IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 15, Issue 11 Ver. II (November. 2016), PP 72-77
www.iosrjournals.org

Evaluation of Micro-Vessel Density (Mvd) and Vascular Endothelial Growth Factor (Vegf) as Possible Indicator of Malignant Transformation in Oral Submucous Fibrosis

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Abstract: *Oral submucous fibrosis (OSMF) is a potentially malignant disorder, prevalent in Southeast Asia, with a malignant transformation rate of 2% to 10%. As the disease progresses, the connective tissue becomes increasingly hyalinised, creating a hostile micro-environment for the initiation and progression of tumour. Angiogenesis is defined as sprouting of new blood vessels from pre-existing ones and is conceded as the prerequisite for tumour initiation, progression and metastasis. VEGF is a prime growth factor involved in the angiogenesis associated with most cancers. In an attempt to evaluate the status of vascularity and the role of VEGF in the malignant transformation of OSMF, we analysed the mean vascular density (MVD) and the expression of VEGF in 65 OSMF biopsies by immunohistochemistry. A significant increase in the MVD was observed between OSMF with no epithelial changes and OSMF with epithelial dysplasia and/or invasion. Similarly, a positive correlation was observed between the expression of VEGF by the epithelial cells and the vascularity beneath. Based on the study, it is concluded that epithelial secreted VEGF could possibly play a pivotal role* in either sustaining or stimulating angiogenesis that support tumour growth and invasion in OSMF patients.

Keywords: *oral cancer, oral submucous fibrosis, squamous cell carcinoma, vascular endothelial growth factor, VEGF*

I. Introduction

Oral cancer is the sixth common cancer worldwidewhich holds high priority inthe health management system. Widespread usage of chewable tobacco and, indigenous ways of using tobacco-related products in most South Asian countries including India, makes it a daunting task for the respective countries to control the disease. Often, oral cancer is preceded by clinically recognizable diseases which are collectively termed as "potentially malignant disorders" (PMD). One such disease, with considerable prevalence in India, is oral submucous fibrosis (OSMF), a chronic deliberating condition caused by chewing areca nut either alone or as an ingredient in tobacco quid. Early studies reported that 40% cases of oral cancers in India had OSMF, supporting the precancerous nature of the disease.[1]Reported incidence of carcinoma in OSMF patients ranged from 4.5% to 7.6%.[2,3] The risk of malignant change inOSMF is often assessed by microscopic recognition of epithelial dysplasia since lesions with epithelial dysplasia carried 15 times higher risk of malignant change.[4]

Any potentially altered oral epithelium to proliferate, breach the underlying basement membrane and transform into an invading carcinoma, requires profuse blood supply which, internnecessitates modulation of existing blood supply and architecture in the stromal tissue. The term "Angiogenesis" is defined as sprouting of new blood vessels from pre-existing ones and is conceded as one of the key steps in tumour growth and progression, failing which the tumour growth is considerably limited.[5]Such pro-angiogenic switch is presumed to be an early event in oral carcinogenesis as demonstrated by increased vascularity noted in the sub-epithelial region of dysplastic lesions.[6]

Among the various factors involved in tumour induced angiogenesis, vascular endothelial growth factor (VEGF) has been identified as the prime signalling molecule that modulates the initiation and progression of oral cancer.[7]VEGF is a group of heparin-binding, dimeric, polypeptide growth factors, a potent mitogen specific to endothelial cells and has a role in enhancing the vascular permeability in the tumour micro environment.[8,9]The tumour angiogenesis and the expression of VEGF have been documented to be important prognostic factors in various malignancies including oral PMDs and cancer, and have a direct effecton tumour progression, local invasion of the tumour and lymph node metastasis.[10,11]Studies on VEGF gained importance in the recent times as VEGF has been targeted by anti-VEGF antibodies as an adjuvant theraputic modality to restrict tumour growth in many solid tumours.[12]

Unlike other PMDs, OSMF is unique in which there is excessive production and irreversible crosslinking of collagen, leading to progressive hyalinization of lamina propria and reduction in vascularity and cellular components, rendering it less conducive to support angiogenesis.The mechanism of tumour induced angiogenesis and the role of VEGF in such hostile micro-environment to support tumour growth remains unclear. The present study was aimed at evaluating the status of vascularity and angiogenesis in the disease process of OSMF leading to carcinoma, and to draw a relation between vascular changes and the expression of VEGF in an attempt to find a suitable biomarker for predicting malignant change in these lesions.

II. Methods

Samples

Sixty-five formalin fixed, paraffin embedded tissue blocks of clinically diagnosed and histopathologically confirmed OSMF patients were retrieved from the archives of oral pathology department, Rajah Muthiah Dental College, Annamalai University. Ten specimens of normal oral mucosa were harvested as control from the patients who underwent prophylactic removal of impacted third molars after informed consent.All the biopsies were processed routinely.

Histopathological And Immunohistochemical Analysis

Three serial tissue sections of 4µ thickness were madefrom each specimen in poly-L-lysine coated slides. One section was stained with routine haematoxylin and eosin stain (H&E) and the rest were used for immunohistochemistry. All the H&E stained sections were reviewed by two pathologists independently and after confirmation of the diagnosis, OSMF patients were graded based on the grading system proposed by Utsunomiya et al (2005). [13] The epithelial component of each sample was analysed separately for the presence of dysplasia and micro-invasive carcinoma.

The remaining sections of all the samples were sequentially dewaxed through a serious of xylene, graded alcohols and water immersion steps. Antigen retrieval was carried out by immersing the slides in Tris EDTA buffer (pH-9) and boiling it in a pressure cooker for 15 minutes. After a cool off period of 10 minutes, the sections were incubated in freshly prepared 3 % hydrogen peroxide for 10 minutes to block endogenous peroxidase activity and incubated with 0.04 % casein in PBS for 10 minutes to block unwanted protein binding. The sections of each sample were incubated in Anti-human CD34 antibody (clone-Qbend-10, Biogenex laboratories Inc, USA)and Anti humar vascular endothelial growth factor (VEGF-1, Clone 165, 121, 189; Dakopath laboratories, Denmark) for 30 minutes each.The detection of antigen-antibody reaction was carried out using super sensitive polymer HRP detection system (Biogenex laboratories Inc, USA) and DAB chromogen. Throughout the procedure the sections were washed thoroughly in 2 changes of PBS, 5minutes each, between the subsequent steps. Finally the sections were counterstained with Mayer's haematoxylin and mounted with DPX for visualization. Samples of breast adenocarcinoma were used as positive control for VEGF. For negative control, all the steps were followed omitting the primary antibody.

Mean vascular density (MVD)

Anti-CD34 antibody labelling was used to highlight the endothelial cells in the tissue sections. For the analysis of vascularity and mean vascular density (MVD), the method advocated by **Weindneret al (1993)** was followed.[14]The slideswere visualized and appropriate areas were recorded under x 200 magnificationusing a high resolution photomicrographic system (Nikon Eclipse E-200 microscope attached with 8.4 mega pixel digital camera) and the photomicrographs wereanalysed using the image analysis software "Image J" to count the blood vessels. Cluster of cells and individual cells, with or without lumen, stained with CD34 were counted as vessel. The vessels were counted from 3 different areas of highest density and the average was noted as the MVD for the particular case.

Assessment of VEGF expression

VEGF expression was noted as brown cytoplasmic staining in the epithelial cells, inflammatory cells and endothelial cells. However, the expression in epithelial cells is noted for the study. All the samples were assessed by two pathologists independently. The expression of VEGF was recorded using a 4-point scoring system presented by **Moriyama et al (1997)** as, 0 –negative, 1, 2 and 3, if the intensity of VEGF staining in epithelial cells is less than, equal to, or more than the staining intensity seen in endothelial cells respectively.[15]

III. Statistical analysis

Statistical analysis of the results was performed using SPSS software. X^2-test and ANOVA were performed to analyse the MVD. X^2- test was performed to evaluate the VEGF positivity among samples and Kruskal-Wallis test was performed to analyse the degree of expression of VEGF among the samples. The results were considered significant if $P \leq 0.05$.

IV. Results

Histopathology

None of the control samples (n10) showed evidence of notable inflammation or alteration of the surface epithelium. The OSMF samples (n65) were assessed and graded as Grade 1, 2 and 3 based on the inflammatory component, stromal cells, oedema, hyalinization and muscle involvement as proposed earlier.[13] 36.9 % of the samples were grade 1 OSMF whereas, 40 % and 23.1 % were grade 2 and grade 3 respectively. Similarly, assessment of the surface epithelium for dysplastic features showed 38.5 % (n25) to have varying degrees of epithelial dysplasia whereas, 7.7 % (n5) cases showed breach in the basement membrane and/or micro-invasion. Table 1 shows the composite distribution of samples with epithelial changes among the grades of OSMF. Epithelium of each grade of OSMF was individually assessed for presence of dysplasia or invasion and accordingly in Grade 1 OSMF, 33% cases showed epithelial dysplasia whereas 12.5% cases showed breach in the basement membrane in the form of either micro-invasion or invasion. Similarly, in Grade 2 OSMF, 38.5% showed dysplasia and 7.9% cases showed micro-invasion/invasion. In the case of Grade 3 OSMF, 46.7% cases showed epithelial dysplasia. However, none of the cases showed basement membrane breach.

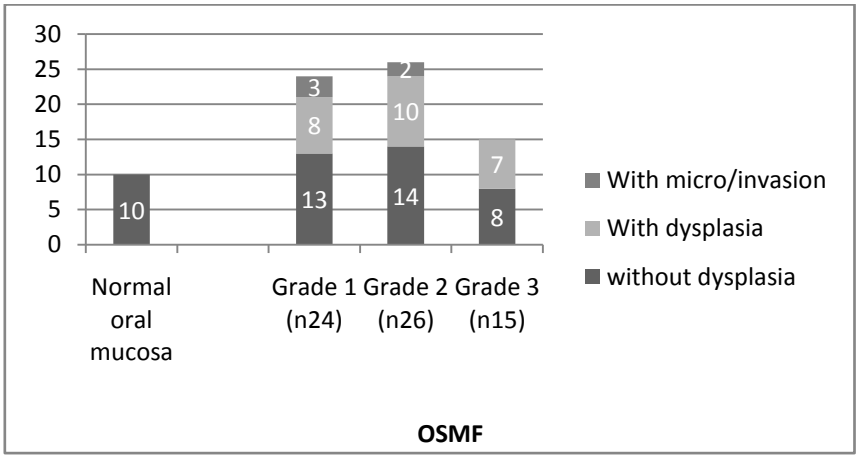

Table 1. Distribution of samples among various histo-pathologic parameters.

Assessment of Mean Vascular Density (MVD)

When MVD of normal oral mucosa (26.87±4.66) and OSMF samples (24.73±9.53) were compared no statistical difference was noted. Conversely, there was a statistically significant reduction of MVD from Grade 1 (33.88±4.75) through grade 2 (22.54±7.12) to grade 3 (13.89±3.02) OSMF. Similarly, when the OSMF samples are grouped based on the nature of epithelium, there was a statistically significant increase in MVD from OSMF without any epithelial dysplasia (23.19±9.35), to OSMF with evidence of epithelial dysplasia (24.04±8.25) and OSMF cases with either micro-invasion or invasion (38.93±5.14).

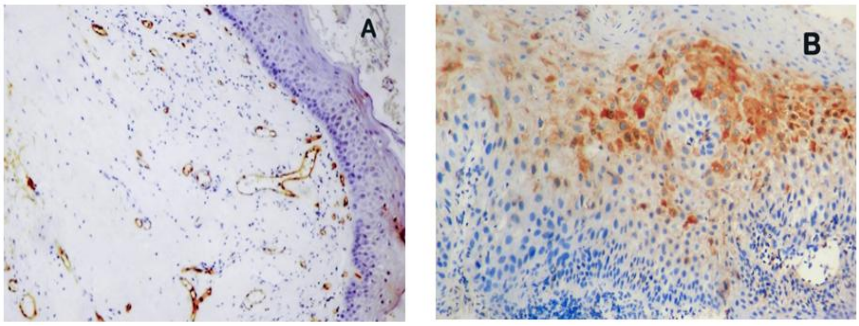

Figure. 1. (A)Stainingof endothelial cells of the connective tissue by anti-CD34 antibody in OSMF(X200). (B) Expression of VEGF in the dysplastic epithelium of OSMF sample (X400).

In order to assess the role of epithelial changes on vascularity, each grade of OSMF is subcategorized based on epithelial characteristics and the MVD was compared within each grade individually. In grade 1 OSMF, samples without evidence of epithelial dysplasia (n13), with epithelial dysplasia (n8) and invasion (n3) showed MVD of 33.82±2.52, 32.92±7.25 and 36.66±4.73 respectively. Similarly, for grade 2, MVD of 19.88±1.05, 22.3±1.34 and 42.34±3.34 were obtained respectively. For grade 3, MVD of 11.71±1.36 and 16.38±2.34 were obtained for samples without dysplasia and with dysplasia. However, there were no samples

with micro-invasion or invasion in this grade. When the results were statistically analysed, no significant difference was observed in grade 1 whereas, grade 2 and grade 3 showed significant increase of MVD from samples without dysplasia through samples with dysplasia to samples with micro-invasion /invasion.

	n	VEGF		P-value
		Positive (%)	Negative (%)	
Sample				
Normal	10	3(30.0)	7(70.0)	NS
OSMF	65	34(52.3)	31(47.7)	
Grade				
Grade I	24	13(54.2)	11(45.8)	
Grade II	26	13(50.0)	13(50.0)	NS
Grade III	15	5(33.3)	10(66.7)	
Epithelial status				
No dysplasia	35	23(65.7)	12(34.3)	
With dysplasia	25	8(32.0)	17(68.0)	0.003
Micro invasion/carcinoma	5	0(0)	5(100)	

NS- Not significant

Table 2. The correlation of VEGF expression with histologic grading and epithelial status in OSMF samples (χ^2 –test)($P \leq 0.05$)

Comparison of VEGF expression and histological parameters

The expression of VEGF was observed as browned cytoplasmic staining in the epithelial cells, endothelial cells andinflammatory cells of the samples. The expression was assessed only in the epithelial cells since there was considerable reduction of both endothelial cells and inflammatory cells with the advancing grades of the disease process. The expression of VEGF was assessed for two parameters, 1) positivity or negativity and 2) the intensity of expression. The expression of VEGF positivity with various histo-pathological parameters is summarized in table 2. When statistically compared, there were no significant differences between normal oral mucosa and OSMF, and also among different grades of OSMF. However, statistically significant differences exist among OSMF without epithelial dysplasia, OSMF with epithelial dysplasia and OSMF with micro-invasion/invasion. A similar trend was observed when the intensity of the staining was compared, the results of which are summarized in table 3.

	n	Staining intensity	*P*-value
Sample			
Control	10	0.3 ± 0.5	NS
Study	65	0.9 ± 1	
Grade			
Grade 1	24	0.8 ± 1	
Grade 2	26	0.8 ± 0.9	NS
Grade 3	15	1.3 ± 1.1	
Epithelial status			
No dysplasia	35	0.5 ± 0.7	
With dysplasia	25	1.1 ± 1	0.00
Micro invasion/carcinoma	5	2.6 ± 0.6	

NS – Not significant

Table 3. Expression of VEGF intensity among histologic grading and epithelial status in OSMF (Kruskal Wallis Test) ($P \leq 0.05$)

V. Discussion

An early Indian study reported that there is increased incidence of leukoplakia and oral cancer in patients with OSMF when compared to others and also, 40% of the oral cancer patients had OSMF thereby instigating the precancerous nature of OSMF.[16,17]Till then, many reports of malignant transformation of oral epithelium in OSMF were reported and reviewed in the literature.[3,4,18]Today, OSMF is a well-documented potentially malignant disorder with a transformation rate of 2% to 10%. We analysed 65 OSMF samples out of which 38.5% samples exhibited varying grades of dysplasia. This may be an exaggerated value since many of

the early cases reported to the hospital are not biopsied for the diagnosis. The presence of dysplasia increases the risk of acquiring cancer by15 times. In spite of excluding cases with clinically noticeableleuko-erythroplakic or carcinomatous changes, 7.7% of the samples showed evidence of either micro-invasion/invasion of dysplastic cells into thesuperficial stromal tissue. This also augments the fact that early carcinomas may not be clinically visible and all suspicious looking areas in OSMF require biopsy. The present study failed to note any grade 3 OSMF exhibiting invasion/micro-invasion, an observation noteworthy of further investigation and such stromal condition either retards tumour initiation/progression or an alternative mechanism exists, is an overstatement at this point.

Yet another interesting observation is that the stromal hyalinization, in few cases, is seen a distance away from the sub-epithelial region. The possible reason could therefore, be an optimum concentration of the alkaloids and flavonoids,which often diffuse through the oral mucosa from the chewing areca nut, is necessary to initiate the process of fibrosis.

Folkman J (1971) first articulated the hypothesis that tumour growth is "angiogenesis dependent" and later (1990) enumerated various indirect studies in the form of evidence to prove that tumour growth and progression is indeed angiogenesis dependent.[19,20] Studies indicate that the onset of angiogenesis is believed to be an early event in carcinogenesis and this process may facilitate tumour progression and metastasis.[21]In the present study, no significant difference in MVD was observed between normal oral mucosa and OSMF. Similar findings were observed by Rajendran et al (2005).[22]The results are justifiable because, unlike other PMDs, the histopathologic changes in OSMF are heterogeneous, with early lesions dominated by inflammatory phase involving inflammatory and stromal cell derived cytokines, resulting in more capillaries and later, by fibrosis and hyalinization of the stromal connective tissue due to increased production, abnormal cross-linking and decreased degradation of the collagen resulting in strangulation and reduction of capillaries and a state of ischemia. However, when the MVD was compared across the grades of OSMF, there was a significant increase in the number of blood vessels with increasing grades. It is logical to believe that, as the disease advances, there is increasing hyalinization of the stromal tissue and reduction in blood vessels, particularly capillaries, in the sub-epithelial region and a compensatory dilation of the larger blood vessels in the deeper region. Further, vascularity also makes one of the criteria in most OSMF grading systems.[23]

In an attempt to answer the question of whether the epithelial changes in the form of dysplasia had any influence on the underlying vascularity, MVD was compared among different epithelial characters for each grade of OSMF. In grade 2 and grade 3 OSMF, marked increase in vascularity was observed in OSMF with epithelial dysplasia whereby supporting the role of dysplastic epithelium in modulating the stromal vasculature. Contrarily, we failed to observe the same, in grade 1 OSMF, possibly due to the early inflammatory phase and associated cytokines result in the increased vascularity seen in grade 1 OSMF.

In the oral mucosa, the pro-angiogenic shift occurs much before the appearance of the cancerous lesions evidenced by early expression of pro-angiogenic growth factors and increase in the blood capillaries beneath the dysplastic epithelium of various PMDs.[24] Vascular endothelial growth factor has been identified as one of the prime factors secreted by the tumour cells that stimulate the existing endothelial cells to proliferate by paracrine mechanism and thereby facilitates the growth, invasion and metastasis of the tumour.[7]Expression of VEGF was also considered an early event in the process of malignant transformation in PMDs such as leukoplakia. In most of the PMDs, the alteration of stromal micro-environment plays a crucial supporting role in the invasion and metastasis of the tumour.

In early OSMF, expression of VEGF was observed in the endothelial cells, inflammatory cells and epithelium. Considering the fact that there is reduction of inflammatory cells, stromal fibroblasts and blood vessels in advancing disease process, with grade 3 OSMF showing literally no inflammatory cells and limited number of large blood vessels in the stromal tissue, it is logical to presume that these cells have limited role in sustaining angiogenesis andtherefore, altered epithelial cells could be the only possible source of VEGF at later stages of the disease process. Results of the present study suggested that OSMF, neither as a single entity compared to normal oral mucosa nor among differing grades, had any significant difference in VEGF expression. Whereas, there was a significant increase of VEGF between OSMF with no dysplastic changes in the surface epithelium and OSMF with some degree of epithelial dysplasia, lamenting the notation that morphologically altered epithelium secretes pro-angiogenic growth factors much before invasion. This is also supported by the observation of significantly more number of blood vessels in the sub-epithelial stromal tissue of lesions with epithelial dysplasia. Similarly a marked increase of VEGF secretion was evidenced when the epithelial cells acquire epithelial-mesenchymal transition and become an invasive carcinoma.

VI. Conclusion

To conclude, the epithelial alterations in the form of dysplasia has a role in the initiation and progression of oral cancer in OSMF patients and, epithelial secreted VEGF could possibly play a pivotal role in either sustaining the existing blood vessels or stimulating angiogenesis that support tumour growth and

invasion. Conversely, the severity of the OSMF seems not to increase the risk of malignant transformation. These facts also make VEGF, a molecule to consider as an early marker of malignant transformation in OSMF patients. Finally, lack of micro-invasion/invasion in severe OSMF with its excessively hyalinised hostile stromal micro-environment warrants further investigation.

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