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Original Research

Correlation between blood and lymphatic microvascular density and cell proliferation in mouth floor and tongue squamous cell carcinoma

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ABSTRACT

Objective: Despite the advances in medicine, the oral squamous cell carcinomas still have high incidence and are very important to study topics such as their lymphatic and microvessel density. This research assessed the correlation between histological grading, formation of new lymphatic and blood vessels and cell proliferation in squamous cell carcinomas (SCC).
Methods: Twenty-nine oral floor SCC and 30 tongue cases were assessed for their clinical characteristics and histological grading of malignancy.
Results: The presence of VEGF (vascular endothelial growth factor VG1), VEGF-C (vascular endothelial growth factor-C), CD105, to determine blood microvascular (MVD), and D2-40 to determine lymphatic density (LMD) was investigated using immunohistochemistry. Histological grading revealed that 73.3% of tongue and 96.67% of floor cases were classified as highly aggressive. Cell proliferation was greater on the floor; however, no significant difference was observed. Most carcinomas were VEGF negative (tongue 63.3% and floor 70.0%) and VEGF-C positive (tongue 73.3% and floor 79.3%). LMD was considerably greater on the tongue. High MVD values occurred in cases with greater cell proliferation. No relationship was determined between the growth factors VEGF and VEGF-C and MVD and LMD, respectively.
Conclusion: Floor of mouth SCC cases was morphologically more aggressive than tongue cases; however, in tongue carcinomas a greater quantity of lymphatic vessels could represent potential ways for locoregional cell dissemination of the neoplastic cells, independent of histological grading. Blood vascularization presented correlation with cell proliferation in intraoral squamous cell carcinoma and could be useful in the prognostic assessment of this neoplasm.

1. Introduction

Oral squamous cell carcinoma (SCC) representing 3% of all malignant tumors is considered the 6th most frequent cancer [1], with regional variations in incidence and mortality rates [2]. These neoplasms are mostly diagnosed at an advanced stage and are therefore regarded as a serious problem by local health authorities [3,4]. With the exception of cases involving the lips, the most common sites for mouth cancer are the tongue, mouth floor, and soft palate with most cases occurring in men over 5–6th decades of life [5]. The same places were also preferable sites for oropharynx SCC [6]. Persistent mucosal erythroplasia rather than leukoplakia is the earliest visual sign of oral and oropharyngeal carcinoma. These lesions should not be regarded merely as precancerous changes. The evidence indicates that these lesions in high-risk sites should be considered to be invasive carcinoma or carcinoma in situ unless proven otherwise by biopsy [6].

Despite the advances in medicine, the prognosis of these types of cancer remains unfavorable considering a survival rate of 56% over 5 years [7,8]. Prognosis depends on numerous factors, including early detection and histological grading [9].

☆ Asian AOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants.

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In order to define a more precise estimate regarding the biological behavior of SCC, several histological grading systems have been developed [5,10–12]. However none of them alone are able to predict neoplastic behavior. Thus, the search for prognostic markers is very important, and can contribute to determine treatment and estimate a patient's survival time.

A correlation often exists between the proliferation rate of neoplastic cells and the biological behavior of tumors [13,14]. Ki-67 antibodies are commonly evaluated to detect and quantify cell proliferation [15,16].

Angiogenesis is fundamental for tumor development because vessels carry nutrient to neoplastic cells during the division process, resulting in favorable conditions for tumor cells. The progression process of a normal cell to malignancy involves numerous mechanisms; one of which is the capacity to stimulate angiogenesis through the increased secretion of vessel-inductors and suppression of inhibitors [17]. Additionally, head and neck cancer is known to result in locoregional metastasis via lymphatic network, so, lymphangiogenesis is also considered an important process for tumor metastasis spread [18–21].

Owing to the importance of angiogenesis in the growth of neoplasm and lymphangiogenesis in lymph node metastasis, several studies have assessed these phenomena as prognostic factors for neoplasms [22,23].

Considering that cell proliferation has been proposed as a prognostic factor for squamous cell carcinoma, this study assessed the correlation between endothelial growth factors and proliferation, also exploring the relationship between neoformation of blood and lymphatic vessels and histological grading of malignancy.

2. Material and methods

This study followed the Ethical Principles for Research on Humans and was approved by the Local Research Ethics Committee, under protocol no. 093/2007-PH/CEP.

Among the 59 cases of intraoral SCC selected, 30 of them originated from the anterior two thirds of tongue and 29 from floor of the mouth. The clinical data (skin color, gender and age); sections stained with hematoxylin–eosin (H–E) and paraffin embedded blocks were obtained from the archives of the Oral Pathology Laboratory.

2.1. Histological grading

To evaluate malignancy, the histological grading proposed by Bryne et al. [12] was used. This is based on analysis of morphological features presented by the cells and their relationship with connective tissue in the region of tumor invasive front; i.e., the most invasive portion visualized by the microscopic cut. The degree of keratinization, nuclear polymorphism, number of mitoses per field, invasion pattern and the leukocyte infiltration were analyzed, each with a score varying from 1 to 4. The analyses were performed by two examiners.

2.2. Immunohistochemical analysis

For cell proliferation analysis, an immunohistochemical reaction was performed using the LSAB system (peroxidase immunostaining method, Dako Corp., Carpinteria, USA) using an antibody against Ki-67. Vascular endothelial (VEGF-VG1) and endothelial lymphatic growth factors (VEGF-C) were also assessed. The blood and lymphatic microvascular density were analyzed by endothelial antigens marking CD105 and D2-40, respectively. The incubation period for all antibodies was 1 h. Details concerning the immunohistochemical techniques and antibodies used are listed in Table 1. Positive controls were obtained according manufactures instructions. For Ki-67, it was used a case of SCC, for D2-40 and VEGF-C it was used a case of lymphangioma, for CD-105 a lymph node and for VEGF a case of lymphoid hyperplasia. As negative controls, reactions were carried out in the same way of samples, except for the incubation using only antibody diluent, without primary antibody.

For analysis of Ki-67 expression (proliferation index), microscopic fields considered to have the greatest density [hot-spots] were identified using 100× magnification. Positive staining for Ki-67 was verified in nuclei of neoplastic epithelial cells. The proliferation index is the percentage of positive cells of a total of 1000 tumor cells, counted by a single examiner in approximately 5 fields of each neoplasm, under a 400× magnification.

The expression of VEGF and VEGF-C was assessed by an examiner using a semiquantitative method of scores, based on the sum of proportion and intensity of tumor cells with positive marking, as previously proposed by Soini et al. [24]. For each selected field, the positive cell percentage was assessed and classified in five scores: **0** = absence of positive tumor cells; **1** = less than 25% of positive tumor cells; **2** = 26–50% of positive tumor cells; **3** = 51–75% of positive tumor cells and **4** = more than 75% of positive tumor cells. The intensity was classified by the following scores: **0** = absence of marking; **1** = weak marking; **2** = moderate marking; **3** = strong marking and **4** = intense marking. The percentage and intensity scores were summed resulting in the following: **0** (sum zero) = absence of marking; **1** (sum from 1 to 4) = weak marking and **2** (sum 5–8) = strong marking.

The blood and lymphatic vessel counts were performed by one researcher, in accordance with Maeda et al. [25]. Any cell or endothelial cell group positively stained by markers CD105 and D2-40, separated from the adjacent micro vessels, from tumor cells and from other elements of connective tissue was considered a unitary vessel, together with vessels containing lumen. Branched structures were counted as unitary vessels. Hot spot regions were selected using 200× of magnification. In these regions, the vessels were manually counted in 5 fields at 400× magnification and the result was expressed by the average number of vessels in each histological cut.

Table 1
Details of the primary antibodies used.

Antigen specificity	Clone	Dilution	Visualization system	Pre-treatment Antigen recovery
CD105	SN6h[a]	1:50	Histofine[c]	Proteinase K[a] (room temperature), 6 min
D2-40	M3619[a]	1:100	Histofine	Double boiler (95 °C), 45 min – Citrate
Ki-67	MIB-1[a]	1:100	LSAB[a]	Double boiler (95 °C), 45 min – Citrate
VEGF	VG1[a]	1:50	Histofine	Double boiler (95 °C), 45 min – Tris EDTA Target Retrieval Solution[a]
VEGF-C	H-190[b]	1:20	Histofine	Double boiler (95 °C), 45 min – Citrate

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