Histomorphometric Study to Compare Histological Changes Between Oral Squamous Cell Carcinoma and Apparently Normal Adjacent Oral Mucosa

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Abstract Despite the advances in surgery, radiotherapy and chemotherapy the annual death for oral squamous cell carcinoma (OSCC) is rising rapidly. The carcinoma has propensity to develop in a field of cancerization. Clinically may it be apparently normal mucosa (ANM) adjacent to squamous cell carcinoma which harbours certain discrete molecular alteration which ultimately reflects in cellular morphology. Hence the aim of the study is to assess histomorphometric changes in ANM adjacent to OSCC. A prospective study was done on 30 each of histologically diagnosed cases OSCC, ANM at least 1 cm away from OSCC, and normal oral mucosa (NOM). Cellular and nuclear morphometric measurements were assessed on hematoxylin and eosin sections using image analysis software. Statistical analysis was done using analysis of variance test and Tukey's post hoc test. The present study showed significant changes in cellular and nuclear area in superficial and invasive island of OSCC compared to ANM. The basal cells of ANM showed significant decrease in cellular and nuclear areas and nuclear cytoplasmic ratio when compared to NOM. Histomorphometry definitely can differentiate OSCC from ANM and NOM. The basal cells of ANM showed significant alterations in cellular area, nuclear area and nuclear cytoplasmic area when compared to NOM suggesting change in the field and have high risk

of malignant transformation. These parameters can be used as indicator of field cancerization.

Keywords Oral squamous cell carcinoma · Apparently normal mucosa 1 cm away from squamous cell carcinoma · Normal oral mucosa · Histomorphometry

Introduction

Oral Cancer is a global health problem with rising incidence and mortality rates. Around 300,000 patients are annually sketched to have oral cancer worldwide. In India oral cancer represents a foremost problem constituting up to 42 % in males and 18 % in females [1, 2].

The cause of oral squamous cell carcinoma (OSCC) is multifactorial and not a single factor (carcinogen) has been clearly defined [3]. It is proposed that principle etiological factor such as oncogenes and tumor suppressing responsible for DNA mutation which leads to change in normal cellular physiology [4]. Studies have shown that carcinogenic exposure leads to multiple foci of malignant transformation in the same patient which is termed as “field cancerization”. The concept was put forward by Slaughter et al. in the year 1953.

They hypothesized that cancer develops multifocally as a result of diffused and repeated carcinogenic assault and progress at various rates within an entire field [5, 6]. To simplify it suggest that the whole oral mucosa is affected by carcinogens and certain changes persists in the exposed mucosa which may not be clinically visible, may result in the development of carcinoma. This concept of field cancerization is supported by various histological, cytological and molecular evidence in which normal epithelium has been compared with adjacent tumour tissue which

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demonstrated similar subcellular and biochemical changes [7–9]. The histopathological and cytologic diagnosis of oral cancer is based on epithelial dysplastic features which has subjective variation, with wide variation between observers in the subjective evaluation of epithelial dysplasia has and been commented on by several authors [10]. Also the molecular methods are very expensive [8]. To overcome unreliability in the subjective examination of these features a more objective approach would be the value which is mainly based on image analysis techniques.

Morphometry is a quantitative structural image analysis technique which enables objective information to be acquired from structures of cells and tissues. The advantage of morphometric analysis is its objectivity and reproducibility which enables direct comparisons from person to person and centre to centre [10–12]. Many morphometric studies have been done on the exfoliative cytology of premalignant and malignant lesions, but not many studies are there on stained tissue section [13–16]. The keratinocytes of basal layer of oral stratified squamous epithelium make up the various layer of epithelium. Changes in basal cells may have serious implications of future cell behaviour including malignant transformation. The measurement of their area, nuclear cytoplasmic ratio may be important prognostic markers [12]. Hence the present study was designed to determine histologically changes in OSCC and apparently normal mucosa (ANM) at least 1 cm adjacent to OSCC which helps to understand the changes in the field in which carcinoma may develop. Study is also planned to establish diagnostic criteria using morphometry which may aid in the treatment and prognosis.

Materials and Methods

After obtaining ethical committee approval from the institute a prospective morphometric study was conducted. Informed consent was obtained from each individual before incisional biopsy was performed from the patients who were clinically diagnosed as OSCC patients. Only after histological confirmation of the same who underwent surgical treatment were considered for the study. One specimen from the tumor site and other specimen from clinically looking ANM at least 1 cm away from the tumor were included in the study. Patients treated with chemotherapy and radiotherapy, distant metastatic disease and recurrence were excluded from the study. Normal oral mucosa (NOM) from patients without any habit was obtained from patients undergoing extraction of impacted canine and third molar tooth.

The biopsy tissues were formalin fixed, processed, sectioned and stained with Haematoxyline and Eosin. Stained 4 μ m thick sections were subjected to histomorphometric

analysis using Q-Win standard (LiecaTM) image analysis software (version 3.5.0) attached to research microscope (LiecaTM DM2500).

The images of five representative fields from each section were captured in a stepwise manner by moving the microscope stage from left to right using CCD color video camera (Lieca DFC 320) attached to the research microscope. The images were visualized and stored in a computer for further analysis. The cellular and nuclear measurements were made from one side of field to the other. A curser was used to outline the basal and suprabasal cells and their nuclei at a magnification of X400. 25 basal cells and 25 suprabasal cells per slide were analyzed for NOM, ANM 1 cm adjacent to OSCC superficial areas, and OSCC. Also 50 cells for combined basal and suprabasal cells were analyses for OSCC invasive front for the groups.

Group 1: Normal oral mucosa (NOM).

Group 2: Oral squamous cell carcinoma superficial areas (OSCCSA).

Group 3: Oral squamous cell carcinoma invasive island (OSCCII).

Group 4: Clinically looking apparently normal mucosa 1 cm adjacent to OSCC (ANM).

Basal cells were defined as those cells in contact with the underlying lamina propria. Suprabasal cells were defined as those cells that were just above the basal cells which showed no signs of flattening in the plane perpendicular to the epithelial surface. The cells showing mitosis and whose outlines were not clearly made out were not considered for counting.

Details of Morphometric Parameters Analyzed

The primary parameters like cellular areas (A_C) and nuclear areas (A_N) was determined. From these data, secondary parameter of nuclear-cytoplasmic ratio (N/C) was generated.

Nuclear area (N_A) was measured in square microns (μm^2) by tracing nuclear boundary with curser using the binary edit tool and measured using measure features tool. Cellular area (A_C) was also measured in similar way by tracing the cellular boundary. Nuclear Cytoplasmic ratio (N/C) was calculated using formula

$$N/C = \frac{\sum A_N}{\sum A_{CELL} - \sum A_N}$$

Data was calculated on individual case basis. The means and standard deviations of each parameter were obtained for each of the groups investigated. The measurements were carried out using measure feature tool of the image analysis software. The measurements were in microns. All

measurements along with the images of sections were saved in Microsoft word the measurements were later transferred to Microsoft excel spread sheet for further statistical analysis.

Statistical Analysis

Statistical analysis was performed using analysis of variance test (ANOVA) and post hoc test (Tukey's test).

The mean and standard deviation were calculated using the formula. Mean, $\bar{x} = \sum x_i/n$; where $i = 1, 2, 3 \dots n$

$$n = \text{Number of cases}$$

$$\sum x_i = \text{Sum of values}$$

$$\text{Standard deviation, SD} = \sqrt{\sum (x_i - \bar{x})^2 / n - 1}$$

Results

A total of 30 surgically excised cases of OSCC and ANM were obtained. Also 30 cases of NOM were obtained as mentioned previously. The mean age of SCC patients were 47.96 years with 22 males and females. The site of involvement were buccal mucosa (53.3 %), tongue (23.3 %), alveolar region (10 %), retromolar region (9.99 %) and buccal vestibular region (3.33) (Table 1a, b).

Comparison of 25 basal and suprabasal cells was done between ANM, NOM, OSCCSA. The mean of A_C 25 basal cells in ANM was significantly reduced when compared with NOM and OSCCSA. The A_C 25 for suprabasal cells in ANM increased significantly when compared to OSCCSA. The mean A_N showed significant reduction in ANM when compared with NOM and OSCCSA but did not show any difference between NOM and OSCCSA. The mean N/C ratio significantly reduced in ANM and OSCCSA when compared with NOM. The parameters were compared using ANOVA test and post HOC test showed significant

difference between each with $p < 0.05$ (Table 2a, b; Figs. 1a, b, c, d, e, f, 2a, b, c, d, e, f.)

The mean of 50 A_C combined basal and suprabasal cells showed differences among the groups. The mean of in 50 A_C ANM showed significant increase when compared with OSCCII, and NOM. ANM showed significant decrease when compared to OSCCSA. The mean of 50 A_C was significantly decrease in OSCCII and increase in OSCCSA when compared with NOM. The mean of A_N in ANM showed significant increase when compared to OSCCII and NOM. The N/C ratio in ANM showed significant reduction NOM and did not show any when compared with OSCCSA. (Table 3a, b; Figs. 1a, b, c, d, e, f, 2a, b, c, d, e, f, 3a, b, c).

Discussion

Despite advances in diagnosis and therapy long term survival of head and neck squamous cell carcinoma patients has not improved significantly. It has been observed that even histopathologically tumor free surgical margins give rise to local recurrences in 10–30 % cases of OSCC. This is mainly due to the presence of second primary tumor in which the basic defect of any cell begins at the molecular level, and triggers a series of reactions which affect the entire cell system and ultimately reflects in the cellular morphology [17–22], and [23]. Morphometric analysis appears to be an efficient and acceptable method of examining features such as structural and morphologic quantitative characteristics of cells and tissues in 2-dimensional planes and it can allow the identification of variations from the norm, making possible objective and reliable information to be acquired. Currently, the application of quantitative techniques together with the recent progression in image analysis system has allowed improving the potential accuracy of morphometric studies [11].

In the present study cellular and nuclear measurements of basal and suprabasal cells were considered for analysis. The reason for this categorization is keratinocytes of the basal cell layer of the oral stratified squamous epithelium represent the progenitor cells that make up the various layers by production of other cells. Suprabasal cells forms the cells of differentiating compartment. Changes in the basal and suprabasal cells may have serious implications on future cell behaviour, including malignant transformation and can be used as prognostic markers [12].

The mean of 25 A_C for basal cells in ANM was significantly reduced when compared with NOM and OSCCSA. The mean of 50 A_C in ANM showed significant increase when compared with OSCCII and NOM. ANM showed significant decrease when compared to OSCCSA.

Table 1 Clinical details of patients with OSCC

Lesion	No. of males	No. of females	Mean age
a			
OSCC patients	22	8	47.96
Site	Percentage		
b			
Buccal mucosa	53.3		
Tongue	22.3		
Alveolar region	10		
Retromolar region	9.99		
Buccal vestibular region	3.33		

Table 2 Comparison of various parameters of 25 each basal and suprabasal cells of the study groups

Parameter	NOM		OSCC SA		ANM		F Value	p Value
	Mean	SD	Mean	SD	Mean	SD		
(a) ANOVA test								
A _C Basal	26.68	3.79	32.01	5.27	20.38	6.27	37.399	0.000
A _C Suprabasal	37.34	8.16	32.01	5.27	43.30	14.77	9.186	0.000
A _N Basal	13.05	2.19	14.45	2.14	8.80	3.29	38.493	0.000
A _N Suprabasal	15.54	2.84	14.45	2.14	15	4.4	0.837	0.837
N/C Basal	1.01	0.25	0.87	0.18	0.79	0.24	7.014	0.001
N/C Suprabasal	0.75	0.16	0.87	0.18	0.57	0.17	22.877	0.000
Parameters		NOM & OSCC SA			ANM & NOM		OSCC SA & ANM	
		p value			p value		p value	
(b) Post HOC test								
A _C Basal	0.000 (S)			0.000 (S)			0.000 (S)	
A _C Suprabasal	0.138 (NS)			0.079 (NS)			0.000 (S)	
A _N Basal	0.116 (NS)			0.000 (S)			0.000 (S)	
A _N Suprabasal	0.837 (NS)			0.837(NS)			0.837 (NS)	
N/C Basal	0.069 (NS)			0.000 (S)			0.502 (NS)	
N/C Suprabasal	0.23 (NS)			0.000 (S)			0.000 (S)	

$p < 0.05$ = statistically significant(S), $p > 0.05$ = statistically non significant (NS), SD Standard deviation, p = probability

$p < 0.05$ = Statistically significant(S), $p > 0.05$ = Statistically nonsignificant (NS). SD = Standard deviation

Fig. 1 Histomorphometric analysis for 25 basal cells of OSCCSA. **a** Photomicrograph showing cellular outline of basal cells in OSCCSA. **b** Photomicrograph showing nuclear outline of basal cells in OSCCSA. **c** Photomicrograph showing cellular and nuclear outline

of basal cells in OSCCSA. **d** Photomicrograph showing cellular outline of suprabasal cells in OSCCSA. **e** Photomicrograph showing nuclear outline of suprabasal cells in OSCCSA. **f** Photomicrograph showing cellular and nuclear outline of suprabasal cells in OSCCSA

A cytomorphometric analysis on three group normal (no lesions), normal (tumor patients) and tumour patients showed significant reduction in the mean A_C in normal (tumor patients) when compared with normal smears from patients with no oral lesions but not in the tumor smears compared with normal mucosa [24]. Another study

reported that patients with oral cancer showed significantly reduced A_C with respect to patients without oral cancer [9]. This is in contrast to the study done by Shabana et al. [12] who studied morphometry in normal mucosa, traumatic keratosis, lichen planus, leukoplakia, candidal leukoplakia, squamous cell carcinoma and the risk group comprising of

Fig. 2 Histomorphometric analysis for 25 basal cells of ANM. **a** Photomicrograph showing cellular outline of basal cells in ANM. **b** Photomicrograph showing nuclear outline of basal cells in ANM. **c** Photomicrograph showing cellular and nuclear outline of basal cells in ANM. **d** Photomicrograph showing cellular outline of suprabasal cells in ANM. **e** Photomicrograph showing nuclear outline of suprabasal cells in ANM. **f** Photomicrograph showing cellular and nuclear outline of suprabasal cells in ANM

Table 3 Comparison of various parameters of 50 basal and suprabasal cells of the study groups

Parameter	NOM		OSCC SA		OSCC I I		ANM		F Value	<i>p</i> Value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
(a) <i>ANOVA test</i>										
A _C	32.01	5.27	54.33	13.73	31.83	9.53	50.77	16.73	29.405	0.000
A _N	14.45	2.14	19.38	5.93	12.90	5.04	19.61	9.61	12.357	0.000
N/C	0.87	0.18	0.59	0.13	0.67	0.16	0.64	0.14	17.849	0.000
Parameter	NOM & OSCC SA		NOM & OSCC I I		NOM & ANM		OSCC SA & OSCC I I		OSCC SA & ANM	OSCC I I & ANM
	<i>p</i> value		<i>p</i> value		<i>p</i> value		<i>p</i> value		<i>p</i> value	<i>p</i> value
(b) <i>Post hoc test</i>										
A _C	0.000 (S)		0.000 (S)		1 (NS)		1 (NS)		0.000 (S)	0.000 (S)
A _N	0.003 (S)		0.002 (S)		1 (NS)		1 (NS)		0.000 (S)	0.000 (S)
N/C	0.000 (S)		0.000 (S)		0.000 (S)		1 (NS)		0.418 (NS)	1 (NS)

$p < 0.05$ = Statistically significant(S), $p > 0.05$ = Statistically nonsignificant (NS). SD = Standard deviation

$p < 0.05$ = statistically significant(S), $p > 0.05$ = statistically non significant (NS), SD Standard deviation, p = probability

white lesion that underwent malignant change or lesions coexisting with carcinoma and from buccal mucosa adjacent to the carcinoma, where they found mean values of A_C increased steadily from normal to risk group to their highest values.

The mean of 25 suprabasal cells in ANM was significantly increased when compared with OSCC. The mean A_C in ANM was higher than the basal cells. These changes may be due to epithelial cells which respond to a wide range of stimuli which may appear similar but may vary in various compartments. In quantitative study in hamster

cheek pouch epithelium after application of carcinogen Everson and Mac Donald showed progressive increase in the thickness of progenitor cell compartment which is due to increase in size and number of progenitor cells [25].

The increase in basal cellular area has been shown to be associated with lesions having malignant change and these changes in basal cells might indicate an increased metabolic activity prior to the invasion of the underlying connective tissues [12]. The reduction in A_C which was observed suggests that the changes in A_C may reflect general debilitation. Study by Frost et al. suggested that the

Fig. 3 Histomorphometric analysis for 50 basal and suprabasal cells of OSCCII. **a** Photomicrograph showing cellular outline of suprabasal cells in OSCCII. **b** Photomicrograph showing nuclear outline of

suprabasal in OSCCII. **c** Photomicrograph showing cellular and nuclear outline of suprabasal in OSCCII

ability of cytoplasm to mature diminishes in cells with increased activity and the amount of cytoplasm the cell makes in relation to the nucleoplasm is less, thus resulting in reduction of A_C [26].

The changes of A_C in ANM are a result of systemic effects of the tumour, and/or of “field cancerization” [24]. A study by Jonathan et al. suggested that reduction in A_C which occurred irrespective of use of either tobacco or alcohol mirrors smears taken from lesions that became malignant which provides evidence for true change. Similarly, Ogden et al. were in consistent with the concept of field cancerization with the reduction of A_C . They suggested the degree of reduction in A_C was independent of whether or not the patient smoked or consumed alcohol. This suggests field cancerization i.e. both generalized changes in A_C and development of tumor at specific location are attributable to the some underlying pathogenesis. [9, 24].

The mean A_N for 25 basal cells in the study group showed significant reduction in ANM compared with NOM and OSCC. Ogden et al. found no change in the A_N but decrease in A_C in cancer and cancer free groups. Yen and Pardee in their study found the earliest cells to enter DNA synthesis were those with the largest nuclei, whereas cells with the smallest nuclei were among the latest. And they suggested regulation of cellular transit from G0 to the S phase, at least in part, deterministic, since all G0 cells did not have equal probabilities of entry into S at a given moment [28]. The other possible reasons for reduced A_N could be terminally differentiated and metabolically inactive cells, the nuclei become pyknotic, and the nuclear size decreases drastically [29]. Also the causes of shrinkage of cell are decreased workload, loss of innervation, a diminished blood supply, inadequate nutrition, a loss of endocrine stimulation, and aging although some of these stimuli are physiologic and others pathologic [30]. These could be the possible reasons for change in cell size in our study.

In the present study the suprabasal A_N did not show significant difference between any of the groups. The

reasons for this non-significant difference could be due to shrinkage or swelling of the tissue during processing, osmolality of the fixative, method used for estimating the mean nuclear diameter, errors in tracing cell outlines by hand, the assessment of exact cell and nuclear outline by the observer is highly subjective and other factors like age, friction, tobacco smoking and iron deficiency anaemia.

The mean of 50 A_N in ANM showed significant increase when compared to OSCCII and NOM. These findings were in correlation with previous studies. Shabana et al. reported increase in basal A_N in risk lesions and carcinomas. The mean of A_N in ANM showed significant increase when compared to OSCCII and NOM [12]. Another study they found increased A_N of keratinocytes group II (OSCC lesion) smears than other three groups. Authors concluded that increased A_N is due to increased DNA synthesis [26].

The mean N/C of 25 basal and suprabasal in study groups were significantly reduced in ANM when compared with NOM and OSCCSA. Similar results were found by White, Jin and Yang who found decrease in N/C for fibrous hyperplasias, inflammatory lesions, hyperkeratosis, squamous cell papillomas, lichen planus, leukoplakias with dysplasia and for dysplasias from the edge of malignant lesions as well as in malignant lesions themselves [31]. Another study also showed decrease in the N/C in the risk group than in the normal group, the opposite of what might have been anticipated in the risk lesions. This decrease in N/C was attributed to the enlargement of the cell to 1.71 times the normal value, whereas the increase in the nuclear area was 1.68 times [12]. Morten Boysen and Albrecht did a morphometric study for objective classification of pseudostratified, various types of metaplastic and dysplastic epithelium. They found that the mean N/C ratio decreased significantly from pseudostratified epithelium and metaplastic epithelium to dysplasia ($p < 0.05$) which is similar to our study [32].

In our study we found significant reduction of suprabasal N/C in ANM when compared with OSCCII. Fascinatingly the supra basal N/C in OSCCII was

significantly increased when compared with ANM. There was no significant difference between OSCCII and NOM. Studies conducted to estimate the N/C ratio ultrastructurally for different layers of normal stratified squamous epithelium [33, 34]. They all demonstrated progressive decrease in N/C during differentiation. Basal cells which are least differentiated, showed a higher value when compared to spinous and granular cell layers which was decreased progressively. An increased N/C is one of the cytological features taken into account when the histopathologist subjectively assesses whether an epithelial lesion is likely to become malignant. F. H. White and K. Gohari have worked on DMBA-treated hamster cheek pouch model with a view to assess whether N/C alterations present a valid indicator of malignant transformation. They did not find any significant differences between normal and premalignant lesions [27].

The mean of 50 N/C of basal and suprabasal in ANM showed significant reduction when compared with NOM and did not show any significant difference when compared with OSCCSA. These findings were similar to the previous studies where they found decrease in N/C in risk group which is attributed to the enlargement of the cell value when compared to normal cell value [32].

Conclusion

From the present study, it can be concluded that morphometry definitely showed significant alterations in A_C , A_N and N/C among the groups which reflects change in cellular morphology. Hence these markers can be used to detect the field cancerization. In OSCC A_C , A_N and N/C was more compared to the ANM which suggests OSCC is malignant tumor. ANM also showed significant changes A_C , A_N and N/C when compared with NOM. This suggests that there are definite changes taking place in cellular level.

Further studies using histomorphometry and molecular and genetic studies have to be conducted with larger sample sizes to confirm field cancerization in ANM adjacent to OSCC which will aid in treatment and prognosis of the patient.

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Conflict of interest None.

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