ORIGINAL RESEARCH

Grading angiogenesis in oral squamous cell carcinoma: A histomorphometric study

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ABSTRACT

Context: Like normal tissues, tumors require an adequate supply of oxygen, metabolites and an effective way to remove waste products. This is achieved by angiogenesis, which is defined as the process by which new blood vessels are produced by sprouting from preexisting vasculature. There is a large spectrum of physiological and pathological processes in which angiogenesis occur, ranging from tissue hypertrophy, wound healing, and inflammation to tumors.

Aims: The present study was designed to morphometrically evaluate the angiogenesis in different grades of oral squamous cell carcinomas (OSCCs) under light microscope by the use of H and E stained sections and to assess that whether the parameters of vascularity like mean vascular density (MVD), mean vascular area (MVA), and total vascular area (TVA) can be used to histologically grade the tumors.

Subjects and Methods: A total of 10 cases each of well-, moderately- and poorly-differentiated SCC cases were retrieved from the archives of the Department of Oral Pathology and Microbiology and were morphometrically analyzed for mean vascular density (MVD), MVA, and TVA. Ten cases of normal oral mucosa were taken as Control. Statistical analysis was done using SPSS 19.0 version (IBM, Armonk, NY, USA) software for windows. Group mean for MVD, TVA and MVA were calculated for 10 cases of each group. "Student's *t*-test" was applied to assess the intergroup variation of mean values of MVD, TVA, and MVA.

Results: Our results showed significant differences between all the three parameters, that is, MVD, MVA and TVA when poorly differentiated OSCC was compared with the normal mucosa, well- and moderately-differentiated OSCC. However, when comparison was made between the well- and moderately-differentiated OSCC, the differences in the three parameters were present but not statistically significant.

Conclusion: There was an increased MVD, MVA and TVA in poorly differentiated OSCC, which could be used as an additional criterion to histologically grade the tumor.

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Squamous cell carcinomas (SCCs) amount to more than 90% of malignant tumors of the oral cavity and oropharynx. According to World Health Organization it is defined as "An invasive epithelial neoplasm with varying degrees of squamous differentiation and a propensity to early and extensive lymph node metastases, occurring predominantly in alcohol and tobacco-using adults in the 5th and 6th decades of life."[1] India has one of the highest incidences of oral cancer (age-standardized rate of 9.8/10,000) making it the most common cancer among men (men: women ratio 2:1) and accounts for about 30% of all new cases annually.[2,3]

Despite the advances made in diagnosis and treatment, the mortality rate has not changed significantly probably because of the different biological behavior of these tumor cells, which show a variable aggressiveness independent of clinicopathological parameters of prognostic importance.[4] Histological grading has been used for many decades in an attempt to predict the biological behavior of SCC in head and neck region. Broder in 1920 initiated quantitative grading of carcinoma based on the differentiation of squamous cell. However, a lack of correlation between Broder's degree of differentiation and prognosis has been reported due to the

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heterogeneous cell population in tumors.[5] Later another histological grading system was introduced by Jacobsson in 1973 which not only included morphological parameters but also included "mode," "stage of invasion," "vascular invasion" and "degree of lymphoplasmacytic infiltration" as additional criteria. His grading system has been modified by various authors like Fisher in 1975, Lund *et al.* in 1975, Willen *et al.* in 1975, Crissman *et al.* in 1980 and Anneroth *et al.* in 1987 as multifactorial grading systems. Later Bryne *et al.* in 1989 further modified Anneroth grading system and presented a hypothesis suggesting that molecular and morphological characteristics at the invasive front area of various SCCs may reflect tumor prognosis better than other parts of the tumor. He proposed invasive tumor front (ITF) grading system better for predicting the prognosis for oral SCC.[6,7]

From last few decades, both clinical and experimental studies have shown that like normal tissue, tumors require a blood supply for growth and dissemination. Thus, tumor-induced angiogenesis is defined as the generation of new blood vessels from the existing vasculature.[8-10]

This has been seriously implicated as a major factor leading to cancer invasion and progression. It is now considered as one of the hallmark of carcinogenesis including that of oral SCC (OSCC).[11] The present study was conducted to evaluate, compare and correlate the morphometric parameters of microvessels in different grades of OSCCs and its applicability as additional criteria in histopathological grading for predicting tumor prognosis.

SUBJECTS AND METHODS

The present study was carried out in the Department of Oral and Maxillofacial Pathology and Microbiology, Subharti Dental College and Hospital, Uttar Pradesh, India. Study consisted of 30 cases of histologically diagnosed SCC and 10 cases of normal mucosa as control, which were retrieved from department archives with relevant clinical details from patient's record. On the basis of histological grading, 30 cases of SCC were further divided into 10 each cases of well-, moderate- and poorly-differentiated SCC. Specimen showing tumor invasive front in underlying connective tissue stroma were included in the study cases.

All formalin-fixed, paraffin-embedded tissue were sectioned at 4 μm and stained with routine H and E procedure.

Morphometric analysis of tissue

- Three parameters were considered for the analysis of blood vessels in each case
- Mean vascular density (MVD) - number of vessels per high power field
- Total vascular area (TVA)
- Mean vascular area (MVA).

Microvessels were identified by the presence of red blood cells and endothelial cells (ECs).

Microvessels were identified using research Microscope (Olympus-CX 31). The slides were first examined at low magnification (×10) for identification of highly vascularized areas (hot spots) based on the criteria given by Weidner *et al.*, areas close to invasive front of tumor was preferred. Finally, images of three fields per case were acquired at ×40 magnification [Figure 1]. Images were captured using Olympus-C5060 and then saved to the computer and morphometric analysis was performed using Image Pro Express ver. 6.0 (Media Cybernetics Inc. Rockville, Maryland, USA) analysis software.

RESULTS

The mean vascular density (MVD) for the normal oral mucosa (NOM) was 3.6 ± 0.8 vessels per high power field which was comparable to the vascular density of well differentiated OSCC (3.7 ± 1.2 vessels per high power field) whereas the MVD for moderately differentiated OSCC and poorly differentiated OSCC was increased which was 4.6 ± 1.3 and 6.9 ± 3.0, respectively. On the application of the Student's *t*-test of significance, it was observed that the MVD in poorly differentiated OSCC was statistically significantly increased in comparison to moderately differentiated OSCC and well differentiated OSCC. The MVD was slightly increased between moderately differentiated OSCC and well differentiated OSCC, but the increase was not statistically significant [Table 1].

The MVA for the normal mucosa was 1499.2 ± 589.6 μm and the MVA for well differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC was 3151.3 ± 1060.3 μm, 3197.4 ± 1027.7 μm, and

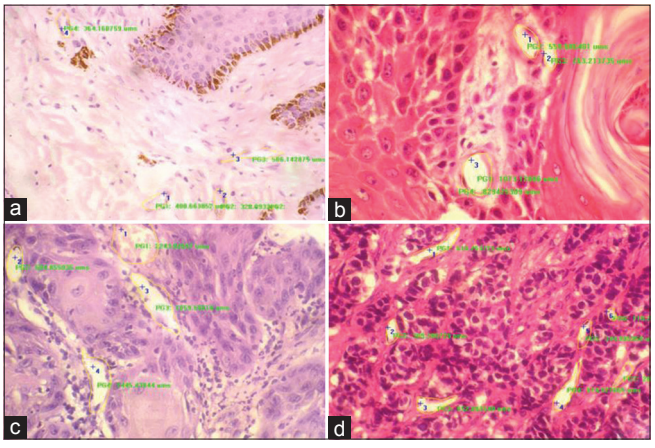

Figure 1: (a) The vascular channels in normal oral mucosa. (b) The vascular spaces marked with software in well differentiated oral squamous cell carcinoma (OSCC). (c and d) The vascular spaces marked with software in moderately- and poorly-differentiated OSCC, respectively

4902.5 ± 1614.2 μm, respectively. There was a slight increase in MVA between moderately differentiated OSCC and well-differentiated OSCC, but the increase was not statistically significant. The MVA for poorly differentiated OSCC, when compared with other groups, showed a statistically significant correlation [Table 2].

The TVA for the NOM was 4497.6 ± 1768.9 μm whereas the TVA for well differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC was 9453.9 ± 3181.0 μm, 9592.2 ± 3083.2 μm, 14707.5 ± 4842.6 μm, respectively. There was a slight increase in TVA between moderately differentiated OSCC and well-differentiated OSCC, but the increase was not statistically significant. However, the TVA for poorly differentiated OSCC, when compared with other groups, showed a statistically significant correlation [Table 3].

When the MVA and TVA for the well differentiated OSCC and moderately differentiated OSCC was compared to NOM, it showed statistically significant increase whereas the MVD showed slight increase in well differentiated OSCC, but the increase was not statistically significant. There was statistically significant difference between the three parameters, that is, MVD, MVA, and TVA for poorly differentiated OSCC when compared with the NOM.

Table 1: Mean vascular density in NOM, WDSCC, MDSCC, PDSCC

Groups	Number of cases	Mean±SD
NOM	10	3.6±0.8
WDSCC	10	3.7±1.2
MDSCC	10	4.6±1.3
PDSCC	10	6.9±3.0

NOM=Normal oral mucosa, WDSCC=Well differentiated squamous cell carcinoma, MDSCC=Moderately differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma, SD=Standard deviation

Table 2: MVA in NOM, WDSCC, MDSCC, PDSCC

Groups	Number of cases	Mean±SD
NOM	10	1499.2±589.6
WDSCC	10	3151.3±1060.3
MDSCC	10	3197.4±1027.7
PDSCC	10	4902.5±1614.2

MVA=Mean vascular area, NOM=Normal oral mucosa, WDSCC=Well differentiated squamous cell carcinoma, MDSCC=Moderately differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma, SD=Standard deviation

Table 3: TVA in NOM, WDSCC, MDSCC, PDSCC

Groups	Number of cases	Mean±SD
NOM	10	4497.6±1768.9
WDSCC	10	9453.9±3181.0
MDSCC	10	9592.2±3083.2
PDSCC	10	14707.5±4842.6

TVA=Total vascular area, NOM=Normal oral mucosa, WDSCC=Well differentiated squamous cell carcinoma, MDSCC=Moderately differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma, SD=Standard deviation

DISCUSSION

As early in 1972, Brem *et al.* proposed a microscopic angiogenesis grading system to assess the angiogenic status of the tumor vasculature. Based on the analysis of the vascular density, the number of EC nuclei and endothelial cytology, an angiogenic score was determined and used to establish an angiogenic rank order of different human brain tumors.[12]

In 1991, Weidner *et al.* developed a new method to perform microvascular density (MVD) counting studies within tumors. The first step in Weidner's approach is the identification by light microscopy of the area of highest neovessel density, the so called hot spot, by scanning the whole tumoral section at low power, then, individual microvessels are counted at a higher power (×200 field) in an adequate area (e.g. 0.74 mm^2 per field using ×20 objective lens and ×10 ocular).[13]

Following the study of Weidner, there were many retrospective studies conducted on different tumor such as non-small cell lung carcinoma, prostatic carcinoma, melanoma and SCC based on same criteria. It has been shown that assessment of microvessel could represent a valid independent prognostic factor for overall survival and disease-free survival in primary tumor showing a significant correlation between high intratumoral micro-vasculation, the presence of metastasis and poor prognosis.[14]

Most of the literature evaluation of angiogenesis as a parameter to account for the biological behavior of OSCC were mainly done on their clinic-pathological parameters like site of lesion, TNM stage, different histological grades which had come out to be of prognostic importance.[15] Pujari *et al.* in 2013, Kalra *et al.* in 2012, Ascani *et al.* in 2005, Bôas *et al.* in 2013 used various immunohistochemical markers for angiogenesis on well-, moderate- and poorly-differentiated cases of SCC and correlated them with clinical parameters for prognostic significance. Their studies showed a significant increase in values related to histological grade of differentiation.[4,16-18] Similar results have been shown in our study. In the present study, we used three parameters for microvessel evaluation in different grades of SCC that is mean vascular density (MVD), TVA and MVA. A statistically significant difference was observed between control and test groups and with increasing grade OSCC there was a significant increase in the values of all 3 parameters was also noticed.

In 1920, Broder's proposed a system of histological grading for tumors which was according to the proportion of differentiated cells within the entire tumor. He gave four grades ranging from Grade I lesion which was highly differentiated (its cell were producing much keratin) to Grade IV which was poorly differentiated (the cells were highly anaplastic and showed practically no keratin

Table 4: Bryne's (1989) ITF grading system

Morphologic feature	1	2	3	4
Degree of keratinization	Highly keratinized (>50% of the cells)	Moderately keratinized (5-20% of the cells)	Minimal keratinization (5-20% of the cells)	No keratinization (0-5%)
Nuclear polymorphism	Little nuclear polymorphism (>75% mature cells)	Moderately abundant nuclear polymorphism (50-75% mature cells)	Abundant nuclear polymorphism (25-50% mature cells)	Extreme nuclear polymorphism (0-25% mature cells
Number of mitoses (high power field)	0-1	2-3	4-5	>5
Pattern of invasion	Pushing, well delineated infiltrating borders	Pushing, well delineated infiltrating borders	Small groups or cords of infiltrating cells (n>15)	Marked and widespread cellular dissociation in small groups of cells (n<15) and or in single cells
Host response (lymphoplasmacytic infiltrate)	Marked	Moderate	Slight	None

ITF=Invasive tumor front

formation). However, it has been repeatedly evident in the literature, the poor association between Broder's grades and patient survival.[6,19]

Hence, multifactorial malignancy grading systems were developed to more precisely evaluate the growth potential of SCCs in head and neck region. These include grading systems given by Jacobsson's in 1975, which stated that not only tumor cell morphology, but the reaction of host to the tumor needs to be graded to give more prognostic information. His multifactorial parameters included for grading were structural differentiation, nuclear pleomorphism, mitosis, mode of invasion and lymphocytic infiltration.

In 1989 Bryne *et al.* appraised the Broder's grading with a multifactorial grading system, they suggested that tumor cells are a heterogeneous population with variable degree of differentiation in deep invasive front than the superficial part of tumor. They further stated that several molecular events of importance for tumor spread such as gains and losses of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of angiogenesis occur at the tumor-host interface; consequently they had developed a simple morphological malignancy grading system that restricts the evaluation to the deep invasive front of the tumor.[6,5,20] All studies performed so far show that invasive front grading is a valuable supplement to clinical staging.[7]

Features included in Bryne's ITF grading system are shown in Table 4. As it has been repeatedly demonstrated in literature and in present study that microvessel assessment is an established mean to assess the prognostic outcome of OSCC cases, these observations have suggested that angiogenesis activity could exert effects upon the clinical course, thus can be used as an additional parameter to the Bryne's grading system for better prognostic indicator.

Although, the results of the present study showed differences in MVD, TVA, MVA according to the degree of tumor differentiation, the literature has not yet reached a consensus regarding the influence of angiogenesis on the rate of recurrence, tumor invasion and regional metastasis. Not all studies have shown a statistical relation between angiogenesis and oral SCC.[21-24] Hence, further studies involving distinct phenotypes of microvessels should be performed for better understanding of the influence on tumor progression and prognosis.

CONCLUSION

There was an increased MVD, MVA, and TVA in poorly differentiated OSCC which can be used as an additional criterion to histologically grade the tumors and it may prove to be very valuable in predicting prognosis and also provide objective assessment for therapeutic strategies. However, further studies are required on large sample size with clinical correlation to further elucidate its role in tumor prognosis and posttherapeutic outcome.

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