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Original article

The role of the VEGF-C/-D/flt-4 autocrine loop in the pathogenesis of salivary neoplasms

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ABSTRACT

Salivary gland cells produce and secrete VEGF under normal conditions, but this property has not been studied in salivary gland neoplasms. The aim of this study was to evaluate the expression of VEGF-C/VEGF-D/flt-4 in salivary gland tumors.

Thirty-one salivary gland tumors (19 with and 12 without myoepithelial differentiation) were examined. Immunostaining for VEGF-C/VEGF-D/flt-4, p63 and SMA was carried out. The chi-square distribution and the Pearson correlation were applied.

A statistically significant relationship ($p < 0.05$) was found in the group of tumors with myoepithelial differentiation regarding simultaneous positive staining for VEGF-C/VEGF-D and flt-4. All pleomorphic adenomas (PA) exhibited a statistically significant coexpression of the three antibodies. p63 and SMA were strongly expressed in the same areas as VEGF-C, VEGF-D and flt-4.

The cells responsible for the strong expression of VEGF-C, VEGF-D and flt-4 in PAs are myoepithelial cells. Coexpression of flt-4 and its ligands in all PAs suggests the presence of a dominant VEGF-C/VEGF-D/flt-4 axis in this tumor.

Introduction

The vascular endothelial growth factor (VEGF) family and their respective receptors play a significant role in tumor growth, due to their angiogenic and lymphangiogenic properties. The human VEGF family consists of five members: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D and placental growth factor PLGF. Their ligands are VEGFR-1, VEGFR-2, VEGFR-3, Neuropilin-1 and Neuropilin-2. VEGF-C and VEGF-D, which are relevant to our study, bind to VEGFR-3 (or flt-4) [10]. However, the role of the VEGF family is not restricted to the formation of blood and lymphatic vessels [13,19]. In effect, the VEGF-C/flt-4 axis is expressed by a variety of human tumor cells [12,14,16,17,21,31,32,34,35]. Furthermore, salivary gland cells are capable of producing and secreting VEGF, either under normal conditions, or during the development of pleomorphic adenomas [33], but this property has not been extensively studied in salivary gland neoplasms.

Salivary gland tumors have been documented to account for 1–4% of all human neoplasms [28]. The histologic appearance of many salivary gland tumors results from a number of morphogenetic processes often linked to myoepithelial differentiation [2]. In effect, myoepithelial cells are critical components of benign and malignant salivary gland tumors [20], and salivary tumors with myoepithelial differentiation are most likely a distinct entity in terms of pathogenesis. Pleomorphic adenoma (PA) is a benign salivary gland tumor with evident myoepithelial differentiation; it displays unique histomorphic heterogeneity and a poorly vascularized stroma [9,25,33]. Although the PA is the most common type of benign salivary gland tumor with a frequency of 40–70% of all salivary gland tumors [8,11,18,22], its pathogenesis has not yet been fully understood.

The aim of this study was to evaluate the immunohistochemical expression of flt-4 and its ligands VEGF-C and VEGF-D in tumors with and without myoepithelial differentiation, and to examine any interrelation among these immunomarkers, as well as any correlation with a specific histologic subtype.

Materials and methods

Sample

The sample used in this study was derived from the records of the ENT clinic of the University of Athens in Greece and

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Table 1
Sample.

Case	Gender	Age (years)	Tumor localization	Tumor type	VEGF-C	VEGF-D	flt-4
1	F	42	Parotid gland	Myoepithilioma	+	–	+
2	F	75	Parotid gland	Myoepithilioma	+	+	–
3	M	63	Parotid gland	Pleomorphic adenoma	+++	+++	+++
4	F	22	Parotid gland	Pleomorphic adenoma	+++	+++	+++
5	F	38	Submandibular gland	Pleomorphic adenoma	+++	+	+
6	F	43	Parotid gland	Pleomorphic adenoma	++	+	+
7	M	48	Parotid gland	Pleomorphic adenoma	++	+	+
8	M	68	Parotid gland	Pleomorphic adenoma	+++	+++	+++
9	F	81	Parotid gland	Pleomorphic adenoma	+	++	+++
10	F	46	Parotid gland	Pleomorphic adenoma	++	+++	+++
11	M	56	Parotid gland	Basal cell adenoma	–	–	+
12	M	73	Parotid gland	Basal cell adenoma	–	+	++
13	F	68	Parotid gland	Basal cell adenoma	+	+	+
14	F	72	Parotid gland	Basal cell adenoma	–	+	++
15	F	31	Parotid gland	Basal cell adenoma	+	+++	+++
16	M	67	Parotid gland	Warthin's tumor	–	+	+++
17	M	59	Parotid gland	Warthin's tumor	–	+	+++
18	M	65	Parotid gland	Warthin's tumor	–	+	+++
19	M	37	Parotid gland	Warthin's tumor	–	+	+++
20	M	65	Parotid gland	Warthin's tumor	–	+	+++
21	F	27	Parotid gland	Adenoid cystic carcinoma	+	–	+
22	M	64	Parotid gland	Adenoid cystic carcinoma	+	+++	+++
23	M	71	Submandibular gland	Adenoid cystic carcinoma	–	–	++
24	F	42	Parotid gland	Adenoid cystic carcinoma	+	+	+++
25	F	53	Parotid gland	Mucoepidermoid carcinoma	–	+	+
26	F	75	Parotid gland	Mucoepidermoid carcinoma	+	–	+++
27	M	72	Parotid gland	Mucoepidermoid carcinoma	+	++	+
28	F	52	Parotid gland	Adenocarcinoma	–	–	++
29	M	77	Parotid gland	Adenocarcinoma	–	+	++
30	F	38	Hard Palate	Adenocarcinoma	++	++	++
31	M	61	Parotid gland	Adenocarcinoma	+	+	+++

consisted of 31 salivary gland tumor cases, classified on the basis of the 2009 World Health Organization (WHO) classification of salivary gland tumors. The sample was divided into two groups. Group A included 19 tumors exhibiting myoepithelial differentiation, immunohistochemically verified. More specifically, Group A included 2 myoepitheliomas, 8 pleomorphic adenomas, 5 basal cell adenomas and 4 adenoid cystic carcinomas. Group B included 12 tumors with no distinct myoepithelial differentiation. Group B consisted of 5 Warthin's tumors, 3 mucoepidermoid carcinomas and 4 adenocarcinomas (Table 1).

Four-micrometer-thick serial sections were obtained from paraffin-embedded samples. A three-staged immunohistochemical technique was applied to study the expression levels for vascular endothelial growth factor-C (VEGF-C), vascular endothelial growth factor-D (VEGF-D) and their receptor VEGFR-3 (or flt-4).

Immunohistochemistry

All samples were fixed in buffered formalin and embedded in paraffin. Four-micrometer thick sections were obtained from each tumor and mounted on silane-coated slides. Subsequently, they were submitted to antigen retrieval solution (DAKO, citrate buffer, ph 6.0), and the following antibodies were applied: (a) flt-4 (DBS Rb Polyclonal RP135, dilution 1/100), (b) VEGF-C Invitrogen Rb and (c) VEGF-D clone 78923 R&D MAB286. Tissue sections from placenta and colon carcinoma served as positive controls.

All antibodies were incubated overnight at 4 °C at dilutions of 1:100. The reaction was amplified using the Envision Polymer Two-step Kit (DAKO, K5007) immunostaining method. Diaminobenzidine was used as a chromogen, followed by counterstaining with Gills hematoxylin. The primary antibody was omitted as a substitute.

Immunohistochemistry for p63 was carried out on an automated system (Bond Visionbiosystems), using anti-p63 (Leica PA0478, Ready to Use Clone 7JUL). Since p63 is a nuclear and not a cytoplasmic myoepithelial cell (MEC) marker, it made studying the presence or absence of the MEC layer easier. The identification of p63-positive cells as myoepithelial was assessed by their concomitant immunoexpression of smooth muscle actin (SMA). The expression of VEGF-C, VEGF-D and flt-4 was evaluated, considering cytoplasmic and nuclear staining. Staining of weak intensity was disregarded. The slides were analyzed by two pathologists using a double blind method, using the following semiquantitative grading system, as applied elsewhere [15]:

Negative (–): absence of expression.
Focal positive staining (+): expression in up to 10% of cells.
Moderately positive staining (++): expression in over 10–50% of cells.
Strongly positive (+++): expression in over 50% of cells.

Inferential statistics were completed by applying the chi-square distribution (SPSS version 16.0).

Results

Immunostaining was evaluated in the tumor cells in all cases. The immunohistochemical results for each case are presented in Table 1. An initial correlation between degrees of staining for the antibodies used, irrespective of tumor type, was investigated. A moderately positive correlation between VEGF-C and VEGF-D staining ($r = 0.57$, $p < 0.01$), as well as between VEGF-D and flt-4 staining ($r = 0.44$, $p < 0.05$), was found. No Pearson correlation was observed between the degree of staining of VEGF-C and flt-4.

When examining tumors with myoepithelial differentiation (Group A) and tumors without myoepithelial differentiation (Group B), no statistically significant differences were found between them, in relation to VEGF-C, VEGF-D or flt-4. These results are presented in Table 2.