RESEARCH COMMUNICATION

Morphometric Analysis in Potentially Malignant Head and Neck Lesions: Oral Submucous Fibrosis

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Abstract

Objective: The objective of this study is to analyze cases of oral submucous fibrosis (OSMF) Grade I, II, III and IV morphometrically with regard to epithelium, vasculature and fibrosis and determine any correlation with histological grading after Pindborg and Sirsat. Materials and methods: Eighty three oral submucous fibrosis cases were analyzed morpometrically using an interactive image analysis system in the Department of Pathology, M.L.N Medical College, Allahabad, U.P, India. Paraffin embedded sections of 3-4 μm thickness were stained with hematoxylin/eosin, Van Gieson's picric acid and acid fuchsin stain and Masson's trichrome stains. Image analysis was performed with specific software (Image –Pro Plus 6.0) and data obtained were finally transported to Excel sheets for calculation of average values for each parameter. Results:With the grading criteria applied, 9 cases of OSMF were grade I, 32 grade II, 39 grade III and 3 grade IV. Clinical trismus was most frequent in Grade IV followed by Grade III, II and I respectively. OSMF Grade I cases did not show any measurable amount of collagenization, whereas it showed a significant increase with OSMF I and II grades [Pearson's χ 2test= 85.72; p= 0.051] and OSMF-III and IV [Pearson's χ2test=188.74; p< 0.001]. Numbers of endothelial cells per low power field consistently decreased with the increasing grade. Conclusions: We concluded that mean blood vessel area and the mean vessel diameter showed a marked increase in grade II and a marker decrease in grade IV and the grade III, collagen thickness (μm) increases according to increasing grade while density of endothelial cells decreases .

Key Words: Oral submucous fibrosis -grading - morphometric analysis

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Introduction

Oral submucous fibrosis (OSMF) is a potentially malignant condition that typically affects the buccal mucosa, lips, retro molar areas and the soft palate. It presents as a whitish-yellow discoloration involving the buccal mucosa, the oropharynx and rarely in the larynx. It has a chronic, insidious biological course (Chen et al., 2006). Out of all cancer i.e. oral cancer itself it is the sixth most common cancer in world causing nearly five percent incidence rate of all cancers world wide and in Indian subcontinent it constitutes its largest group of malignancy with an incidence rate as high as 30-40%.Over the years, the incidence of OSMF has increased manifold in India - especially among the younger generation (Gupta et al., 1998). Areca nut is the main causative factor for the development of OSMF. Commercially freeze-dried products such as paan masala, dohra, gutka and mava have a high concentration of areca nut and appear to cause OSMF (Tilakaratne et al., 2005). It is mainly seen among Asian communities, as a result of the increased popularity of the habit of chewing pan masala with areca nut, gutkha, tobacco chewing and smoking.

Over the years, the incidence of OSMF has increased manifold in various parts of the Indian subcontinent including Allahabad (Mehrotra et al., 2008). Pandya et al reported that the widespread habit of chewing dohra/paan masala is a major risk factor of OSMF, especially in the younger age group North India Allahabad and also reported that an increase in histopathological grading was found with severity and duration of addiction habit, however no significant correlation was found between clinical staging and histopathological grading (Pandya et at., 2009). Other factors, including viruses, especially human papilloma virus (HPV), may also play a role in the initiation and development of these lesions. (Chaudhary et al., 2009). Fang et al investigated that pathologic change of the microvessel in oral submucous fibrosis (OSMF) and concluded that presence of microvessel hyperplasia occurred in the early stage of OSMF (Fang et al., 2000). Gao et al suggested that the change of the epithelium in OSMF appearing in the spinous cell is specific itself (Gao et al., 1995) and another study of Gao et al also suggested that the pathological grade of OSF in between normal mucosa and mild epithelial dysplasia (Gao et al., 1992). Recently, Safwat

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et al concluded that the expression of microvascular density (MVD), vascular area ratio (VAR) and to a lesser extent vascular endothelial growth factor (VEGF) might be reference predictors for the biological behavior and prognosis of breast carcinoma (Gotoh et al., 2009; Safwat et al., 2009) Only single studies from India have been reported regarding characterization and quantification of mucosal vasculature in OSMF by image analysis (Rajendran et al., 2005). There are no studies on image analysis of OSMF regarding epithelial thickness, number of koilocytes and thickness of collagenization, blood vessels and collagen thickness in the literature.

Thus the objective of the study was to analyze cases of OSMF Grade I, II, III and IV morphometrically with regard to epithelium, vasculature and fibrosis and assess correlations with the histological grading.

Materials and Methods

A total of 83 histological sections of OSMF were analyzed morpometrically by using interactive image analysis software system (Image pro plus 6.0). Cases were diagnosed in the Department of Pathology, M.L.N Medical College, Allahabad, U.P, India. Before making the diagnosis, the grading criteria of OSMF were made which were circulated among two pathologists to avoid subjective error. We used the criteria proposed by Pindborg and Sirsat (Pindborg and Sirsat., 1966). There were 9 cases of grade I, 32 of grade II and 39 of grade III and 3 cases of grade IV. Paraffin embedded sections of 3 – 4 μm thickness were stained routinely with Hematoxylin/Eosin, Van Gieson's picric acid and acid fuchsin stain and Masson's Trichrome stains. The later two special stains impart different colours to the different connective tissue elements and made the analysis easy as well as less erroneous by highlighting the area of interest (eg. endothelial cells, collagen fibres etc.)

Image analysis (Nikon Eclipse E2 100):

Examination was under Nikon Eclipse E2 100 microscope: Nuclei were black. Muscle, cytoplasm, keratin were red while collagen appeared blue or green. Photographs were captured with the help of a camera fitted to the microscope and directly displayed on the computer monitor. Scanner (4X) was used for measurement of epithelial thickness as well as collagen thickness, low power (10X) for number of endothelial cells, number of koilocytes, area of blood vessels and the high power (40X) for measuring vessels diameter. These magnifications were changed where felt necessary. Before proceeding for morphometric analysis, these captured photomicrographs were adjusted according to their magnification with the help of the software. Then the areas of interest were selected and analyses of desired parameters were preformed.

A total of six parameters were used for each section: (1) Epithelial thickness; (2) Number of koilocytes per low power field; (3) Collagen thickness; (4) Number of endothelial cells per low power field (directly proportional to the number of blood vessels); (5) Mean blood vessel area; and (6) Average mean diameter of the blood vessels.

Data analysis

Data obtained from the analysis were finally transported to the excel sheet and the average value of each parameter was calculated. Chi square test was applied for each parameter within the grades of OSMF to judge the statistical significance of the differences in data. Finally these data were compared with the histological grading criteria used for histological diagnosis.

Results

We used the grading criteria of OSMF proposed by Pindborg and Sirsat (Pindborg and Sirsat, 1966). There were 9 cases of OSMF grade I, 32 of grade II and 39 of grade III while 3 cases of grade IV OSF. Demographic and histopathological grading distribution of patients and controls are mentioned in Table 1.

Clinically trismus was most frequent in Grade IV (100%), followed by Grade III, II and I (56.4%, 53.1% and 44.4 respectively). However, most of the patients of Grade IV and of Grade II had stage II trismus (66.6% and 47.0% respectively) and maximum patients of Grade I and Grade III had stage I trismus (50% and 45.4% respectively). Stage III trismus was most frequently observed among Grade I patients (25%) (Table 2).

The epithelial thickness did not show any consistent result (increase/ decrease) with the grades of OSMF- the epithelium may be hyperplastic or atrophic in any grades of OSMF; however all the cases of grade IV showed atrophy (least thickness). The number of koilocytes (see Figure 1) per low power field found to be gradually increasing with increasing grades, these differences were highly statistically significant ($p<0.001$).

Grade I cases did not show any measurable amount in case of collagenization. In others hand, comprising the six morphometrical parameter, it showed a significant increase with OSMF I and II grades (Pearson's $\chi 2$test= 85.725; p= 0.051). Number of endothelial cells per low power field consistently decreased with the increasing grades of OSMF.

Within the grades, grade I and grade II OSMF cases

Table 1. Demographic and Histopathological Grading Distribution

Diagnosis	Number	Age (Yrs)		Sex	
		Mean	Range	Male	Female
Normal	85 (100)	30	18-42	63 (74.2)	22 (25.0)
OSMF					
Grade I	9 (10.8)	25	20-30	6 (66.7)	3 (33.3)
Grade II	32 (38.6)	28	22-34	22 (68.5)	10 (31.5)
Grade III	39 (46.9)	30	21-39	28 (72.0)	11 (82.0)
Grade IV	3 (3.6)	36	24-48	2 (66.7)	1 (33.3)

Table 2. OSMF Grading and Clinical Staging of Trismus

	Total (%)	Trismus present (%)	Clinical staging		
			I	II	III
Grade I	9 (10.8)	4 (44.4)	2 (50.0)	1 (25.0)	1 (25.0)
Grade II	32 (38.6)	17 (53.1)	6 (35.3)	8 (47.0)	3 (17.6)
Grade III	39 (46.9)	22 (56.4)	10 (45.4)	8 (36.3)	4 (18.1)
Grade IV	3 (3.6)	3 (100)	1 (33.3)	2 (66.6)	0 (0.0)

Table 3. Morphometric Analysis of Different Grades of OSMF in Different Parameters

Parameter OSMF	I (9)	II (32)	III (39)	IV (3)
Koilocytes (no/lpf)	50.0	68.5	72.3	99.0
Collagen thickness (μm)	10.0	419.9	519.6	594.8
Endothelial cells (no/lpf)	409.2	252.6	208.7	85.7
Blood vessel Area (Sq.μm)	33.6	575.2	161.0	7.8
Average mean diameter (μm)	5.6	13.0	8.5	1.0

Fisher exact test 2x5 Table; between different morphometric parameter of OSMF I and II grades; Chi squares: Pearson's= 85.725 (p= 0.051) and Likelihood Ratio= 48.426 (p= 0.06) and OSMF III and IV grades Chi squares: Pearson's= 188.7 (p= 0.000) Likelihood Ratio= 220.923 (p= 0.000)

showed significant differences (p= 0.00), while grade III and grade IV showed a highly significant statistical difference [Pearson's _2test=188.748; p= 0.000]. As the number of blood vessels are directly proportional to the number of endothelial cells, these findings can also be expected for the number of blood vessels [Table 3 and Figure 2].

Discussion

In the present study the number of koilocytes per low power field found to be gradually increasing with increasing grades and these differences showed that statistically significant (P=0.00). That means the human papilloma vius (HPV) viral load may correlate with the disease progression mentioned in Table 1. Rather, it may be the type of the HPV (high risk or low risk) that can contribute to its progression to increasing the grading.

Collagen thickness (μm) increases according to increasing grading OSMF-I =10.00780; OSMF II= 419.97006; OSMF-III=519.6797; OSMF-IV=594.80089). In others it showed a proportionate increase with OSMF grades. Pindborg and Sirsat also described the very early stages by presence of fine fibrillar collagen dispersed with marked oedema with increasing thickness in higher grades of disease (Pindborg and Sirsat., 1966). Huang et al reported that accumulation of collagen fibers increases with the severity of the disease (Huang and Shieh., 1989). Number of endothelial cells per low power field consistently decreased with the increasing grades of OSMF mentioned in Table 3. As the number of blood vessels are directly proportional to the number of endothelial cells, these findings can also be expected for the number of blood vessels. The mean blood vessel area and the mean vessel diameter showed a marked increase in grade II and a marker decrease in grade IV and the grade III. The luminal diameter in grade IV showed near obliteration of the lumen. These findings are similar to the study results of Fang et al who showed the increase in microvessel quantity and quantity density in the early stage and the decrease in micro-vessel quantity, density, micro-vessel area and area density in the middle stage and the late stage. The authors concluded that presence of micro-vessel hyperplasia occurred in the early stage of OSMF (Fang et al., 2000).

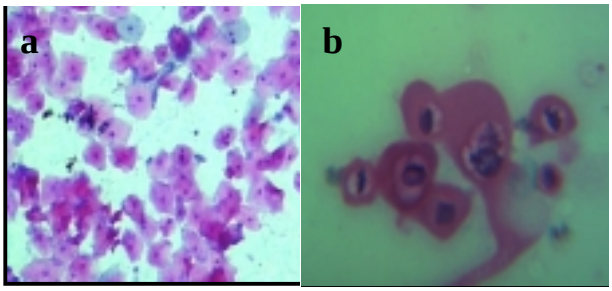

Figure 1. a) Benign Koilocytic Cells and b) Malignant Squamous Cells with Koilocytic Change

The above findings differ very much rather just an opposite to that of Rajendran et al who assessed quantitatively the mucosal vascularity in OSMF by image analysis. They found the mean vascular density to be more or less same in the test and control samples (F = 0.82, P>0.05). The mean vascular percentage area and the mean vascular luminal diameter showed an increasing trend as the disease progresses (F = 8.63, p<0.01 and F = 34.1, p<0.001 respectively). They concluded that the mean vascular dilatation occurred as a result of adaptive response to compensate tissue ischaemia/hypoxia (Rajendran et al., 2005).

Overlying epithelium is either hyperplastic or atrophic regardless of the grades of OSMF. However, atrophy is most frequent in grade IV. Although HPV may have a role in pathogenesis of OSMF, the viral load may correlate with the disease progression. It may be the type of the virus (high risk or low risk) that can contribute to its progression. Therefore, the common belief that trismus occurs due to increase in sub epithelial collagen deposition. It probably depends on the invasion of the muscle fibres by collagen (i.e. muscle to collagen ratio) rather than the simple increase in collagen thickness. At the later

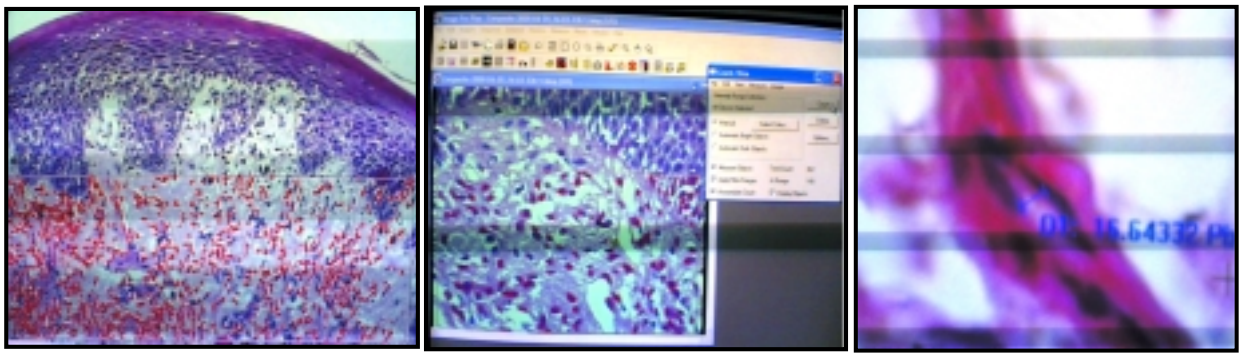

Figure 2. Morphometric Analysis of OSMF Cases. a) Endothelial cells in the sub epithelial zone were selected (red colour) and their numbers were counted with the help of software Image pro plus 6.0; b) Endothelial cell and vascular dilatation; and c) Collagenization and decrease in endothelial cells

advanced stages probably these mechanisms become decompensated due to persistent insults resulting in constriction or obliteration of the blood vessels along with decrease in their number.

We concluded that mean blood vessel area and the mean vessel diameter showed a marked increase in grade II and a marker decrease in grade IV and the grade III, collagen thickness (μm) increases according to increasing grading OSMF and number of endothelial cells per low power field consistently decreased with the increasing grades of OSMF.

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