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Assessment of Tumor Cell Morphology and Peri-Tumoral Stromal Changes in Oral Squamous Cell Carcinoma

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Abstract

Background: *Oral squamous cell carcinomas are tumors with dissimilar behavioural patterns. Several cellular and extracellular changes take place throughout the process of carcinogenesis, which convey diagnostic and prognostic significance. This is an attempt to recognize significant indicators that convey the impending course of the tumor.*

Aims: *To evaluate the alteration in the morphology of tumor cells and the stromal changes occurring in OSCC. To correlate these features with clinicopathological variables of each case*

Material and Methods: A total of 47 cases were included in the study. Oral squamous cell carcinomas (n=27), Leukoplakia with dysplasia (n=10), oral submucous fibrosis (n=10) cases were selected. 3 morphometrical parameters were assessed namely nuclear area (NA), cell area (CA) and their ratio (NA/CA). In addition, the birefringence patterns of collagen fibers in the peritumoralstromawere assessed.

Results: *Nuclear area (P=.014) and nuclear to cell area ratio (P<.0001)showed statistically significant increase from dysplasia to increasing grades of OSCC. Collagen fibres showed a higher preponderance of the orange red (OR) birefringence in the well (66.6%) and moderately differentiated (77.7%) OSCC cases. Among the poorly differentiated OSCC cases, green yellow (GY) birefringence predominated.Follow up analysis was done.*

Conclusion: *Substantial nuclear and cellular morphometric alterations occur in tumor cells and in the peri-tumoralstroma during the advancing grades of oral squamous cell carcinoma. Morphometrical analysis of nuclear area and nuclear to cell area in association with birefringence patterns of stromal collagen fibres, can serve as an adjunct for more efficient prognostication of OSCC.*

Key words: *Oral squamous cell carcinoma (OSCC), morphometry, image analysis, collagen, picrosirius red stain, birefringence, polarising microscopy.*

INTRODUCTION

Accounting for 90% of all the oral malignancies, oral squamous cell carcinoma (OSCC) has been established as the most common type of malignancy of the oral cavity.Despite the therapeutic advances, the 5 year survival rate is about 50 % and continues to stand poor [(1)]. Furthermore, the malignancy frequently remains overlooked during its initial stages. This may be due to patients' negligence owing to its inconspicuous progression, or due to its inappropriate diagnostic and prognostic assessment at the time of initial presentation. The latter may be a result of insufficient accuracy of the techniques employed for the same. This encourages further research on the factors that play a prominent role in modifying the disease outcome. Unambiguously, early detection of oral OSCC is imperative in bringing down the high morbidity and mortality rate associated with it(1).This can be a daunting task as OSCCs are tumors which boast dissimilar behavioural patterns. However, the assessment can be simplified by focussing on the two discrete sections that constitute as well as display major variations in OSCC, i.e. the malignant epithelial cells and the stroma in which they reside[(2)].

Regarding the cellular changes, considerable disparity persists among pathologists in terms of subjective routine histopathological evaluation. To resolve this, computer-assisted morphometry can be used for a more accurate and reliable quantification of the nuclear and cellular changes in the advancing stages of OSCC [(3 - 6)].In addition, the changes in the tumorstroma are also strongly associated with the tumor progression. Research indicates that biological aggressiveness of OSCC may be influenced by the reactive changes in the collagen fibres of the tumorstroma [(7,8)]. The crucial changes in the collagenous material can be specifically and reliably determined by employing picrosirius red stain along with polarised microscopy [(9,10)].

Assessing thesefundamental changes displayed by the epithelial cells and the peri-tumoralstroma can

help in more efficient diagnosis and management of the disease, along with a more accurate assessment of the patient prognosis. This study attempts to quantitate the nuclear and cellular morphological features in various grades of OSCC using computer- aided image analysis. In addition, the changes in the collagen fibres in the peri-tumoralstroma have also been analysed using Picrosirius red stain. The purpose of this study isto interpret an association of morphological changes occurring in tumor cells and the changes in the peritumoral stromal tissues during the progression of the tumor and to apply this awareness in individualized treatment planning and in prognostication.

MATERIALS AND METHODS

Following the approval from the Institutional EthicsCommittee (IEC444/2013), 47formalin fixed paraffin embedded tissue blocks were retrieved from the departmental archives, which consisted of9 cases each of well, moderate and poorly differentiated histopathologically diagnosed cases of oral squamous cell carcinomas, 10 cases of leukoplakia with dysplasia and 10 cases of oral submucous fibrosis as controls. The clinicopathological and follow up details were retrieved from the medical records files of the patients.

Study design: A retrospective cohort study. This is a pilot study and additional studies using morphometry along with picrosirius red stain can be tried on more number of patients with oral squamous cell carcinoma to further reinforce the findings.

Morphometrical analysis: Fresh sections of 5 µm thickness were cut using a soft tissue microtome from the formalin-fixed, paraffin-embedded tissue blocks of OSCC cases and dysplastic cases. The sections obtained were stained with Harris's Hematoxylin and Eosin and morphometrically analysed. Morphometrical parameters considered: Nuclear area (NA), cell area (CA) and nuclear area-cell area ratio (NA/CA).

The scale for morphometrical analysis was standardized using an eye piece graticule and a stage grid in 40X magnification.For each case, pictures of 3 fields were taken under 40X magnification.Ten clearly defined cells were analyzed from each field. Image analysis was done using – "Image J 1.34 software" available at website: http://rsb.info.nih.gov/ji/.**The**images were captured onto the hard drive of the computer, following which they could be opened in ImageJ for evaluation, using the various tools provided in the panel.The software compiles all the data into Microsoft Excel worksheets to give a summated set of results for a single slide.

Picrosirius red staining: Sections of 5µm thickness were cut using a soft tissue microtome from formalin-fixed, paraffin-embedded tissue blocks of the OSCC cases and oral submucous fibrosis cases. The sections were stained with the modified Picrosirius red stain. Following deparaffinization and hydration in distilled water; the sections were incubated in 0.1% (w/v) Sirius red F3B (C.I.35780) in saturated Picric acid solution for 1 hour at room temperature. This was

followed by rinsing with distilled water, staining with Mayer's haematoxylin, differentiation in 1% HCl, alkalinisation with tap water, dehydration and mounting with Dibutylphathalate xylene (DPX). The sections were examined under 40x magnification using Olympus polarized microscope [(11)].Color change and content of birefringence of the collagen fibres, both were analyzed in this study.

Statistical analysis: Statistical analysis was carried out using the SPSS package (version 15.0). One- way ANOVA (Analysis of Variance) was used for comparing the parameters for multiple groups. Comparison of the mean nuclear and cellular area and diameter values between groups was made using multiple comparison test by Tukey-HSD procedure. The results were considered significant when *P*-value <0.05.

RESULTS

Nuclear area: The mean nuclear area of well differentiated OSCC was found to be 226.22μ^2 and the value showed progressive rise along the increasing grades of OSCC, being the highest in poorly differentiated OSCC which is 260.11μ^2(P=.014). The increase in the nuclear area was found to be statistically significant with the p-value being .014. (Table 1).

Cell area: The mean cell area of well differentiated OSCC was found to be 452.00 μ^2 which decreased progressively from along the increasing grades of OSCC, being the lowest in poorly differentiated OSCC. The increase in the nuclear area was found to be statistically insignificant. (P-value 0.68) (Table 1)

Nuclear area to cytoplasmic area ratio (NA/CA): The mean NA/CA of well differentiated OSCC was found to be 0.50 which showed an increase along the progressing grades of OSCC, being the highest i.e. 0.63 in poorly differentiated OSCC. The increase was found to be highly significant with the P<.0001. (Table 1)

Picrosirius red staining: The birefringence colors as seen under polarising microscope were classified as orange - red (OR), yellow - orange (YO) and green - yellow (GY). On analysing the variation in the colors of birefringence among the different grades of OSCC, it was observed that 6 out of 9, i.e. 66.6% cases of well differentiated OSCC, showed OR birefringence and remaining all the cases had YO birefringence. Among moderately differentiated OSCC cases, 7 out of 9, i.e. 77.7% showed OR birefringence and rest of the cases had YO birefringence. None of the well and moderately differentiated OSCC cases had GY birefringence (Fig. 2a, 2b). On the other hand, majority of the poorly differentiated OSCC cases, i.e. 4 out of 9 (44.4%)cases showed GY birefringence (Fig. 2c). 22% poorly differentiated OSCC had OR birefringence and 33% had YO birefringence. The OSCC values were also compared with 10 OSMF cases used as controls, since the condition discretely comprises of the changes involving collagen in the connective tissue. The OSMF cases primarily showed OR and YO birefringence (Fig. 3). Table 2 gives the details of the color changes observed among the

cases. The content of picrosirius red staining was also studied under polarizing microscope and categorized as: low, moderate and high, depending on the extent of areas demonstrating birefringence under the field of 4x magnification. Out of 9 well differentiated OSCC cases, 4 had low and 5 cases had moderate content. Among moderately differentiated OSCC cases, 5 had low content, 2 had moderate and 2 showed high content. 5 of the 9 cases of poorly differentiated OSCC showed low content while the rest had moderate content. In comparison, out of 10 OSMF cases, which formed the control group, majority of the cases showed high content. (Table 3)

Table 1: Comparison of the morphometrical variations

Criteria	**Grade of OSCC**	**Mean**	**Standard deviation**	**P-value**
Nuclear Area	Dysplasia	157	13.34	.014 a<b b<c c<d
	Well differentiated OSCC	226	76.80	
	Moderately differentiated OSCC	243	36.83	
	Poorly differentiated OSCC	259	52.04	
Cell Area	Dysplasia	462	88.13	.68
	Well differentiated OSCC	452	126.84	
	Moderately differentiated OSCC	428	70.9	
	Poorly differentiated OSCC	411	100.09	
Nuclear Area/Cell Area	Dysplasia	.30	.04	.000 a<b b<c c<d
	Well differentiated OSCC	.50	.116	
	Moderately differentiated OSCC	.57	.111	
	Poorly differentiated OSCC	.6	.094	

With respect to Post Hoc analysis: **a=Dysplasia, b=Well differentiated OSCC, c=Moderately differentiated OSCC, d=Poorly differentiated OSCC**

Table 2: Variation in the color of picrosirius birefringence

Categories	**Green-yellow (GY)**	**Yellow-orange (YO)**	**Orange-red (OR)**
OSMF (n=10)	1	7	2
Well differentiated (n=9)		3	6
Moderately differentiated (n=9)		2	7
Poorly differentiated (n=9)	4	3	2

Table 3: Variation in the content of picrosirius birefringence

	Low	**Moderate**	**High**
OSMF (n=9)		4	6
Well differentiated (n=9)	4	5	
Moderately differentiated (n=9)	5	2	2
Poorly differentiated (n=9)	5	4	

Table 4: Correlation of the color and content of birefringence with the recurrence period

Grade of OSCC	**Morphometry**	**Content of picrosirius red stain**	**Color of birefringence of the stain**	**Recurrence period**
Case 1 (well differentiated OSCC)	NA=152.86 CA=358.66 NA/CA=.42	Low	Reddish orange	15 months

Case 2 (moderately differentiated OSCC)	NA=230.15 CA=373.54 NA/CA=.61	Moderate	Reddish orange	6 months
Case 3 (moderately differentiated OSCC)	NA=301.27 CA=502.99 NA/CA=.59	Moderate	Yellow orange	4 years

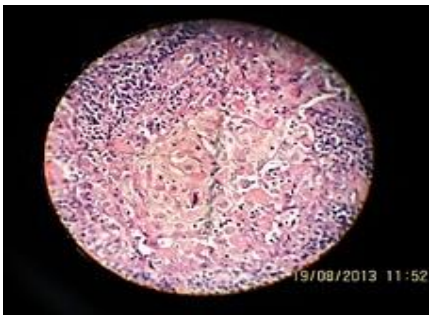

Figure 1a: Calibration using an eye piece graticule and a stage grid in 40X magnification.

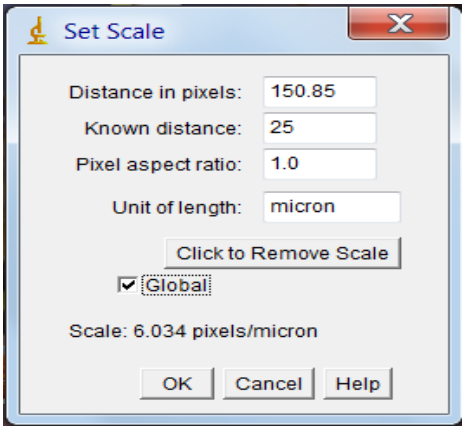

Figure 1b: Standardisation (1pixel=6.034micron)

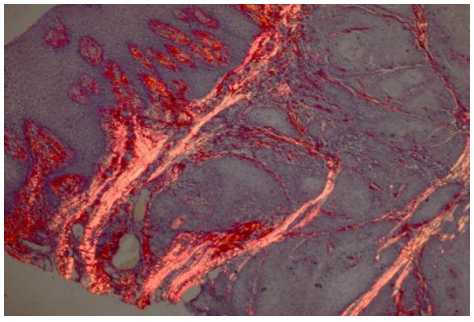

Figure 2a: Photomicrograph showing red-orange (RO) birefringence around tumor islands in well differentiated OSCC. [Picrosirius red stain, 4X

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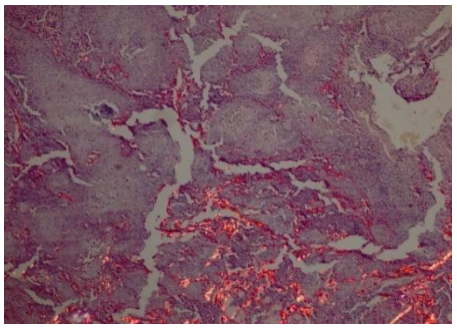

Figure 2b: Photomicrograph showing yellow orange (YO) birefringence around tumor islands in moderately differentiated OSCC [Picrosirius red stain, 4X]

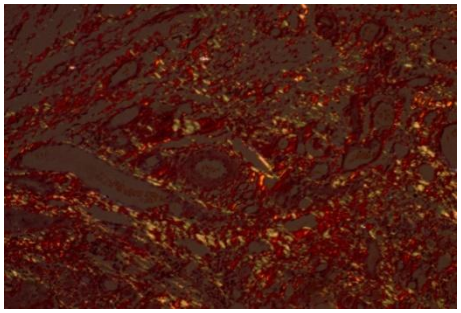

Figure 2c: Photomicrograph showing green yellow (GY) birefringence around tumor islands in poorly differentiated OSCC [Picrosirius red stain, 10X]

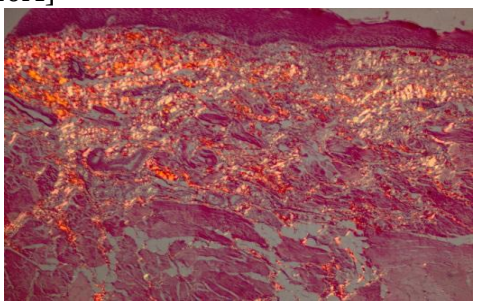

Figure 3: Photomicrograph of OSMF showing yellow orange (YO) birefringence demonstrating the thickness and packing of collagen fibres under polarizing microscopy. [Picrosirius red stain, 4X]

DISCUSSION

Oral squamous cell carcinoma (OSCC) is leading cancer of the Indian population and is known for its unpredictable course of progression,constantly leading to serious impairment of the tissues involved.Presently, clinical staging and histological grading are used to evaluate OSCC, but they tend to be subjective and not efficiently reproducible. This necessitates going beyond the routine histological methods to detect and control this disease in its early stages.

Quantitative histo-morphometric techniques help to detect certain important features that may be overlooked by routine staining. Image J one such computer aided image analysis softwarewhich can help in objective and reproducible assessment of OSCC.

A study by Natarajan et al has employed the technique of nuclear morphometry in OSCC and has stated that morphometric analysis can be effectively used to assess nuclear features preoperatively and enable early detection of cellular changes efficiently [(12)].

A study was carried out by T Smitha et al.[(13)]applying morphometric analysis in oral leukoplakia and well-differentiated oral squamous cell carcinoma. Theresults revealed that the values of nuclear perimeter and area, along with cellular perimeter and area, gradually increased as they moved from the normal buccal mucosa to leukoplakia, reaching the highest value in OSCC.

DB Nandini and RV Subramanyam[(14)] conducted a study using computer-assisted microscopy on nuclear features in oral squamous cell carcinoma and highlighted the reliability of computer-assisted nuclear morphometry in OSCC grading.

These studies coordinate with our morphometrical results where nuclear area (NA) andnuclear to cell area ratio (NA/CA)were found to be statistically significant parametersamongthe progressive grades of OSCC. The analysis was easy and quick and highly reproducible between the two different observers.

The stromal component of a tumorhas a considerable influence on the development of cancer [(2)]. The key components of the connective tissue are the collagen fibres which display the property of birefringence with polarising microscopy.This study employs the use of Picrosirius red (PSR) stain to study collagen, which is a special stain capable of demonstrating both the thick and thin collagen fibresefficiently.Itis easy to use and gives remarkable results under a polarising microscope, revealing many additional details which go unnoticedin routine H and E staining [(15).]Although collagen fibres can be detected by stains such as van- Geison and various forms of trichrome, these are not as effective in detecting the thin collagen fibres.In addition, it is rather futile to continue employing hemtoxylin and eosin staining to assess collagen when the collagen possesses no specificity for eosin [(16)].

According to a study conducted by Junqueira et al.[(17),] Type-I collagen fibres exhibit an intense birefringence of red, orange and yellow colour by polarizing microscopy and a weak birefringence

of green when the fibres are thin, thus constituting Type-III collagen.In addition, as stated in the study conducted by Singh et al.[18], tightly packed collagen fibres appear reddish orange whereas loosely appear greenish yellow.Thus the thickness and the molecular packing of the collagen fibres dictate their color profile.In the present study we observed the color changing from reddish orange to green yellow between progressing grades of OSCC and the results demonstrate that PSR staining may be used to evidently demarcate poorly differentiated OSCC and guide the prognosis.However, an association between morphometry and PSR staining was not significant.

Among the 27 cases of OSCC, the follow up status of 15 patients was available with us. Among the 15 cases, 3 patients showed recurrence, 2 patients had radiation induced morbidity, 2 patients developed OSMF and 1 patient developed lymph node metastasis. 7 patients had no complaints in follow up.

The patients showing recurrence were further studied to investigate a relation of the color and intensity of birefringence with recurrence period.Among the 3 cases that recurred, one was well differentiated with the NA/CA ratio of 0.42 while the other two were moderately differentiated with NA/CA ratio 0.61 and 0.59. The morphometrical findings and peritumoral details are given intable 4. It was observed that well differentiated showed lower intensity than moderately differentiated. The two moderately differentiated cases exhibited similar intensity, so we studied NA/CA ratio which clearly showed that higher NA/CA ratio had an earlier recurrence as compared to the lower values, indicating that higher N/C ratio may help in predicting the possibility of recurrence. However, the color of the birefringence did not show significant association with morphometry (Table 4).

CONCLUSION

Significant cellular and extracellular changes occur throughout the process of carcinogenesis which hold diagnostic and prognostic relevance. Morphometrical analysis of nuclear and cellular changes in OSCC along with the interpretation of stromal changes in collagen fibersfunction as simple yetreliable adjunctto routine clinical and histological evaluation of OSCC, aiding in formulationof an effective treatment plan according to the individual treatment needs of the patient. To the best of our knowledge, this is a first study analyzing the cellular as well as peri-tumoral stromal changes in OSCC and aiming at finding an association between the two aspects.Since this is a pilot study, further studies with a larger sample size and more elaborate follow up details would do justice to the present findings.

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