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Pattern of tumor invasion, stromal inflammation, angiogenesis and vascular invasion in oral squamous cell carcinoma – A prognostic study

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ABSTRACT

Introduction: Oral cancer is one of the leading causes of mortality, and its worsening impact on the society has revealed the danger it poses in the coming future. Several researchers proposed and investigated the prognostic implications of various clinicopathologic and histopathologic parameters.
Aim and objectives: The aim of this study––assessing significance of histopathological features like pattern of tumor invasion, stromal inflammation, angiogenesis and vascular invasion on the clinical outcome of oral squamous cell carcinoma any possible correlations between the parameters, TNM Staging and prognosis were assessed and evaluated for a 5-year period.
Materials and methods: This study includes description of 50 diagnosed cases (mean age: 61.40, 29 males, and 21 females) of oral squamous cell carcinoma and their characteristics collected at baseline and at a 12-month follow up. The cases were grouped on the basis of their histological grade (well–differentiated, moderately differentiated, and poorly-differentiated).
Results: All the data collected was tabulated in a baseline descriptive table, and all the parameters were compared between the 3 different histological groups. On cross-tabulations we found statistical significant difference the parameters of stromal inflammation with recurrence, clinical stage with T-stage, T-stage with N-stage, and N-stage with clinical stage. On analysis of the follow up we found 16 patients (32%) with recurrence and 9 patients (18%) succumb to the disease.

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Conclusion: This study provides a significant insight on the importance of a combined histopathological analysis and clinical staging process to deliver an accurate prognostic opinion and also subsequently effect the treatment protocol.

ARTICLE INFO

Keywords: Oral cancer; Angiogenesis; TNM staging; Inflammation; Prognosis; Immunohistochemistry; Invasion

Introduction

Neoplasm with malignant characteristics and of epithelial origin is classified as carcinomas, in which the disease progression is regulated by the well-orchestrated cross talk between the malignant epithelial cells and specialized stroma.[1]

Several researchers proposed and investigated the prognostic implications of various clinicopathologic and histopathologic parameters.[2,3] Strong correlations have been found with respect to the clinical outcome of oral cancer, but still abundant research ensues in this field to define a more concrete prognostic forum. The parameters which have been suggested include tumor size, margins, thickness, patterns of invasion, stromal contents, eosinophils, chronic inflammatory cells, and many more.[4]

The various patterns in which tumor cells invade the stroma and infiltrate the host and tumor interface have been suggested to play a significant role in predicting the prognosis and effects the treatment plan.[5] The stromal response to the invading neoplasm includes a chronic inflammatory infiltrate which has also been implicated positively in determining survival of patients.[4] The growth and invasive metastatic potential of the neoplasm is largely governed by the source of oxygen and nutrients which includes the proliferating blood vessels, termed as angiogenesis.[6] Anti-CD34 antibody is an antibody targeting the transmembranous sialo-protein, CD34 has been detected in precursors of (undifferentiated) endothelial cells to differentiated endothelial cells and this protein has been successfully used to demonstrate the angiogenic potential of tissues.[7] The evidence of tumoral invasion of vessels (vascular and lymphatic) is an indication of spread of the tumor cells to an extracapsular space which could be an important prognostic factor.

A collective united assessment of the histological and clinical staging of the patients might serve as a more precise measure for predicting the outcome of the neoplasm and for determining the treatment. Hence, this study was undertaken to assess the prognostic significance of various histopathological factors including grade, pattern of tumor invasion, stromal inflammation, angiogenesis, and vascular invasion in cases of oral squamous cell carcinoma. This is the description of baseline data collected and year 1 of follow up, this study aims to further follow up patients for at least the next 4 years.

Materials and methods

A total of 50 cases of oral squamous cell carcinoma (OSCC) formed the sample, which were grouped on the basis of the histological grading. Group I included cases of well-differentiated OSCC (WDSCC, 21 cases), Group II included cases of moderately differentiated OSCC (MDSCC, 22 cases), and Group III included cases of poorly differentiated OSCC (PDSCC, 7 cases). Cases of OSCC were included in the study with the intention of further follow up of the status of recurrence for next 5 years to complete a follow up of each patient. In cases with recurrence only the sample of the primary lesion was included for histopathological examination. Those cases/ subjects with recurrence in which sample from primary lesion were unavailable were

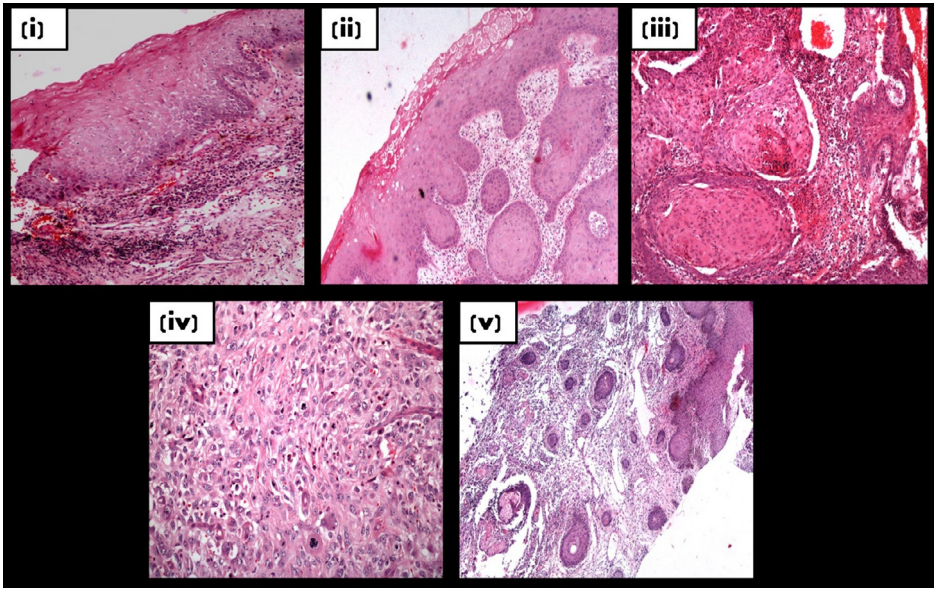

Fig. 1. (i) score 1 for pattern of tumor invasion- Broad pushing invasive front (ii) score 2 for pattern of tumor invasion––Broad pushing fingers/ separate tumor islands (iii) score 3 for pattern of tumor invasion––Invasive islands (>15 cells/ island) (iv) Invasive islands (<15 cells/ island), including single cell invasion (v) score 5 for pattern of tumor invasion––Tumor satellites with >1 mm distance from tumor.

excluded from the study. All cases were from the oral cavity (specifically tongue, buccal mucosa, labial mucosa, and palate) and any lesions of the oropharynx were excluded from the study. Each sample was also verified for human papillomavirus negativity (p16-immunoexpression) and were then included in the study. The approval from the Ethics committee of the university was taken prior to collection of data.

Three tissue sections each of 4 µm thickness were obtained from paraffin embedded blocks of the tumor tissue, the tissue sections were subjected to 1 set albumin coated slides for Hematoxylin and Eosin staining and other 2 sets of poly-L-lysine coated slides for immunohistochemical staining. The sections were stained using the following staining techniques:

1. Standard Hematoxylin and Eosin staining.
2. Immunohistochemical staining using CD34 antibody (Monoclonal Mouse CD34 class II clone QBEnd 10; Dako).

The assessment of various parameters was done using standard established grading and staging systems, the pattern of tumor invasion and stromal inflammation was scored at the host/tumor interface as suggested by Brandwein Gensler in 2005[5]; where they used to score pattern 1-4 according to previously described systems by Byrne[8] but introduced pattern 5 for the first time (Figs. 1,2). We assessed the stromal inflammation by a 3 tiered system (1, scanty inflammation; 2, intense localized inflammation and 3, intense diffuse inflammation). For evaluating the microvessel density immunohistochemical CD-34 stained sections were scanned at low magnification to identify the most vascular areas (hot spot areas). Analysis was performed under 20x objective and 10x ocular lens (x200 magnification). An average of 10 hot spots was analyzed (Fig. 3). The selection of hotspots was done blindly and the CD34 reactions were evaluated considering the cytoplasmic staining in endothelial cells. Evaluation of positive reactions was performed using the criteria defined by Weidner et al.[9] For the parameter of vascular invasion on examination of the slide the larger vessels with visible lumen were observed and

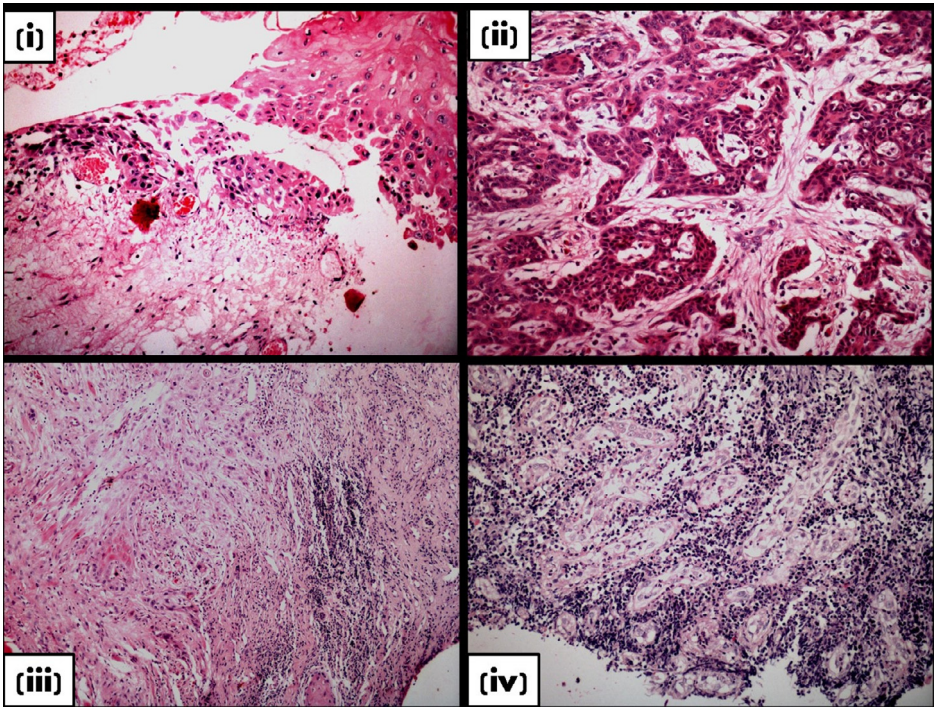

Fig. 2. (i) score 0 for stromal inflammation––Absence of inflammation (ii) score 1 for stromal inflammation––scanty inflammatory infiltrate (iii) intense localized inflammation (iv) intense diffuse inflammation.

presence of tumor epithelial cells was interpreted as presence of vascular invasion and was marked as positive (Fig. 4). Data was collected at baseline (Table 1) and at 12 months interval, and suitable statistical comparisons were performed (Table 2). Data was further examined for statistical significance (*P*-value) using appropriate tests (Chi-square tests) and, "*P*" value $<$ 0.05 was considered as statistically significant.

Results

Our sample consisted of 50 cases, aged from 24 years to 82 years (mean – 61.4 years); with 29 males and 21 females. On the whole the cases were grouped on the basis of the grade of differentiation as well differentiated (42%), moderately differentiated (44%), and poorly differentiated (14%).

The characteristics of all patients were collected (Table 1) and then grouped on the basis of the histologic grade (Table 2). The most common pattern of invasion was type 4 (invasive islands (<15 cells/ island), including single cell invasion) with 38% of the cases showing it. Angiogenesis was assessed by calculating the mean microvessel density in all the cases and the maximum MVD (Mean vascular density) value was found to be 177.4, minimum MVD value was 5.9, and the mean value was found to be 84.30 $\pm$ 38.77. In Group I, the mean of 21 cases was calculated to be 89.881 $\pm$ 6.197 with maximum and minimum values as 41.6 and 128.4 respectively. In Group II, the mean of 22 cases was found to be 81.859 $\pm$ 43.76; with minimum and maximum values of 157.5 and 5.9 respectively. In Group III the mean of 7 cases was calculated to be 75.271 $\pm$ 51.56; with minimum and maximum values of 177.4 and 23.5 respectively. On

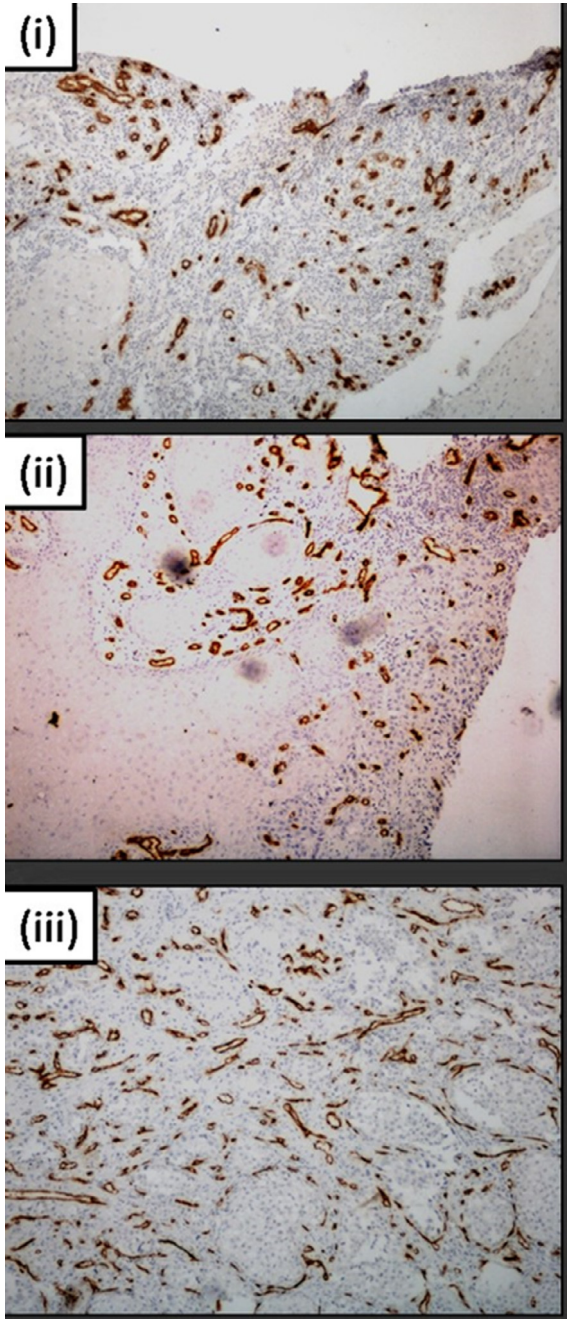

Fig. 3. (i) hot spot at 20x magnification in a case of WDSCC (ii) hot spot at 20x magnification in a case of MDSCC (iii) hot spot at 20x magnification in a case of PDSCC.

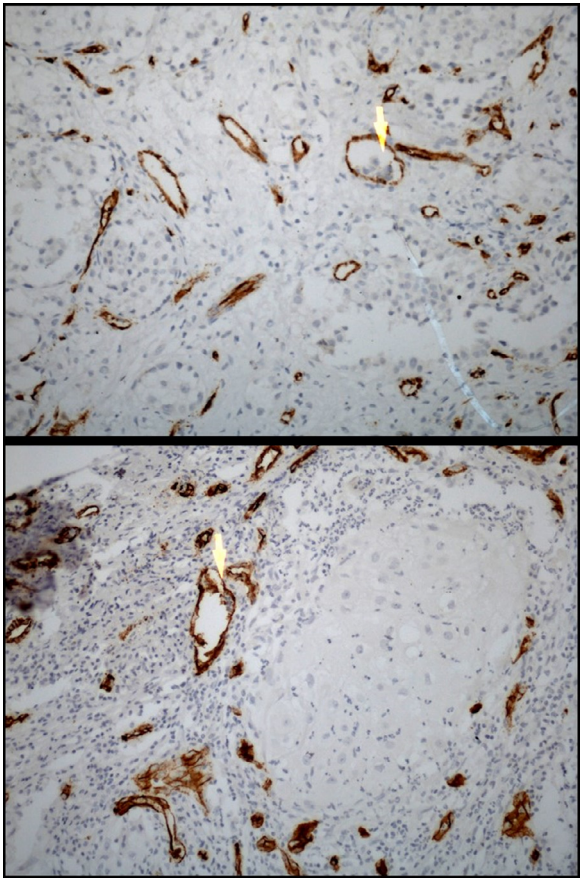

Fig. 4. (i) and (ii) areas showing positive vascular invasion.

assessment of invasion of tumoral vessels by malignant epithelial cells, 64% of the total cases showed vascular invasion and 36% showed absence of vascular invasion. Only 1 case showed extra nodal metastatic spread, the case was histologically categorized as poorly differentiated, and the metastatic deposits were found in the lung of the patient.

On the 12 month follow-up, we found that 50% of the patients showed no signs of recurrence, 34% showed recurrence and 16% has died.

On cross tabulating (chi-square test) we found no significant differences of any of the histopathological or clinical parameter with histological grading of tumor. (Table 2)

No statistical significant association was found between the histological parameters of pattern of tumor invasion and stromal inflammation ($P = 0.112$); pattern of tumor invasion and angiogenesis ($P = 948$); pattern of tumor invasion and T-stage ($P = 0.403$); stromal inflammation and angiogenesis ($P = 0.461$); stromal inflammation and T-stage ($P = 0.714$); stromal inflammation and N-stage ($P = 0.563$); stromal inflammation and clinical staging ($P = 0.448$); angiogenesis and T-stage ($P = 0.085$); angiogenesis and N-stage ($P = 0.371$), and angiogenesis and clinical staging ($P = 0.306$).

Statistical significance was observed between stromal inflammation and recurrence, Clinical staging with T-stage and N-stage, N-stage and T-stage.

Table 1
Clinical data of all cases included in the study.

Variable	No. (%)
Age at diagnosis	
Range	24-82
Mean	61.4
<60 years	20 (40%)
>60 years	30 (60%)
Gender	
Male	29 (58%)
Female	21 (42%)
Histologic grade	
Well-differentiated OSCC	21 (42%)
Moderately-differentiated OSCC	22 (44%)
Poorly-differentiated OSCC	7 (14%)
Pattern of tumor invasion	
Broad pushing invasive front	1 (2%)
Broad pushing fingers/separate tumor islands	13 (26%)
Invasive islands (>15 cells/ island)	15 (30%)
Invasive islands (<15 cells/ island), including single cell invasion	19 (38%)
Tumor satellites with >1 mm distance from tumor	2 (4%)
Stromal inflammation	
Scanty	10 (20%)
Intense localised	25 (50%)
Intense diffuse	15 (30%)
Mean vascular density	
Mean	84.30
Range	5.9-177.4
<80	23 (46%)
>80	27 (54%)
Vascular invasion	
Absent	18 (36%)
Present	32 (64%)
T-stage	
T1	14 (28%)
T2a	25 (50%)
T2b	8 (16%)
T3	3 (6%)
N-stage	
N0	21 (42%)
N1	15 (30%)
N2a	6 (12%)
N2b	5 (10%)
N3	3 (6%)
M-stage	
Absent	49 (98%)
Present	1 (2%)
Clinical stage	
I	12 (24%)
II	8 (16%)
III	16 (32%)
IV	13 (26%)
VI	1 (2%)
Prognosis (12-month follow-up)	
No recurrence	25 (50%)
Recurrence	17 (34%)
Death	8 (16%)

OSCC, Oral Squamous Cell Carcinoma.

Table 2
Cross tabulations of all parameters on the basis of histologic differentiation grade and follow-up status.

	WD-OSCC ($n=21$)	MD-OSCC ($n=22$)	PD-OSCC ($n=7$)	NO REC ($n=25$)	REC ($n=17$)	DEATH ($n=8$)
Age						
<65 years	14 (66.7%)	11 (50%)	1 (14.3%)	11 (44.0%)	11 (64.7%)	4 (50.0%)
>65 years	7 (33.3%)	11 (50%)	6 (85.7%)	14 (56.0%)	6 (35.3%)	4 (50.0%)
POI 1, 2, 3	7 (33.3%)	5 (22.7%)	2 (28.6%)	7 (28.0%)	7 (41.2%)	0 (0%)
POI 4, 5	14 (66.7%)	17 (77.3%)	5 (71.4%)	18 (72.0%)	10 (58.8%)	8 (100%)
SI 1	3 (14.3%)	14 (63.6%)	3 (42.9%)	19 (76.0%)	11 (64.7%)	5 (62.5%)
SI 2 and 3	18 (85.7%)	8 (26.4%)	4 (57.1%)	6 (24.0%)	6 (35.3%)	3 (37.5%)
MVD						
<80	5 (23.8%)	6 (27.3%)	3 (42.9%)	7 (28.0%)	5 (29.4%)	2 (25.0%)
<80	16 (76.2%)	16 (72.7%)	4 (57.1%)	18 (72.0%)	12 (70.6%)	6 (75.0%)
VI negative	8 (38.1%)	8 (36.4%)	2 (28.6%)	11 (44.0%)	6 (35.3%)	1 (12.5%)
VI positive	13 (61.9%)	14 (63.6%)	5 (71.4%)	14 (56.0%)	11 (64.7%)	7 (87.5%)
T1 & T2a	15 (71.4%)	19 (86.4%)	5 (71.4%)	19 (76.0%)	14 (82.4%)	6 (75.0%)
T2b & T3	6 (28.6%)	3 (13.6%)	2 (28.6%)	6 (24.0%)	3 (17.6%)	2 (25.0%)
N0 & N1	17 (81%)	20 (90.9%)	5 (71.4%)	21 (84.0%)	15 (88.2%)	6 (75.0%)
N2a, N2b, & N3	4 (19%)	2 (9.1%)	2 (28.6%)	4 (16.0%)	2 (11.8%)	2 (25.0%)
M0	21 (100%)	22 (100%)	6 (85.7%)	25 (100.0%)	17 (100.0%)	7 (87.5%)
M1	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)		
0 (0%)	1 (12.5%)					
CS 1 & 2	7 (33.3%)	12 (54.4%)	1 (14.3%)	10 (40.0%)	7 (41.2%)	3 (37.5%)
CS 3, 4, & 6	14 (66.7%)	10 (45.5%)	6 (85.7%)	15 (60.0%)	10 (58.8%)	5 (62.5%)
No rec	11 (52.4%)	13 (59.1%)	1 (14.3%)	-	-	-
Rec	7 (33.3%)	7 (31.8%)	3 (42.9%)			
Dead	3 (14.3%)	2 (9.1%)	3 (42.9%)			

CS, Clinical stage; MD-OSCC, Moderately-differentiated oral squamous cell carcinoma; MVD, Mean vascular density; NO REC, No recurrence; PD-OSCC, Poorly-differentiated oral squamous cell carcinoma; POI – Pattern of tumor invasion; REC, recurrence; SI, Stromal Inflammation; VI, Vascular invasion; WD-OSCC, Well-differentiated oral squamous cell carcinoma.

Discussion

The last few decades have been marked with tremendous progress in the field of cancer research, treatment, management, and assessment. It has been shown time and again that the most important aspect in predicting prognosis of an oral cancer patient is the identification of various significant parameters; clinical and histopathological. The foundation of carcinoma diagnosis and staging is laid down primarily by the evaluation of histopathological parameters in the tissue sections. Recurrence and metastasis are the main etiological factors which result in the collapse of the treatment plans and management strategies particularly in the case of OSCC. Thus, the recognition of histological parameters which have a positive or negative impact on the prognosis of oral cancer is of utmost importance.[3,10]

Two morphological and functional variants of collective migration have been identified in tumors; the first variant can be described as the result of protruding sheets and strands that have maintained contact with the primary site of occurrence and yet they show evidence of local invasion and the second variant clusters of cells (nests) which are distinctly detached from the tumor mass and extend though perineural and vascular structures (path of least resistance).[11] In the present study, in group I (WDSCC) the predominant pattern of tumor invasion was by invasive islands (<15 cells/island), inclusive of single cell invasion exhibited by 42% cases; in group II (MDSCC) the predominant pattern of tumor invasion was by invasive islands (>15 cells/ island) exhibited by 40% cases and in group III the predominant pattern observed was invasive islands (>15 cells/island) along with single cell invasion exhibited by 57% of cases. A contrasting result in group I is observed, as the tumor cells are completely differentiated but still predominantly show score 4 which includes single cells invasion and tumor islands (<15 cells/island). This con-

tradiction can be attributed to the fact that OSCC usually exhibit a heterogenous cell population with probable differences in invasive and metastatic behavior.[10] Our study is in accordance with previous studies[12-14] and pattern of tumor invasion has been suggested to be the single most important parameter in predicting survival.[12]

The features of suppressed immunity, invasive growth and metastasis associated with malignancies are of major prognostic importance; the inflammatory cells infiltrating the tumor along with their associated cytokines (TGF-β, IL-1, and NF-κB) are thought to have a major contribution in regulating these features.[15-18] 71% cases of group I shows a localized intense inflammatory response, 40% cases of group II show a localized intense response and 57% cases of group III show a diffuse intense inflammatory response. It has been suggested that with an increased proliferation of keratinocytes is associated with inflammatory cells, angiogenesis and inflammatory cytokines; also they have an effect on the size, shape, and growth of these tumors, that is, in cases of a reduced inflammatory response the tumors would show a more aggressive clinical behavior.[4,17,19]

As mentioned above growth and metastasis of tumor is also regulated by angiogenic factors and inhibitors. The mechanisms of angiogenesis in tumors closely resemble the physiologic mechanisms, the only difference being its lack of regulation.[6,20,21] We have observed an evident but statistically insignificant increase in the mean microvessel density with increase in differentiation of cells. Studies showing similar results[20] and contradictctory results,[23,24] both have been published and reviewed. It was suggested that due to the profuse vascularity of the tongue and the oral cavity, carcinomas of this region might be less dependent on neovascularization[22] for growth and metastasis.[25]

Owing to the weak immature basement membranes of the proliferating vascular channels in an aggressive neoplasm, we have frequently observed invasion of malignant neoplastic cells invading the vascular lumens. Many investigators have omitted this parameter in determining the prognostic implications due to the inability to identify invasion with certainty.[18,26-28] In the current study in group I 61% cases show vascular invasion, in group II 63% cases show vascular invasion and 71% cases in group III show vascular invasion. An evident increase in comparative number of cases showing positive vascular invasion was observed with decrease in differentiation of cells. Cells showing least differentiation and more anaplastic characters are devoid of features like adhesion with an increased motility, which could explain the pattern observed in the current study.

OSCC has a great predisposition to produce metastasis in lymph nodes. In clinical practice, the treatment plan and prognosis of OSCC is mainly based on the TNM (primary tumor, regional lymph node metastasis, and distant metastasis) staging system. The most recent staging system is the AJCC TNM staging system 2017.[29] In the present study, the same system was employed to record the TNM staging of the patient. The TNM and clinical staging showed no correlation with histological grading of the tumor in our study. In a previous study,[30] 40% of the cases with abundant keratinization showed metastases, whereas in our study a total of 61% cases of WD-SCC showed lymph node involvement; thus, pointing to a relation with the maturity of these keratinizing cells and their spread into the vascular and lymphatic vessels and manifesting as lymphadenopathy. As we know that poorly differentiated cells are mostly associated with the single cell invasion pattern and they have lost their cohesive property, hence there will be an inherent increase in chances of lymph node metastasis with decrease in differentiation of cells. A strong association between the presence of extracapsular spread and clinical N-stage was found in other studies.[31,32] In this study out of 50 cases of OSCC, only 1 case showed extra nodal metastatic spread. In accordance with other published research work,[12,26,27,33,34] this case was histologically categorized as poorly differentiated, and the metastatic deposits were found in the lung of the patient. In a study of OSCC,[12] they found that the histologic grade reflected the aggressiveness of the individual neoplasm and that there was a clear relationship between the grade and cure rate, stage of disease, and metastatic involvement. The present study failed to show any significant correlation between clinical staging and histological grading, as also observed in other publications.[5,18,27,28,33]

The histological parameter of pattern of tumor invasion and the clinical parameter of N-stage have shown a significant correlation in a few studies.[5,35-37] The molecular basis of this connection has been attributed to MMP-7, MT1-MMP,[37] cellular motility, and proteolysis[39,40] involving interactions between tumor cells and extracellular matrix. Another noteworthy association was observed between the pattern of tumor invasion and clinical staging. There was a statistically significant difference between angiogenesis and vascular invasion in the current study and we can thus conclude an apparent correlation between these 2 entities. The cases showing vascular invasion showed a mean MVD score of 94.07 ± 37.35 and the cases showing no evidence of vascular invasion had a mean MVD of 66.944 ± 35.90. A highly significant difference was observed between T-stage and N-stage and a strong correlation is suggested, the cases without nodal metastasis steadily decreased as the size of the tumor increased, hence a connection between the size of the tumor and the nodal metastasis incidence was inferred. Reports in the literature showed that tumor thickness was a better prognostic parameter compared with the T stage or largest diameter in the prediction of nodal metastasis in oral cavity carcinomas.[38,41-48] Between the 2 parameters of T-stage and clinical staging a significant correlation was suggested, we observed that an increase in tumor size lead to a poorer prognosis and more advanced clinical stage of patient, as larger tumor tend to be more aggressive, invasive, and damaging.

With reference to the prognosis of the patients no statistical significance was observed between the histopathological groups. Most authors have established significant correlations between lower histologic differentiation and poorer prognosis.[49-53] but others did not find such association.[54-56] There was a statistically significant difference observed between the prognosis of cases and various scores of pattern of tumor invasion (P= 0.043). A gradual decrease in the rate of survival was observed from score 1 to score 5 of pattern of tumor invasion. It was observed that pattern of invasion alone has been reported to be a significant prognostic factor, indicating the importance of removing larger biopsies with inclusion of the connective tissue stroma lying underneath.[26] Over the past 2 decades, POI alone, and as part of weighted scoring systems, has been demonstrated to predict loco-regional recurrences and decreased overall survival.[5] No statistical significant difference between the parameters of prognosis and stromal inflammation, but some previous studies have indicated a positive correlation.[4,5,19] The antitumor effects of the inflammatory infiltrate is postulated to be mediated by cytokine, Pronounced inflammatory infiltration can be due to the antitumor effects of the inflammatory cells mediated by cytokine secretion induced by the response of inflammatory cells to tumor stimulation.

Conclusion

Thus, through thus study, we have tried to summarize the influence of various invasion patterns, the lymphocytic host responses, the neoangiogenic incidence and presence of invaded vascular channels in the prognosis or outcome of patients with OSCC. The efficiency and accuracy of the well-established TNM and clinical staging system was challenged and a possible correlation between the histopathological parameters and clinical staging systems was sought. We may conclude that this study sheds significant light on the importance of a combined histopathological analysis and clinical staging process to deliver an accurate prognostic opinion and also subsequently affect the treatment protocol.

Declaration of Competing Interest

None.

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