

Malignant potentiality assessment of oral submucous fibrosis through semi-quantitative approach

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Abstract

Background: In the context of early diagnosis and prevention of oral cancer, precise assessment of malignant potentiality of the oral potentially malignant disorders, particularly oral submucous fibrosis (OSF) is crucial. Till date, the assessment of malignant potentiality suffers from predictive ambiguity due to the lack of precision in the gold standard techniques. This can be addressed by integrating heuristic domain knowledge with quantitative analysis.

Aim: The aim of this study is to propose an index for enhancing accuracy in malignant potentiality evaluation.

Materials and Methods: The present study analyzes important histomorphometric attributes (epithelial thickness, basal cell nuclear size, nuclear-to-cytoplasmic area ratio of basal cells, chromaticity of basal cell nucleus, thickness of basement membrane, ratio of vasculature in juxta-epithelial connective tissue [i.e., area covered by blood vessels/total area], collagen density in the lamina propria) of oral mucosa in dysplastic and nondysplastic OSF in association with relevant oncopathological appreciations (weightage of different features as suggested by oral pathologists) toward proposing a “Malignant Potentiality Index” (MPI).

Results: Analysis of variance and notch box plot analysis depict statistically significant differences ($P < 0.0001$) in the histopathological features among different study groups (normal oral mucosa, OSF without dysplasia, OSF with dysplasia). Histopathological observation of one OSF patient with calculated MPI is shown.

Conclusion: This newly proposed diagnostic cum prognostic decision-making parameter, the “MPI” may bring a value addition to the conventional diagnostic gold standard.

Keywords: Histomorphometry, malignant potentiality index, oral submucous fibrosis, weightage of features

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INTRODUCTION

Globally, oral cancer (OC) is the 11th most common cancer in the world.^[1] According to the International Agency for

Cancer Research, deaths due to this dreaded disease rank sixth worldwide. Two-thirds of the global incidences of OC s are recorded in low- and middle-income countries,

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and India, in particular, has the highest incidence of OC in the world.^[2]

OC usually develops from preexisting oral potentially malignant disorders (OPMDs) and its high incidence is primarily due to late diagnosis and failure of assessing the malignant potentiality of these OPMDs.^[3] The common oral premalignant disorders are leukoplakia, erythroplakia and oral submucous fibrosis (OSF). Oral leukoplakia affects people across the globe, while OSF is primarily prevalent in the Indian subcontinent and etiologically linked to oral habits such as chewing of betel nut and allied tobacco products.^[4] It has also been reported that OSF is becoming epidemic in India.^[1] The OSF being an insidious, chronic, progressive scarring premalignant disorder of the oral cavity and oropharynx, progresses into OC in a significant number of cases.^[5] Hence, the proper assessment of the malignant potentiality of this disease process at an early stage is very important to combat the incidence of OC.

The clinical features of OSF include discomfort, burning sensation, pain; firm and coarse oral mucosa, depapillation of the tongue, recurrent patchy ulcerations as well as varying degrees of trismus.^[6] Histopathologically, this disease is usually characterized by hyalinized, hypovascular connective tissue along with atrophic overlying surface epithelium, which may reveal variable degrees of dysplasia. Oral histopathologists endorse certain light microscopic attributes in the “gold standard” technique for the diagnosis of premalignant nature of OSF and its progression toward malignancy. This process of qualitative evaluation is primarily subjective and not reproducible as it differs according to pathologist’s acumen.^[7]

As heuristic-based clinical acumen should be given due importance, the present study has been conducted involving both semiquantitative/quantitative histomorphometric attributes of OSF biopsied tissues by considering oral oncopathologists’ empirical domain knowledge in developing a kind of integrated malignant potentiality assessment of this oral premalignant disorder.

MATERIALS AND METHODS

A total of 36 stained OSF sections were collected among which 10 slides belonged to OSF with dysplasia (OSFWD), and 26 were OSF without dysplasia (OSFWTD). The control group (NOM) consisted of 10 tissue specimens that were obtained from surgically excised buccal mucosa during transalveolar extraction of impacted teeth. Biopsies were performed with the informed consent of the patients and with proper ethical approval (GNIDSR/IEC/07/16). All patients had the habit of chewing betel quid and/or areca nut and had characteristic clinicopathological symptoms of OSF.

Tissue processing

Hematoxylin and eosin staining and evaluation

All the above biopsy samples were fixed in 10% phosphate-buffered formalin and subsequently processed for paraffin block preparation. Paraffin blocks were microtomed (LEICA RM 2125 RT) to get 4–5 μ m thick tissue sections on albumin (chickenegg) coated glass slides. The slides containing tissue sections were air-dried overnight. After that, the sections were deparaffinated by 1020 min of xylene treatment and subsequently processed for hematoxylin and eosin (H&E) staining with Harris’ Hematoxylin (Merck, India, Catalog No. 61593800051730) and counterstained with Eosin yellowish (Merck, India Catalog No 60134500251046) [Figure 1].

Van Gieson’s staining

Dewaxed and hydrated tissue sections were first stained with hematoxylin (Hematoxylin-Merck, India, Catalog, No. 61593800051730) and then dipped in 9:1 saturated picric acid: acid fuchsin (1%) for Van Gieson’s (VG) staining [Figure 2].

Periodic acid–Schiff staining

Deparaffinized and rehydrated tissue sections were stained with periodic acid for 10 min and then treated with Schiff’s reagent (Merck, India, Catalog No. 1090330500) in the dark for 5 min. Then, the sections were stained with hematoxylin, dehydrated and mounted [Figure 3].

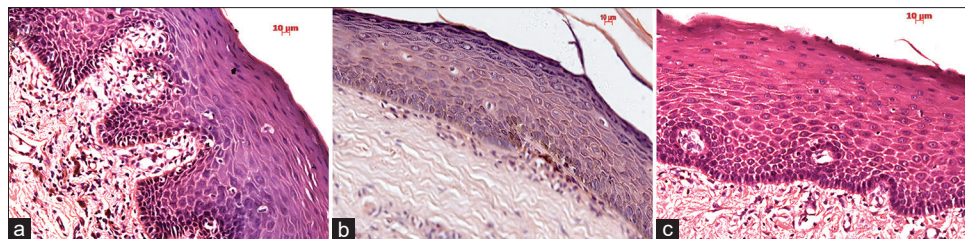


Figure 1: Microphotograph ($\times 200$) of H&E staining of different study groups: (a) normal oral mucosa; (b) oral submucous fibrosis without dysplasia; (c) oral sub-mucous fibrosis with dysplasia

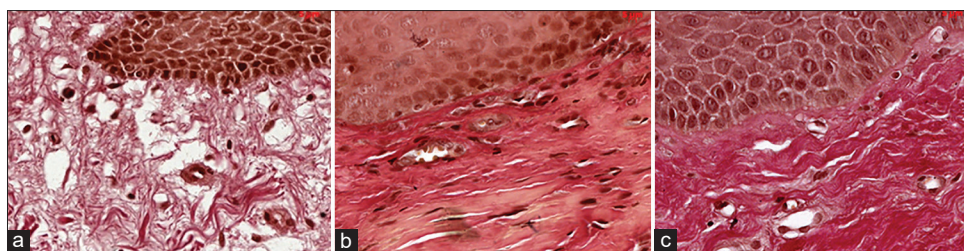


Figure 2: Microphotograph (×400) of Van Gieson's staining of different study groups: (a) normal oral mucosa; (b) oral submucous fibrosis without dysplasia; (c) oral submucous fibrosis with dysplasia

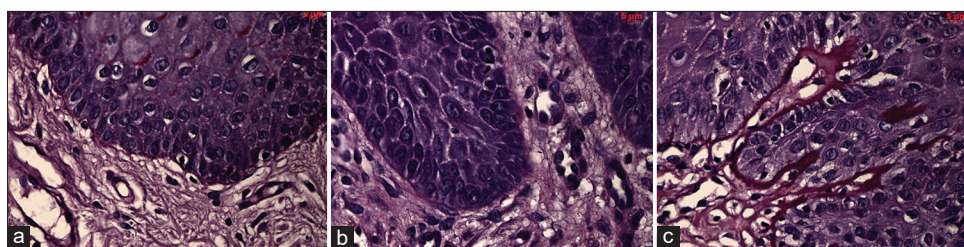


Figure 3: Microphotograph (×400) of periodic acid-Schiff staining of different study groups: (a) normal oral mucosa; (b) oral submucous fibrosis without dysplasia; (c) oral submucous fibrosis with dysplasia

Microscopic imaging

The light microscopic images were grabbed digitally using Zeiss Observer. Z1 Microscope (Carl Zeiss, Germany) associated with CCD camera (AxioCam MRC, Carl Zeiss, Germany) at 1388 × 1040 pixels under various magnifications (epithelial thickness at ×100, basal cell size-×400, ratio of vasculature-×200, collagen intensity in subepithelial connective tissue and basement membrane-×400).

Extraction of histomorphometric attributes from microscopic images

The quantitative dimension of histomorphometric features (epithelial thickness, basal cell nuclear size, nuclear-to-cytoplasmic area ratio of basal cells, chromaticity of basal cell nucleus, thickness of basement membrane, ratio of vasculature in juxta-epithelial connective tissue, collagen density in lamina propria [i.e., VG intensity-I1, I2, I3; where I1 denotes VG staining intensity at the epithelial-mesenchymal junction, I2 and I3 were measured at points 50 μm and 100 μm below the junction, respectively.]) pertinent to malignant potentiality have been extracted from the histological images. The first six features except chromaticity were extracted by AxioVision Rel. 4.7, Carl Zeiss. For chromaticity, HE images were converted to grayscale, and the average grayscale intensity of nuclei was extracted using Matlab R2014a. Higher chromaticity is implied by lower grayscale intensity. Visual analog scoring (0–10 scale) system was used for determining VG staining intensity of collagen.

Statistical analysis

Statistical analysis, i.e., analysis of variance and notch box plot were performed using Excel 2007 and MATLAB R2014a software.

Calculation of malignant potentiality index

All the above-mentioned histopathological features were assigned a weightage in 1–10 scale according to their significance in disease progression and malignant potentiality [Table 1]. The upper and lower boundaries of each feature were determined from the data of OSF patients. Max = median + standard deviation and Min = median – standard deviation.

To calculate the malignant potentiality index (MPI) of a patient, the average values of above-mentioned features were noted. In transforming all variables in the data to a specific range, these values were normalized to 0–10 scale, i.e., $\bar{x}_i = \{(x_i - a_i^{\min}) / (a_i^{\max} - a_i^{\min})\} * 10 \dots\dots\dots (i)$. (where \bar{x}_i = Normalized value of feature x_i , a_i^{\max} = Max value and a_i^{\min} = minimum value of feature x_i).

However, for certain features, namely, the chromaticity of the nucleus, since the lower numerical value indicates more severity of disease, normalized values of these features are subtracted from 10, i.e.,

$$\bar{x}_i = 10 - \{ \{ (x_i - a_i^{\min}) / (a_i^{\max} - a_i^{\min}) \} * 10 \}$$

$$\text{Thus, the calculated MPI} = \sum_{i=1}^9 w_i \bar{x}_i \dots\dots\dots (ii)$$

(\bar{x}_i) = Normalized value of histological feature x_i ; w_i = Weightage of the corresponding histological feature.

RESULTS

Quantitative analysis of histopathological images

Epithelium

Quantitative evaluation of the epithelial atrophic status revealed that the epithelial thickness was significantly decreased ($F = 210.7$ and $P < 0.0001$) in nondysplastic OSF tissues ($143.17 \mu\text{m} \pm 31.45$) than normal ($338.305 \mu\text{m} \pm 90.369$) and dysplastic ones ($296.12 \mu\text{m} \pm 64.68$) [Table 2 and Figure 4]. The basal cells in normal and OSF tissues were analyzed with respect to the total cell area, nuclear area, cytoplasmic area, nuclear-to-cytoplasmic ratio and chromaticity. The nuclear area in the dysplastic tissues ($52.58 \mu\text{m}^2 \pm 15.37$) was increased significantly (F value was 115.4 and $P < 0.0001$) in relation to normal ($34.31 \mu\text{m}^2 \pm 7.08$) and nondysplastic ones ($38.10 \mu\text{m}^2 \pm 11.96$) [Table 2 and Figure 4]. A significant increase (F value was 90.29 and $P < 0.0001$) in the nuclear-to-cytoplasmic ratio was recorded in dysplastic OSF tissues (1.24 ± 0.39) in comparison to normal (0.92 ± 0.28) and nondysplastic (0.74 ± 0.26) states [Table 2 and Figure 4]. While recording the chromaticity in normal and OSF tissues, there was a significant increase (F value of 1862 and $P < 0.0001$) of grayscale intensity of nucleus (reciprocal of chromaticity) in the OSF without epithelial dysplasia (105.92 ± 23.61)

in comparison to normal (80.97 ± 15.79) and dysplastic tissue (54.32 ± 15.50) [Table 2 and Figure 4].

Basement membrane

The thickness of basement membrane assessed through quantization revealed a considerable increase in the thickness in OSFWD ($4.05 \mu\text{m} \pm 1.58$) than normal ($1.42 \mu\text{m} \pm 0.46$) and OSFWTD ($2.16 \mu\text{m} \pm 0.70$) [Table 2 and Figure 4]. Statistical evaluation revealed significant results (“ F ” = 813.8 and $P < 0.0001$).

Connective tissue

The ratio of vasculature (i.e., area covered by blood vessels: Total area of juxta-epithelial connective tissue as assessed from H&E images) was increased significantly in OSF tissues with dysplasia (0.16 ± 0.02) than normal (0.13 ± 0.02) and OSFWTD (0.09 ± 0.02) [Table 2 and Figure 4]. Corresponding “ F ” value was 52.68 and $P < 0.0001$.

Furthermore, to evaluate the juxta-epithelial connective tissue quantitatively as per VG intensity, maximum intensity was noted at the epithelial-mesenchymal junction of OSF tissues with dysplasia which gradually decreased as we moved deeper (I1- 8.8 ± 0.4 ; I2- 8.55 ± 0.63 ; I3- 8.42 ± 0.78) than OSFWTD (I1- 7.32 ± 1.02 ; I2- 7.07 ± 1.37 ; I3- 6.62 ± 1.52). Interestingly, reverse findings were recorded in normal subjects, with maximum intensity in the deeper layers (I1 - 3.6 ± 0.98 ; I2 - 4.5 ± 1.18 ; I3 - 4.75 ± 1.37) [Table 2]. The statistical evaluation revealed “ F ” value for I1, I2 and I3 to be 128.9 , 186.5 and 121.9 , respectively, and $P < 0.0001$.

Table 1: Histopathological features with assigned weightage

Chromaticity of nucleus-10 implies feature named Chromaticity of nucleus is assigned a weightage of 10	Ratio of vasculature in connective tissue (area covered by blood vessels/total area)-5 implies 5 is assigned to this feature
Basal cell nuclear area-9.5	Epithelial thickness-4
Nuclear area/cytoplasm area-9	Collagen density I2 (VG intensity at 50 μm below the junction)-2
Collagen density I1 (VG intensity at the epithelialmesenchymal junction)-7	Collagen density I3 (VG intensity at 100 μm below the junction)-1
Thickness of basement membrane-6	

VG: Van Gieson’s

Table 2: Statistical significance test of the histopathological features among different study groups

Features	NOM	OSFWTD	OSFWD	ANOVA	
				F	P
Epithelial thickness (μm)	338.30±90.37	143.17±31.4	296.12±64.68	210.7	<0.0001
Basal cell nuclear area (μm^2)	34.31±7.08	38.10±11.96	52.58±15.37	115.4	<0.0001
Nuclear area/cytoplasm area	0.92±0.28	0.74±0.26	1.24±0.39	90.29	<0.0001
Grayscale intensity of nucleus (reciprocal of chromaticity)	80.97±15.79	105.92±23.61	54.32±15.50	1862	<0.0001
Basement membrane thickness (μm)	1.42±0.46	2.16±0.70	4.05±1.58	813.8	<0.0001
Ratio of vasculature in connective tissue (area covered by blood vessels/total area)	0.13±0.02	0.09±0.02	0.16±0.02	52.68	<0.0001
Collagen density I1 (VG intensity at the epithelialmesenchymal junction)	3.6±0.98	7.32±1.02	8.8±0.40	128.9	<0.0001
Collagen density I2 (VG intensity at 50 μm below the junction)	4.5±1.18	7.07±1.37	8.55±0.63	186.5	<0.0001
Collagen density I3 (VG intensity at 100 μm below the junction)	4.75±1.37	6.62±1.52	8.42±0.78	121.9	<0.0001

NOM: Normal oral mucosa, OSF: Oral Submucous fibrosis, OSFWTD: OSF without dysplasia, OSFWD: OSF with dysplasia, ANOVA: Analysis of variance, VG: Van Gieson’s

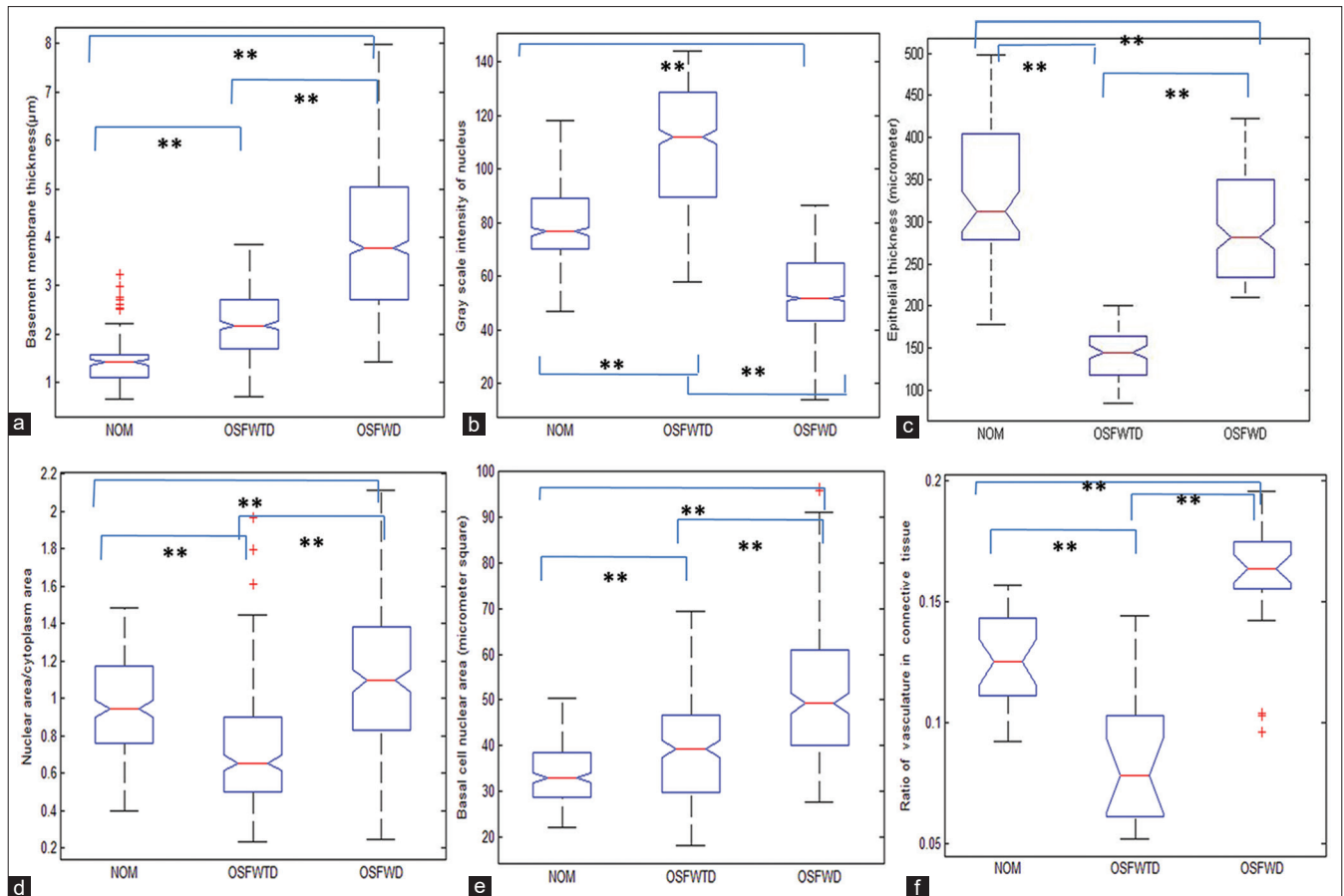


Figure 4: Notch box plot analysis of histopathology features among different study group (normal oral mucosa, oral submucous fibrosis without dysplasia and oral submucous fibrosis with dysplasia): (a) basement membrane thickness; (b) grayscale intensity of nucleus; (c) epithelial thickness; (d) ratio of nuclear area and cytoplasmic area; (e) basal cell nuclear area; (f) ratio of vasculature in connective tissue; Significance between groups: * $P > 0.0001$, ** $P < 0.0001$

DISCUSSION

Improvement of diagnostic accuracy for assessing malignant potentiality of oral premalignant disorders particularly OSF is of paramount importance to combat OC in the Indian subcontinent. Qualitative microscopic analysis of cellular and nuclear features introduces an ambiguity in the conventional process regarding the assessment of the progression of OPMDs. On the other hand, experience-based medical domain knowledge cannot be ignored as it embeds huge vital information in the context of appreciating pathological discriminatory attributes of the target lesion. Hence, the amalgamation of clinical acumen with quantitative histological information will be valuable as has been done in this study.

The biological changes in the basal cells of oral surface epithelial tissue have potent implications in various disease process including OPMDs such as OSF.^[8] Increase in the size of basal cell nuclei, alteration in the nuclear-to-cytoplasmic area ratio are regarded as cardinal

qualitative features in epithelial dysplasia^[9] and the quantitative analysis in the present study also demonstrate an increase of same in dysplastic OSF in comparison to normal and nondysplastic status. Thickness of the surface epithelium has decreased in nondysplastic OSF than normal, but it has increased in dysplastic condition than without dysplasia. Such findings are also corroborative with the related qualitative analysis depicting the increase in thickness of surface epithelium due to hyperplasia in premalignant disorder. Hyperchromatism of the basal cell nuclei is another important qualitative parameter. In the present work, it has been observed that there is increase in the grayscale intensity in nondysplastic OSF tissues as compared to normal whereas there is a decrease in the grayscale intensity in dysplastic conditions indicating a definite increase in chromaticity in OSFWD.

Interestingly, significant increased thickness of the basement membrane in dysplastic status compared to the other two groups as noted in this study indicates a potential protective biological role of the basement

membrane against the progression of dysplastic OSF into frank squamous cell carcinoma. Further, neoangiogenesis characterized by microcapillary proliferation has been regarded as a qualitative observation in premalignant and malignant disorders.^[10] The present study has quantitatively demonstrated reduced vasculature in nondysplastic OSF as compared to normal but a significant increase of the same in dysplastic conditions. Thus, this quantitative finding may reflect the change in the oral mucosa which is conducive for the transformation of precancer into malignancy. Increased hyalinization *vis-a-vis* increase in collagen densities, especially at the epithelial-mesenchymal junction, is a qualitative biological indicator for the progression of OSF. Increased collagen density in dysplastic OSF tissues as compared to the normal and nondysplastic state may have a crucial biological impact in assessing the chances of dysplastic progression *vis-a-vis* malignancy, as this change indicates a plausible increase in subepithelial connective tissue stiffness which has a significant connotation with pro-metastatic transformation.^[11]

CONCLUSION

The proposed MPI is likely to add value to the existing gold standard by reducing predictive ambiguities regarding the assessment of the malignant potentiality of OSF. This proposition of MPI can be applicable for other OPMDs also. Moreover, the present concept of calculation of MPI can further be enriched with the incorporation of extensive clinicopathological and molecular attributes.

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Conflicts of interest

There are no conflicts of interest.

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