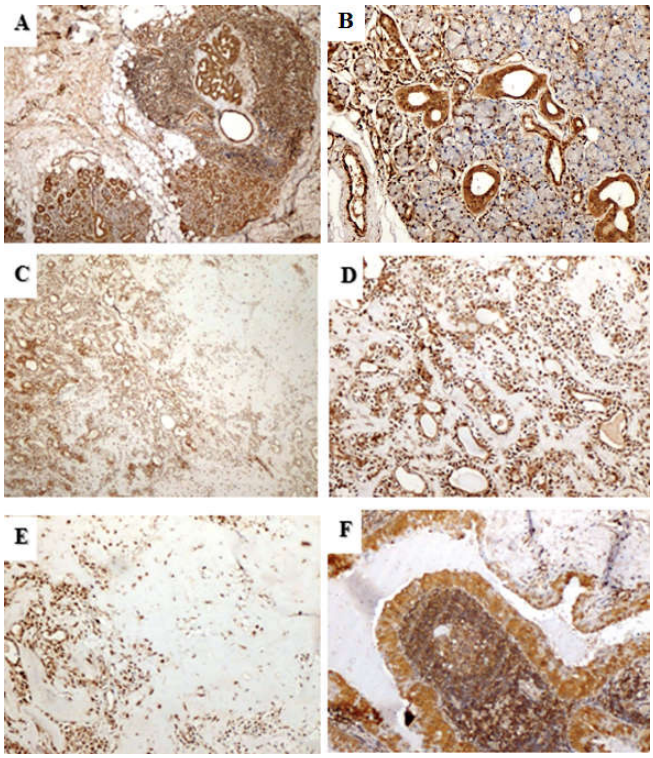

Figure 1 Photomicrograph (A) & (B) showing strong cytoplasmic and nuclear staining in normal salivary gland (VEGF-C; 40X and 100X), (C) low power view of strong positive staining in PA (VEGF-C; 40X), (D) & (E) showing strong to moderate staining in epithelial and mesenchymal component of PA (VEGF-C; 100X) (F) showing moderate positive cytoplasmic staining in WT (VEGF-C; 100X).

All the malignant tumours were strong positive for VEGF-C antibody, only 1 case of MEC was moderate positive.

The staining pattern was mostly nuclear & cytoplasmic (n=29;53.7%) followed by membranous and cytoplasmic 18(33.3%) cases. Only 1 (1.9%) case showed cytoplasmic localization alone. Most cases (n=21; 84%) of AdCC showed cytoplasmic &nuclear staining. Nuclei alone were positive in the abluminal cells lining the cribs and tubules in AdCC. On the other hand most MECs (n=18; 72%) showed membranous & cytoplasmic localization and only 7(28%) cases showed nuclear & cytoplasmic reaction in addition to membranous. Basal cell adenocarcinomas showed membranous and cytoplasmic staining reaction in both cases.One case of carcinoma ex pleomorphic adenoma showed cytoplasmic reaction and the other showed nuclear and cytoplasmic staining.

Stroma in AdCC and MEC was moderately reactive in 13(52%) and 03(12%) cases respectively, strong in 7(28%) & 2(100%) cases of MEC & CEPA respectively. Weak stromal reaction was noted in 2(100%), 12(48%) and 15(60%) cases of BCA, AdCC & MEC respectively.

Vascular endothelial growth factor C staining pattern was significantly associated with the histological patterns in AdCC (p= 0.003) and histological grades in MEC (p<0.001). No significant association of VEFG-C antibody staining was noted with clinical stage, histological grade, lymph node involvement, perineural invasion or vascular invasion in both AdCC and MEC. Figures 2 &3 show anti-VEGF-C staining in grades of AdCC and MEC respectively while Figure 4 shows staining in CEPA (Figure 4A-4B) and BCA (Figure 4C-4D).

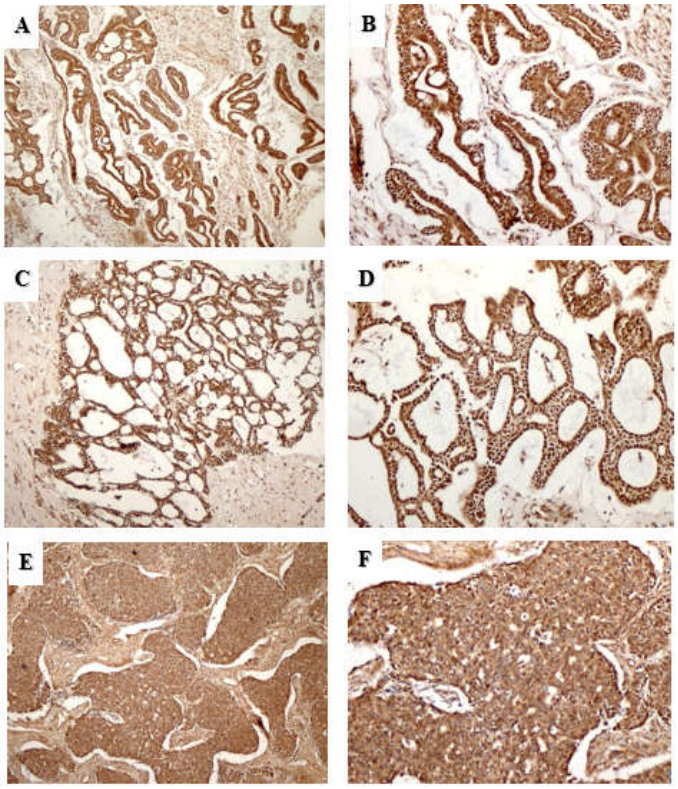

Figure 2 Photomicrograph (A) & (B) showing strong positive anti- VEGF-C nuclear and cytoplasmic staining in tubular pattern of adenoid cystic carcinoma (VEGF-C;40X & 100X), (C)& (D) Strong positive staining in cribriform pattern of AdCC (VEGF-C;40X & 100X), (D) & (E) strong positive cytoplasmic staining in solid pattern of AdCC (VEGF-C ;40X & 100X)

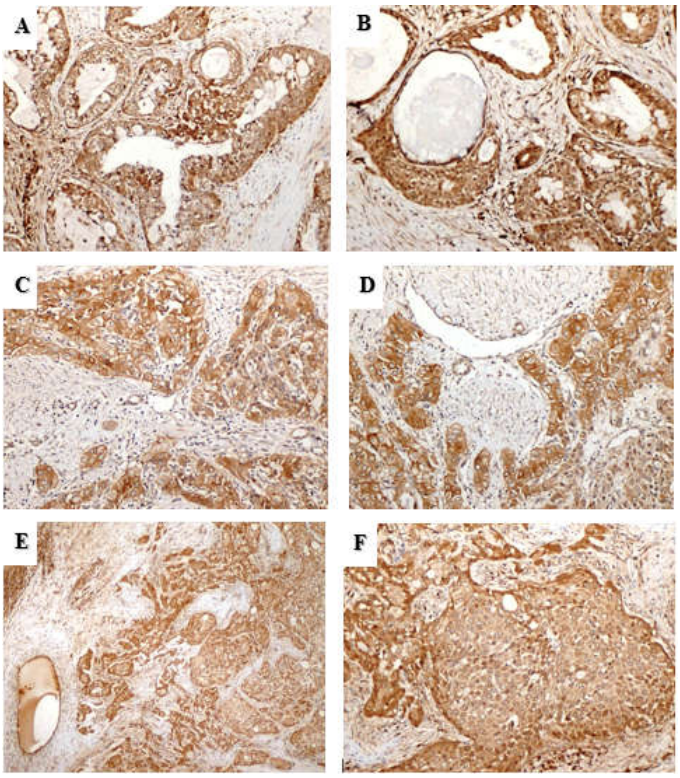

Figure 3 Photomicrograph (A)&(B) showing strong nuclear cytoplasmic positive staining reaction in grade I MEC (VEGF-C; 40X &100X), (C) & (D) Membranous staining in most cells of grade II MEC (VEGF-C; 40X &100X), (E) & (F) Strong positive staining in grade III MEC (VEGF-C; 40X & 100X)

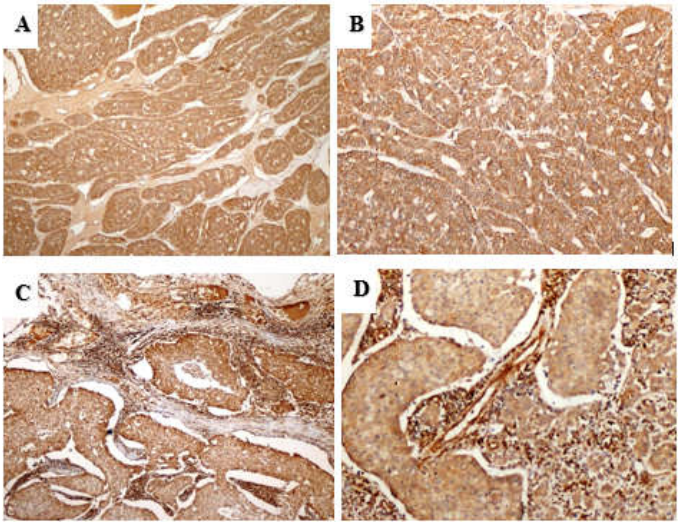

Figure 4 Photomicrograph (A)& (B) showing strong positive cytoplasmic and membranous staining reaction in BCA (VEGF-C ; 40X &100X), (C) & (D) Strong membranous and cytoplasmic staining in most cells of CEPA (VEGF-C;40X & 100X). Note the strong stromal reactivity in CEPA.

DISCUSSION

Salivary gland tumours constitute 1-4% of all tumours occurring in human body [(11)]. Among these, pleomorphic adenoma is the most commonly occurring benign salivary gland tumour in any location mostly involving the parotid gland [(12)]. As regards the commonest malignancy of salivary glands, there is some debate. Some studies have reported adenoid cystic carcinoma to be the most commonly occurring malignant tumour of salivary glands[(4,13)] while others have named mucoepidermoid carcinoma as the commonest[(14,15)].
VEGF-C is one of the lymphangiogenic marker being studied extensively to accurately determine its role in cancer metastasis. In the current study, we determined the expression of VEGF-C in 85 cases (6 different types) of salivary gland tumours. Of these, 31 were benign (n=25 pleomorphic adenoma and n=06 Warthin tumour) and 54 were malignant (n=25 adenoid cystic carcinoma, n=25 mucoepidermoid carcinoma, n=02 basal cell adenocarcinoma and n=02 carcinoma ex pleomorphic adenoma).

The mean age of the patients for benign tumours (32.52± 16.326 years), malignant tumours (35.65±14.130 years) and total salivary gland neoplasms (34.51 ± 14.949 years) studied in the current study is quite lower than other studies conducted worldwide[(13,16)]. Even when these tumours were considered individually (Table 1), they seem to affect younger age group in our population than reported in other studies[(13,16-17)]. These differences may be attributed to geographical, racial or ethnic dissimilarities among the various populations or to the variations in sample size of the study.

In line with the findings of current study, Kızıl[(13)]reported female predisposition for PA and MEC and male predilection for WT and AdCC. Other studies, however, have reported different findings[(17)].

Regarding site distribution of these salivary gland tumours, the present study is in accordance with other national and international studies with parotid being the commonest site for PA, WT, MEC, minor salivary glands (palate) for AdCC, both major and minor glands in basal cell adenocarcinoma and major glands for ca ex PA[(13,16-17)].

Byrd[(18)] reported lymph node involvement in 42.2% cases of MEC which is in accordance with the current study which reports nodal positivity in 48% cases.In contrast, Liu[(19)] reported only 13.8% positive nodes in MEC. Most cases of MEC were stage I & II which is line with the findings of Liu[(19)]who reported 68% cases of stage I/II. Bianchi[(20)] reported 9% positive nodes in AdCC which is almost same as found in the current study. In concordance with the findings of Ko[21] almost 72% AdCC were of stage I/II.

McHugh[(22)] and Agarwal[(23)] reported peri-neural invasion in 28.7% and 32.4% cases of MEC and AdCC respectively which is close to the findings in the current study (MEC:36%, AdCC: 40%).

The staining reaction for anti-VEGF-C in the normal salivary gland tissue was strong nuclear and cytoplasmic in the ductal structures while in the acini the cytoplasmic intensity (when noted) was less profound than ducts however, nuclei of the acinar cells were strong positive. This localization of VEGF-C in normal salivary gland tissue is in accordance with other reported studies[(5)].

We are reporting strong VEGF-C reactivity in all cases of PA both in epithelial and mesenchymal component which is in contrast to the other studies[(24-26)]. Salzman[(25)]reported only 1 VEGF-C positive case of PA.Teymoortash[(26)] studied LYVE-1 stained lymphatic vessels in PA and WT. They reported significantly higher LVD in WT (32) than in PA (2)[(26)]. Soares[(24)] reported that lymph vessel density (LVD) in PA and early CEPA was low when compared to invasive carcinomas.They concluded that in CEPA that has not infilterated outside the confines of PA, the lymphatic network consists of pre-existing channels. Also, they reported that tumours with myoepithelial differentiation showed low invasion of lymphatics by the neoplastic cells[(24)]. Swelam[(27)]

reported enhanced VEGF and HIF-1 in PA and concluded that hypoxia controls the VEGF expression in PA.

In contrast to the current study, Fujita[5] reported that VEGF-C was barely detectable in cases of AdCC. They also reported higher VEGF-C positive reaction in salivary glands than AdCC using real-time reverse transcriptase-polymerase chain reaction[5].

In line with the current study, Gleber-Netto[28] reported moderate to strong VEGF-C expression in all cases of MEC. The staining pattern was nuclear and cytoplasmic[28] which is slightly different from the current study as we also noted membranous expression in few cases. In addition, they also reported low lymphatic vessel density in MEC which cannot be explained by VEGF-C expression.

Mello[29] studied VEGF-C expression in different salivary gland carcinomas. They divided those carcinomas into high risk and low risk depending upon their metastasis to lymph nodes. They reported ≤ 25% positive cells in 29 cases and >25% in 16 cases. In line with the current study, they did not find any significant association between VEGF-C expression and high/low risk salivary gland cancers or peri-/ intra-tumoral lymph vessels. They suggested that either VEGF-C produced by salivary gland cancer cells is non-functional or there may be other markers involved in tumour lymphangiogenesis. They also concluded that low LVD in salivary gland cancers in contrast to head and neck squamous cell carcinomas may depict the different malignant behaviour of these cancers i.e. they may have different capacity to invade the lymphatic vessels in addition to the mere presence of these conduits.

CONCLUSION

As evident from the studies being conducted all over the world, VEGF-C is a potential lymphangiogenic marker in a large number of tumours. Its higher expression in malignant salivary gland tumours may dictate its potential advantage as a biologic marker in determining the lymphatic spread of these tumors. In addition, it may be utilized as a promising therapeutic target to restrict the metastatic spread of malignant salivary gland neoplasms to lymph nodes and distant organs[1].

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