Angiogenesis and Mast Cells Density in Oral and Esophageal Squamous Cell Carcinomas

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Abstract- Despite the similarities between oral and esophageal squamous cell carcinomas (SCCs), the survival rate of this cancer is relatively low in the esophagus compared to the oral cavity. To our knowledge, mast cells and angiogenesis have not been simultaneously compared between oral and esophageal SCCs. However, they have been separately evaluated in each of these locations with conflicting results. Therefore, the aim of this study was to assess and compare mast cell count and microvessel density between SCCs of the esophagus and oral cavity. A total of 46 oral and esophageal SCCs (23 of each) were stained immunohistochemically and histochemically with CD31 and methylene blue, respectively. Statistical analysis was performed using t-test, one-way ANOVA, and Pearson correlation analysis. Microvessel density was significantly higher in oral compared to esophageal tumors (P=0.02). Conversely, esophageal SCCs showed significantly higher mast cell counts than that of oral neoplasms (P=0.04). Pearson correlation analysis showed no association between these two factors in either oral SCC (P=0.51) or esophageal SCC (P=0.34). A significant difference between mean mast cell count and microvessel density in oral and esophageal SCC may be related to inherent differences in the tissues of origin and might, to some extent, be responsible for the different biological behaviors of these cancers.

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Keywords: Oral squamous cell carcinoma; Esophageal squamous cell carcinoma; Microvessel density; CD31; Mast cells

Introduction

One of the most common types of esophageal cancer, particularly in developing countries, is squamous cell carcinoma (SCC) that can cause significant morbidity and mortality (1). The five-year survival rate of this malignancy is reported to be about 25% (2). Despite extensive research and the use of adjuvant therapy, unfortunately, the overall survival of these patients has not improved significantly during the past years (1). Oral SCC is a multifactorial disease with an estimated 5-year survival rate of 50% and encompasses approximately 90% of oral cavity cancers (3). Tobacco use and alcohol consumption are the two major risk factors for SCCs of the upper aerodigestive tract, including the oral cavity and esophagus (4). Despite the similarities between SCCs of these locations, it should be noted that esophageal SCCs have a relatively low survival rate compared with oral SCCs. The difference in survival between these two may be related to tumor biology or diagnostic delays and other factors.

In the past, several studies have demonstrated the importance of the epithelial component of SCC. However, tumor stroma has attracted the attention of many researchers in the last few decades (5-7). Tumor-associated stroma is essential for the preservation, development, and metastasis of cancers, such as SCC (8). The main constituents of the tumor stroma are the

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basement membrane, fibroblasts, extracellular matrix, inflammatory cells, and vessels (9). The sprouting and growth of new capillaries from existing vasculature are defined as angiogenesis. This process is involved in many physiological and biological normal functions in addition to tumor survival, growth, and metastasis (10). One of the inflammatory cells in the tumor stroma is mast cells. Some studies have shown the relationship between these cells and tumor angiogenesis. Mast cells are able to secrete various mediators that regulate angiogenesis and stimulate the proliferation of endothelial cells (10). Both mast cells and angiogenesis have been evaluated in esophageal and oral SCC, separately (10-12). The difference in prognosis between these two sites may be associated with biologic factors or elements like anatomical characteristics and delayed diagnosis. Therefore, the aim of this study was to evaluate and compare the density of mast cells and angiogenesis in SCCs of the esophagus and oral cavity.

Materials and Methods

The study protocol for this research was approved by the Research and Ethics Committee of our University (IR.IAU.DENTAL.REC 93/2/9/477). All patient records with a diagnosis of oral or esophageal SCC were extracted, and H and E slides of those who fulfilled our inclusion/exclusion criteria were selected for evaluation and grading based on the criteria proposed by Broders (13). Our study sample included individuals with complete clinical and demographic data, a histologic diagnosis of conventional SCC confirmed by two oral pathologists, no history of previous neoplastic/systemic diseases, and tumors that had adequate tissue with proper fixation and no evidence of widespread hemorrhage or necrosis.

Sections (3-4 μm) were cut from corresponding paraffin blocks, mounted on coated slides, deparaffinized, and rehydrated, followed by endogenous peroxidase blocking with 3% H_2O_2 for 10 minutes). After washing in phosphate-buffered saline (PBS), the specimens were immersed in 10mM fresh citrate HCL (pH=6) and placed in a microwave oven at 900 W power for 5 minutes, followed by 720 W power for 20 minutes. The slides were allowed to cool for 15 minutes at room temperature after which they were washed in PBS and sequentially incubated with CD31 antibody (DAKO, Ready to use, monoclonal mouse, anti-human, CD31, endothelial cell, clone JC70A) for 60 minutes, and Envision (DAKO REAL Envision/ HRP, Rabbit/Mouse) for 60 minutes. The color was developed with diaminobenzidine (DAKO REAL DAB+Chromogen) followed by hematoxylin counterstaining for 1 minute (MERCK, USA). Positive and negative controls were run simultaneously with each batch and consisted of hemangioma and omission of the primary antibody, respectively. Large pre-existing vessels in the adjacent connective tissue were regarded as internal controls.

For mast cell detection, we used methylene blue histochemical staining as follows: 4 μm sections were cut from paraffin blocks, deparaffinized in xylene, and hydrated using a series of graded alcohol. All sections were covered with a few drops of methylene blue working-solution for 15 minutes, followed by rinsing and mounting with coverslips. Colon mucosal mast cells were used for positive control.

Microvessel density (MVD) was determined based on the method proposed by Weidner *et al*., (14). All brown-stained cells, either singular or in small groups, with or without lumen formation, were counted in 10 high power fields (×400) selected from hotspots identified at ×100 magnification. Large vessels with muscular walls were excluded from the microvessel count. All observations were made on a double-headed microscope by two oral and maxillofacial pathologists, and disagreements were resolved by consensus. The final MVD was expressed as mean±standard deviation.

Metachromatic granules of the mast cells were purple-red on a background of blue, which was the basis of counting these cells in a manner similar to MVD assessment: hot spot areas with a high concentration of mast cells were defined at ×100 and mast cells density was calculated at 400 magnifications.

Statistical analysis was done using *t*-test, one-way ANOVA, and Pearson correlation analysis, and $P<0.05$ was considered significant.

Results

We were able to collect 46 oral and esophageal SCCs, 23 samples of each neoplasm based on our inclusion/exclusion criteria. The age range of patients with oral carcinomas was between 33 and 76, with a mean age of 58.43 years, and tumor sizes ranged from 0.5 cm to 4.5 cm (mean=2.8 cm). Most oral samples were intermediate grade (Table 1).

Table 1. Mean of MVD and mast cell density according to clinical and demographic variables in Oral SCCs (OSCCs)

OSCC		N=23 (100)	M±SD(CD31)	M±SD(Mast cells)
Pathology staging of primary tumor(pT)	T1	7(30.43)	71.57 ±17.11	8.57 ±2.69
	T2	11(47.82)	71±20.60	12.45±9.44
	T3	1(4.34)	99	6
	T4	4(17.40)	93.25±29.98	14.25±7.41
ANOVA			*P*=0.23	*P*=0.53
Differentiation(grade)	Well	9(39.13)	71±21.51	12.22±10.55
	Moderate	11(47.82)	80.36±24.32	11.18±5.26
	Poor	3(13.04)	75.66±20.13	9±4.35
ANOVA			*P*=0.69	*P*=0.82
Sex	Male	12(52.17)	76.08±26.89	13.66±9.58
	Female	11(47.82)	76.45±16.94	8.72±2.83
T-test			*P*=0.96	*P*=0.11
Nodes	N0(1)	15(65.21)	76.86±25.46	10.46±7.94
	N2(2)	8(34.79)	75.12±15.75	12.87±6.72
T-test			*P*=0.86	*P*=0.47
Age(3)	≤45	20(86.95)	77.40±22.94	10.35±6.15
	≤45	3(13.04)	66.66±17.67	17.66±13.61
T-test			*P*=0.53	*P*=0.11
Stage	I	6(26.08)	74.83±15.61	7.66±2.16
	II	5(21.73)	64.20±27	12.20±11.86
	III	1(4.34)	99	6
	IV	11(47.82)	80.45±22.90	13.36±6.97
ANOVA			*P*=0.42	*P*=0.44

(1) No regional lymph node metastasis
(2) Metastasis in a single ipsilateral node more than 3cm but not greater than 6 cm in greatest diameter, multiple ipsilateral nodes, none more than 6cm in greatest ,or bilateral or contralateral nodes, none more than 6cm in greatest diameter
(3) The most common age

The oldest patient with esophageal SCC was 77 years old, while the youngest was a 39-year-old individual (mean=58.39). The mean size of the esophageal SCCs was 4.3 cm, which ranged from 1.5 cm to 11 cm. Esophageal SCCs were also predominantly intermediate grade tumors (Table 2)

Table 2. Mean of MVD and mast cell density according to clinical and demographic variables in esophageal SCCs (ESCCs)

ESCC		N=23 (100)	M±SD(CD31)	M±SD(Mast cells)
Pathology staging of primary tumor(pT)	**T2**	7(30.43)	76±15.77	19.28±10.16
	T3	16(69.56)	55.93±16.64	15.25±8.74
T-test			*P*=0.028	*P*=0.34
Differentiation(Grade)	**Well**	3(13.04)	82±5.65	20.66±4.16
	Moderate	18(78.26)	59.82±21.63	16.11±9.93
	Poor	2(8.69)	61.50±16.70	13.50±7.77
ANOVA			*p=0.75*	*P*=0.66
Sex	**Male**	14(60.87)	63.21±23.33	15.64±9.41
	Female	9(39.13)	60.22±16.24	17.77±9.13
T-test			*P*=0.74	*P*=0.59
Nodes	**N0(4)**	19(82.60)	60.78±20.61	16.89±8.88
	N1(5)	4(17.40)	68±21.77	14.50±11.61
T-test			*P*=0.53	*P*=0.64
Age(6)	**≤50**	4(82.60)	60±21.13	9.50±6.24
	>50	19(17.40)	71.75±15.75	17.94±9.10
T-test			*P*=0.30	*P*=0.09
Stage	**I**	2(8.70)	82±5.65	20±2.82
	II	17(73.91)	59.82±21.63	17.82±9.59
	III	4(17.40)	61.50±16.70	9±5.35
ANOVA			*P*=0.36	*P*=0.19

(4) No regional lymph node metastasis
(5) Metastasis in a single ipsilateral node 3cm or less in greatest diameter
(6) The most common age

There was no significant difference regarding mean age (*P*=0.98) and gender (*P*=0.76) between oral and esophageal carcinomas. MVD was significantly higher in oral tumors as compared to esophageal neoplasms (*P*=0.02) with a mean of 76.26 and 62.04, respectively (Figure 1). Conversely, esophageal (16.47) SCCs showed significantly higher (*P*=0.04) mast cell density than that of oral (11.30) tumors (Figure 2). Pearson correlation analysis showed no association between MVD and mast cell density in either of the study groups (*P*=0.51 for oral SCCs and *P*=0.34 for esophageal neoplasms). Tables 1 and 2 demonstrate mean MVD and mast cell density according to clinical and demographic variables in oral and esophageal SCCs.

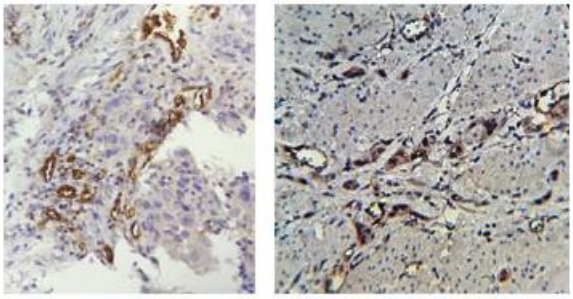

Figure 1. Representative section of CD31 immunostaining, demonstrating microvessel density in oral squamous cell carcinoma (left) and esophageal squamous cell carcinoma (right). (Original magnification: ×400)

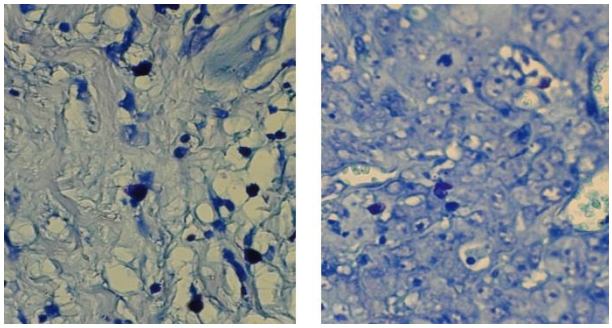

Figure 2. Metachromasia of mast cells shown by arrows following methylene blue staining in oral squamous cell carcinoma (left) and esophageal squamous cell carcinoma (right). (Original magnification: ×400)

Discussion

The mouth and esophagus are functioning organs of the digestive tract, and squamous cell carcinoma is one of the most common malignancies in these areas (15). SCC originates from abnormal and atypical squamous cells and can be locally destructive, invading adjacent tissues, and has the ability to metastasize to lymph nodes and other organs (16). Researchers have focused on the cellular and molecular details of this tumor in an attempt to increase and expand their knowledge about its pathogenesis and biology (15), which may ultimately lead to the development of new treatment strategies and improvement of the patient's survival. Tumor stroma and cancer cells closely interact with each other; this relationship is reflected in many aspects of tumorigenesis, such as tumor growth, development, and progression (17). Mast cells are one of the main types of immune cells in the tumor microenvironment that can contribute to tumor development via the promotion of angiogenesis (18). This is done through the production of angiogenic factors like histamine, heparin, and basic fibroblast growth factor (18). Based on the results of some investigations, stromal mast cells and microvessel density can influence the progression and survival of oral and esophageal SCCs (10-12). Although the mucosa of both organs is histologically similar and the SCCs originating from these areas appear morphologically identical, their behavior is not the same, which may be related to the different biology of these tumors.

For the above reasons, in a previous study, we compared the microvessel density of esophageal and oral SCC by CD34. The results did not show any significant differences between these tumors (19). However, the selection of the immunohistochemistry marker used for the recognition of endothelial cells and/or sample size may have an impact on the results. CD31 is another well-known endothelial marker that has been extensively used in angiogenesis studies (20). The present investigation focused on the evaluation of microvessel density by CD31 and the presence of mast cells in oral and esophageal SCC.

In the current study, the mean mast cell count and MVD showed a significant difference between oral and esophageal SCC. However, the results were contrary to our expectation since, despite the poorer prognosis of esophageal SCC, angiogenesis was higher in oral tumors. On the other hand, the density of mast cells was higher in esophageal SCC in comparison with oral SCC and did not correlate with microvessel density. The mechanism of cancer development and progression is a multistep process that could involve many molecular events and cells during carcinogenesis (21). It seems angiogenesis and mast cells have an impact on the survival difference between oral and esophageal SCC,

but it is difficult to explain and interpret these effects. CD31 is a pan-endothelial marker expressed in both pre-existing and neoformed vessels, and it is impossible to distinguish between these vessel types with this marker (22). Oral cavity tissues are known to have a rich blood supply and high vascularity (23). Therefore, the higher expression of CD31 in our oral SCCs may be related to the rich vascularity of this area and the potential of this marker to react with normal, new, and old tumor microvessels.

The mean mast cell count in this study was lower in oral-compared to esophageal-SCCs. It seems that environmental influencing elements or factors inherent to the tissue can affect the number of mast cells in different organs. Parizi *et al*., (24) evaluated mast cell density in SCCs of the skin and oral cavity and found a higher density of these cells in cutaneous tumors, which was suggested to be due to chronic sun exposure of the skin resulting in activation of mast cells (24). Regarding the higher count of mast cells observed in the current investigation, it should be noted that these cells are known to increase in the esophageal mucosa following infections and non-erosive reflux diseases (25), which may be responsible for our findings.

We did not detect a significant correlation between mast cells and angiogenesis in oral and esophageal SCCs, which was in agreement with some studies (26,27) and in contrast to others (11,12,28). A possible explanation for the opposing findings is the different methods and markers used for the detection of MVD or mast cells in these investigations. In oral SCC, other clinicopathologic parameters such as age, gender, grade, size, lymph node metastasis, and stage did not show any significant correlation with either mast cell density or angiogenesis. The lack of a significant relationship between these variables in the present study may be due to the small sample size of subgroups. Among the studied factors, only tumor size showed a significant correlation with angiogenesis in esophageal SCC, which requires further evaluation and confirmation with future studies.

The limitations of this study include the use of a single pan-endothelial marker for the assessment of angiogenesis and the lack of patient follow-up. Our findings offer preliminary data on the differences in microvessel density and mast cell count between esophageal and oral SCCs, which can be further expanded by future studies with larger sample sizes, multiple angiogenesis-detecting markers, evaluation of TC (tryptase and chymotryptic proteinase) and T (tryptase) mast cells and patient follow-up.

In conclusion, the results of our study showed a significant difference between the mean mast cell count and microvessel density in oral and esophageal SCC, which in part could be due to inherent features of the tissues of origin also affecting the biological behaviors of these cancers, leading to the observed differences. Therefore, further studies should be performed to explore the precise role of mast cells and angiogenesis that can affect the different survival of oral and esophageal SCC.

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