Journal of
Carcinogenesis & Mutagenesis

Mohanta and Mohanty, J Carcinog Mutagene 2016, 7:1
DOI: 10.4172/2157-2518.1000247

Researech article Open access

Cytomorphometric Analysis of Non-keratinized Malignant Squamous Cells in Exfoliated Cytosmears of Human Oral Neoplasm

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Received date: December 17, 2015, **Accepted date:** January 08, 2016, **Published date:** January 12, 2016

Abstract

Objectives: During the present investigation, a unique type of pleomorphic cytological atypia- named as non-keratinized malignant squamous cell (NMSC) was frequently observed in the exfoliated cytosmears of the oral cancer patients. Details of these peculiar atypical, pleomorphic cells have not been reported earlier. Therefore, an attempt has been undertaken to find out the practical utility of NMSCs during difficult diagnoses and in early detection of oral squamous cell carcinoma (OSCC) cases through cytomorphometric analysis.

Materials and Methods: In a hospital-based case-control study exfoliated scrape smears were collected from the clinically diagnosed 136 patients suffering from precancerous lesions and cancerous cases and a parallel set of 136 samples were also collected from the non-addicted and non-cancerous healthy individuals considered as control group. Wet fixed smears were stained by adopting Papanicolaou's staining protocol and counter-stained with Giemsa's solution. Out of one thousand screened cells, frequently observed NMSCs were scored. Cytomorphometry was done by using computer-assisted Cat Cam 1.30 (1.3 Mega Pixel) Microscope Camera® fitted with hund®-H500 research binocular microscope. The findings were statistically analyzed and interpreted by using software package PAleontological Statistics (PAST)® Version 2.17 with respect to age groups, degree of pathogenicity, oral sites and sexes.

Results: Cytomorphometrically, the cellular parameters (mean length, breadth and area) of the NMSC were found to be in decreased state and nuclear parameters are in increased state. The quantitative parameters of the NMSCs were significantly ($p \leq 0.01$) decreased with increasing age groups. Cytomorphometrically, the nuclear-cytoplasmic (N/C) ratio of the NMSC was found to be 1:1 in both sexes.

Conclusion: Extreme reduction of cellular diameter and increased nuclear diameter in each NMSC, lead to cellular non-keratinization, hyperchromasia and increased N/C ratios in both sexes indicate the state of malignancy. Thus, the present finding has a practical utility in early detection and diagnosis of the OSCC patients.

Keywords: Exfoliated cytosmears; Hyperchromasia; Pleomorphism; Neoplasm; Non-keratinized malignant squamous cell (NMSC); Nuclear–cytoplasmic (N/C) ratio

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies as well as a major cause of cancer morbidity and mortality, worldwide [1]. Early detection of premalignant and malignant lesions can significantly reduce the morbidity and mortality associated with oral cancer. In spite of various techniques applied so far, cytopathology has been accepted as a standard protocol for early detection of oral cancer.

Cytological pleomorphism is a common feature during oral carcinogenesis. Pattern of keratinization, cellular and nuclear anomalies are frequently observed in the exfoliated cytosmears of human oral neoplasm [2]. Based on their structural atypias, Broders' has categorised these cells into well differentiated, moderately differentiated and poorly differentiated squamous cells [3]. Out of these, well differentiated and moderately differentiated squamous cells are predominantly observed in benign or precancerous stage; whereas poorly differentiated squamous cells are mostly found in malignant or cancerous stage. But, the cytopathologists very often encounter problems in detection and diagnoses of the OSCC patients as most of the oral squamous cells appear to be either well differentiated or moderately differentiated and mimic to be benign and non-neoplastic. Contrary to that, many benign and non-neoplastic lesions appear to be malignant neoplasms [4-6].

In real sense, early detection of oral cancer is not easy, because at an early stage, it is generally asymptomatic and mimics many benign conditions in the mouth, leading to delay in diagnosis and treatment [7]. But, as it develops in multistep process, it offers the advantage of diagnosing it at an early stage before it manifests as cancer [8]. Although, histopathology has been accepted as the gold standard model for early diagnosis of oral cancer, it has its own limitation so far as psychology of the patient is concerned. Not only that, it is a painful invasive method and not feasible for mass screening also. However, oral cytology is a relatively simple, non-invasive, inexpensive and risk-free technique which is well accepted by the patients. Therefore, in the present investigation, cytomorphometric analysis was undertaken to find out the practical utility of frequently observed non-keratinized

malignant squamous cells (NMSCs) in human oral neoplasm during difficult diagnosis and in early detection of OSCC.

Materials and Methods

The subjects

Clinically diagnosed 136 patients (82 male and 54 female) suffering from precancerous (32 male and 23 female) and cancerous (50 male and 31 female) lesions at different oral sites (International Classification of Diseases, 10th Edition: ICD-10) and registered at the Out-patient Department (OPD) of the Acharya Harihar Regional Cancer Centre, Cuttack, Odisha during May 2007-May 2009 were included in the present study. Prior to the collection of samples, case-history of the patients related to their age, sex,food, habits (addiction to tobacco, alcohol etc.), oral hygiene and occupation were asked and recorded for detail analysis.

The recorded age of the patients varies from 30 to 87 years. Therefore, the collected samples were grouped into three broad age groups, such as 30-49, 50-69 and 70-89 years. Out of 82 males, 33 (40.2%) patients belong to 30-49 years, 34 (41.5%) were between 50-69 years and 15 (18.3 %) patients were under 70-89 years. Out of 54 females, 11 (20.4%) were between 30-49 years, 36 (66.7%) were grouped under 50-69 years and 7 (12.9%) belong to 70-89 years of age group. Thus, the relative proportion of the patients was found to be more (41.5% in males and 66.7% in females) in the age group of 50-69 years than the other two (Figure 1).

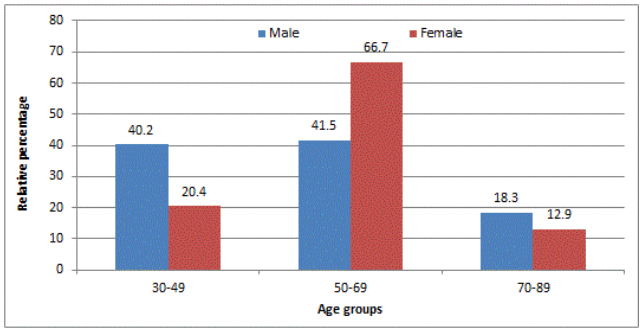

Figure 1: Age groups and sex-wise relative percentage of subjects included.

Considering the nature of addiction, out of 136 cancer affected individuals, 126 (92.7%) were addicted and 10 (7.3%) were non-addicted. Out of 126 addicted individuals, 76 (60.3%) were males and 50 (39.7%) were females and were addicted to different forms of tobacco and alcohol for more than 15 years. Similarly, out of 10 non-addicted individuals 6 (60%) were males and 4 (40%) were females. A parallel set of 136 samples were also collected from the non-addicted and non-cancerous healthy individuals from different regions of Odisha which is considered as the control group. Thus, a total of 272 subjects were included in this case-control study (Table 1).

No	Oral Sites (ICD-10)	Control group		Precancerous				Cancerous				Grand Total
				Leukoplakia		Erythroplakia		Benign		Malignant		
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
1	Lip	5 (6.1)	6 (11.1)	2 (13.3)	2 (14.3)	1 (5.9)	2 (22.2)	2 (5.7)	1 (5.0)	Nil	1 (9.1)	22 (8.8)
2	Tongue	11 (13.4)	7 (12.97)	4 (26.7)	2 (14.3)	3 (17.6)	1 (11.1)	2 (5.7)	2 (10.0)	2 (13.3)	2 (18.2)	36 (13.2)
3	Alveolus and gingiva	16 (19.5)	6 (11.1)	3 (20.0)	2 (14.3)	2 (11.8)	2 (22.2)	8 (22.9)	1 (5.0)	3 (20.0)	1 (9.1)	44 (17.6)
4	Floor of the mouth	7 (8.5)	6 (11.1)	2 (13.3)	3 (21.4)	1 (5.9)	1 (11.1)	3 (8.60)	2 (10.0)	1 (6.7)	Nil	26 (9.5)
5	Palate	6 (7.3)	3 (5.6)	Nil	Nil	3 (17.6)	1 (11.1)	2 (5.7)	2 (10.0)	1 (6.7)	Nil	18 (6.5)
6	Buccal mucosa	37 (45.2)	26 (48.2)	4 (26.7)	5 (35.7)	7 (41.2)	2 (22.2)	18 (51.4)	12 (60.0)	8 (53.3)	7 (63.6)	126 (46.4)
Total		82	54	15	14	17	09	35	20	15	11	272

Table 1: Degree of pathogenicity, oral site and sex-wise collected samples.

Collection of samples

Exfoliated scrape cytosmears were collected from the oral lesions of the respective sites (ICD-10). Smearing was done on the pre-cleaned-coded microslides and the smears were immediately fixed in aceto-alcohol (1 part of glacial acetic acid: 3 part of ethyl alcohol) fixative. Two slides were smeared and prepared from each affected site of the patient.

Staining protocol and scoring

Wet fixed smears were stained by adopting Papanicolaou's staining protocol and counter-stained with Giemsa's solution. One thousand cells were screened and non- keratinized malignant squamous cells along with other cytological atypias were scored.

Morphometric and Statistical Analysis

Cytomorphometry was done by using computer-assisted *Cat Cam 1.30 (1.3 Mega Pixel) Microscope Camera of Catalyst Biotech*®

(Maharashtra, India) fitted with hund®-H500 research binocular microscope. Software package PAleontological Statistics (PAST)® Version 2.17 alongwith Microsoft® Excel was used for statistical analysis (One-way ANOVA followed by Tukey's pair-wise comparison). The measured values were tabulated in the Excel spread sheet. The nuclear-cytoplasmic ratio (N/C) was calculated after taking the area of the cytoplasm (C) and nucleus (N) of the respective cell. The findings were statistically analysed and interpreted with respect to age groups, oral sites and sexes. Photomicrographs were also taken out as supporting evidence.

Ethical considerations

This study was approved by Subject Research Committee of Utkal University, Bhubaneshwar, Odisha, India and necessary permission from the Director, AHRCC, Cuttack, Odisha, India was also obtained for the same purpose.

Results

During this cytopathological investigation, cellular and nuclear architecture of the oral squamous cells were taken into account. Regularity of the cell boundary, pattern of cellular keratinization, nuclear staining, cell division and computer assisted morphometry of the oral exfoliated cells were studied (Figure 2).

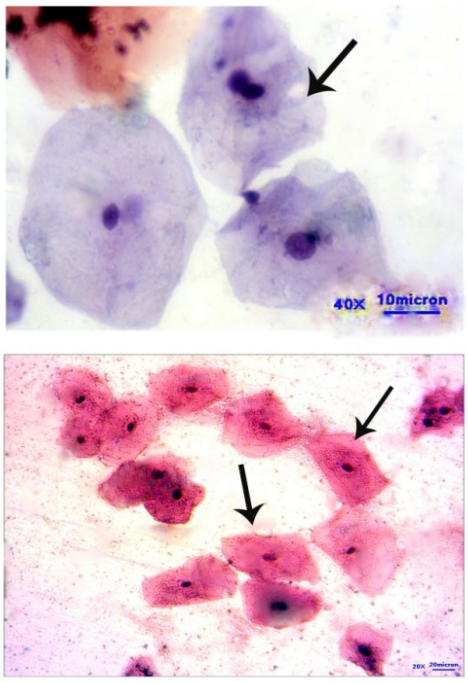

Figure 2: Normal oral squamous cells (a-Papanicolaous' stain × 40X, b- Giemsa stain × 20X).

Irrespective of oral sites, the normal oral squamous cells (NOSCs) are observed to be polyhedral and flattened with more or less centrally placed a round or oval nucleus in each. Cytoplasm appears to be light blue colour in Papanicoloau's stain and magenta in Giemsa's stain.

However, almost all the detected cells in the exfoliated cytosmears from oral lesions of the patients were observed to be round or oval with a regular cyto-contour. Nuclei were hyper chromatic. The Nuclear boundary touches the cell boundary in each cell in such a way that each atypical cell appears to be devoid of cytoplasm and only the nucleus exists. As a result, the atypical cells were found to be hyper chromatic and non-keratinized in Papanicolaou's stain. Morphometricly, the nuclear-cytoplasmic (N/C) ratio was found to be 1:1 in each cell-which is considered as one of the most important features of the malignancy. That's why; the atypical cell was named as non-keratinized malignant squamous cell (NMSC). Transformation of a normal polyhedral cell to a more or less round or oval NMSC in oral neoplasm may be considered as a remarkable event in the long run of oral carcinogenesis (Figure 3).

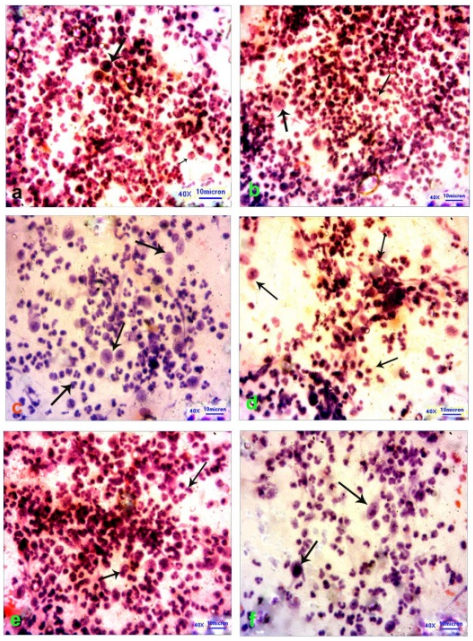

Figure 3 (a-f): Site specific non-keratinized malignant squamous cells (NMSCs- arrow marked).

a. Hyperchromatic two prominent NMSCs in the exfoliated samples from lip (Papanicolaou's stain 400X)

b. Pleomorphic NMSCs in tongue (Papanicolaou's stain 400X)

c. Amitosis in both large and small NMSCs in alveolus and gingiva (Giemsa's stain 400X);

d. Large and small NMSCs in floor of the mouth(Papanicolaou's stain 400X);

e. Amitosis of NMSCs in palate (Papanicolaou's stain 400X);

f. Hypochromasia and hyperchromasia NMSCs in buccal (cheek) mucosa (Giemsa's stain 400X).

The NMSCs are very small and the nuclei of these cells exhibit a great extent of pleomorphism irrespective of different oral sites (Figure 3). Anisonucleosis is a typical feature of these cells. It is important to note that the NMSCs are non-keratinized due to lack of cytoplasm and are not found in normal or control group. But, these cytological atypias

are frequently observed in precancerous lesions (mostly in erythroplakia), carcinoma and in malignant cases. NMSCs appear to be fewer in number and slightly larger in size in precancerous lesions than the cancerous cases.

Although, the number of NMSCs in precancerous cases is significantly very less, its appearance indicates a quick progression towards cancerous stage in the long run of oral carcinogenesis. NMSCs are observed to be clumped in carcinoma in situ and in benign neoplasm. On the basis of Broders' cellular differentiation, these cells may be categorized into poorly differentiated squamous cells.

Numerically, the total number of NMSCs in precancerous cases was recorded to 776 in 32 males and 788 in 23 females. But, in cancerous cases it was calculated to be 18414 in 50 males and 11630 in 31 females (Table 2).

No	Oral Sites (ICD-10*)	Age groups in years	No. of samples		Precancerous						Cancerous					
					No. of samples		NMSCs Scored		Total NMSCs		No. of samples		NMSCs Scored		Total NMSCs	
			Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	Lip	30-49	2	Nil	1	Nil	36	Nil	58 (7.4)	156 (19.8)	1	Nil	842	Nil	1986 (10.8)	1908 (16.4)
		50-69	2	4	1	3	22	124			1	1	684	1216		
		70-89	1	2	Nil	1	Nil	32			1	1	460	692		
2	Tongue	30-49	5	2	4	1	22	17	57 (7.3)	61 (7.8)	1	1	1913	291	3854 (20.9)	1055 (9.1)
		50-69	5	4	3	2	35	44			2	2	1446	604		
		70-89	1	1	Nil	Nil	Nil	Nil			1	1	495	160		
3	Alveolus and gingiva	30-49	4	3	1	2	25	12	170 (21.9)	44 (5.5)	3	1	1183	664	3015 (16.4)	1782 (15.3)
		50-69	8	3	3	2	88	32			5	1	1236	1118		
		70-89	4	Nil	1	Nil	57	Nil			3	Nil	596	Nil		
4	Floor of the mouth	30-49	4	1	2	1	28	172	74 (9.6)	216 (27.4)	2	Nil	1110	Nil	2043 (11.1)	895 (7.7)
		50-69	2	4	1	2	46	44			1	2	528	704		
		70-89	1	1	Nil	Nil	Nil	Nil			1	1	405	191		
5	Palate	30-49	4	1	3	Nil	25	Nil	25 (3.3)	24 (3.1)	1	1	643	267	1080 (5.9)	807 (6.9)
		50-69	1	2	Nil	1	Nil	24			1	1	203	540		
		70-89	1	Nil	Nil	Nil	Nil	Nil			1	Nil	234	Nil		
6	Buccal mucosa	30-49	14	4	6	1	142	33	392 (50.5)	287 (36.4)	8	3	2766	437	6436 (34.9)	5183 (44.6)
		50-69	16	19	5	6	172	202			11	13	2620	3864		
		70-89	7	3	1	1	78	52			6	2	1050	882		
Total		30-89	82	54	32	23	776	788	776	788	50	31	18414	11630	18414	11630

Table 2: Oral sites, age groups and sex-wise enumeration of NMSCs.

Thus, the total number of NMSCs was 19190 in 84 males and 12418 in 54 females. More number of NMSCs was observed in the age group of 50-69 years than the other two age groups. In this study, an exception was observed in a female case of 30-49 year age group, in which 172 NMSCs was recorded from the floor of the mouth- probably due to multifactorial carcinogenic (tobacco and alcohol) effect. With reference to the specific oral sites, the highest relative percentage of NMSC was recorded from the buccal mucosa and lowest from the palate in both precancerous and cancerous cases.

Cytometrically (Table 3), the mean length, breadth and area of the NMSCs were measured to be 16.237 μm (± 5.637 μm), 12.590 μm (± 4.862 μm) and 204.423 μm^2 (± 27.407 μm^2) in male. The mean length, breadth and area of the nucleus were found to be same with respect to the length, breadth and area of the cell. Hence, the N/C ratio of the NMSC was observed to be 1:1(Figure 4).

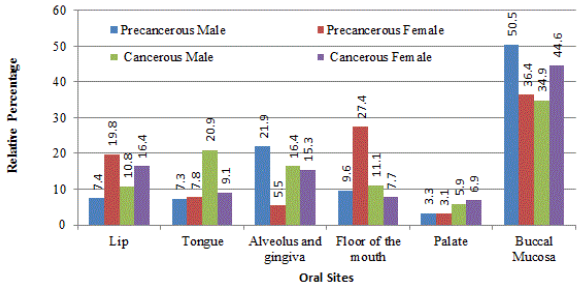

Figure 4: Oral sites and sex specific relative percentage of NMSCs in precancerous and cancerous groups.

In case of females, the mean length, breadth and area of the NMSCs are calculated to be 15.982 µm (± 5.246 µm), 12.537 µm (± 5.013 µm) and 200.366 µm^2 (± 26.298 µm^2) which were also accurately same with that of length, breadth and area of the nucleus. Thus, the N/C ratio of the NMSC was also calculated to be 1:1 in female which signifies the true nature of a malignant cell.

In comparison with the NOSCs, the cellular length and breadth of the NMSCs were observed to be in decreased state; whereas the nuclear length and breadth were in increased state (Table 4). As a result, the N/C ratio of the NMSC was found to be higher (1:1 in both sexes) than the NOSC (1:34.5 in male and 1:34.4 in female). The most important fact is that, the cellular and nuclear parameters are found to be significantly decreasing ($p \leq 0.01$) with increasing level of age groups in both sexes (Table 3).

Cell type	Sex	No. of cells scored	Cytoplasm (C)			Nucleus (N)			N/C Ratio
			Mean length in µm+SD	Mean breadth in µm+SD	Mean area in µm2 + SD	Mean length in µm+SD	Mean breadth in µm+SD	Mean area in µm2 + SD	
NOSC	Male	1000	86.50 ±17.234	64.23	5555.89	12.95	12.43	160.968	1:34.5
				± 12.125	± 208.362	± 3.368	± 3.235	± 10.895	
	Female	1000	85.895	65.012	5582.905	12.92	12.53	161.887	1:34.4
			16.374	11.876	194.475	3.544	3.452	12.233	
NMSC	Male	19190	16.237	12.59	204.423	16.237	12.59	204.423	1:1
			± 5.637	± 4.862	± 27.407	± 5.637	± 4.862	± 27.407	
	Female	12418	15.982	12.537	200.366	15.982	12.537	200.366	1:1
			5.246	5.013	26.298	5.246	5.013	26.298	

Table 3: Morphometric analysis of NOSC and NMSC in both sexes.

Discussion

Oral exfoliative cytology is the microscopic examination of shed or desquamated cells from an epithelial surface. It also includes the study of those cells that have been collected by scraping the tissue surface or collected from body fluids such as sputum, saliva, etc. Papanicolaou and Traut's staining technique for cytology smears were first used in normal oral epithelial smears by Montgomery in 1951 [9]. Continuous exfoliation of epithelial cells is a part of physiological turnover. Deeper cells which are strongly adhered in normal conditions become loose in the case of malignancy and tend to exfoliate or shed along with superficial cells [10,11]. Kumar et al., [12] have opined that exfoliative cytology not only aids in the differential diagnosis of an unidentified oral lesion or a probable benign lesion when the physician or dentist is reluctant to perform a biopsy, but also helps to detect carcinoma in situ and other premalignant lesions in suspicious red, velvety and granular-appearing areas. It is a non-invasive, simple sensitive staining technique which can be used as an adjuvant for biopsy or where the gold standard biopsy is not feasible for mass screening (Figure 5).

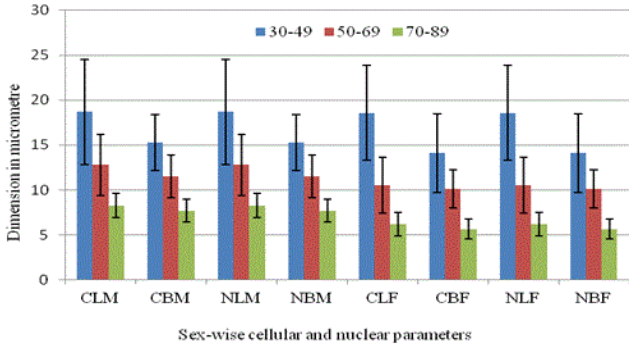

Figure 5: Age groups and sex-wise quantitative parameters of NMSCs.

Oral cytopathology is a simple, non-invasive technique that could be used for early detection of oral premalignant and malignant lesions, but the effectiveness of this diagnostic approach remains controversial. Although many studies have demonstrated the value of oral

cytopathology as a diagnostic tool for OSCC [13-16], other professionals disagree with its application [17,18].

Stromby [19] has reported that clinical cytology, in the hands of well trained personnel now possesses a sensitivity of 92% to 100% while its specificity has risen to somewhere between 97% to 100%. Similarly, Kumar et al. [12] have also found that oral exfoliative cytology is a reliable adjuvant to biopsy with the 69% sensitivity and 100% specificity in oral leukoplakia and 75 % sensitivity and 100% specificity in squamous cell carcinoma.

Based on a comparative study, Fontes et al., [20] have reported that the diagnostic concordance between histopathological (gold standard) and cytopathological examinations was 83.1% for OSCC and 85.7% for non-neoplastic lesions. Specifically, the sensitivity was 83.1%, the specificity was 100%, the positive predictive value was 100%, the negative predictive value was 49.0%, and the accuracy was 85.5%. The results indicate that cytopathological diagnosis had good concordance with histopathological diagnosis and showed high sensitivity, specificity, positive predictive value, and accuracy. They have also concluded that the sensitivity of oral cytopathology is sufficient to justify its use as a diagnostic screening test and to confirm the malignant nature of epithelial cells, mainly for the classification of OSCC. In a current study, Hafez and Fahim [21] reported to have the diagnostic reliability in the form of sensitivity was 93.5%; specificity 96.2%; predictive value (PPV)97.7%; negative predictive value (NPV)89.3% and overall diagnostic accuracy was 94.4%. A statistically significant relation was found between cytological and pathological diagnosis ($p<0.001$). Kappa was 0.882 indicating a good agreement between cytological and histopathological results.

Age group	Sex	No. of Cells	Cytoplasm (C)		Nucleus (N)		N/C Ratio	F-Value
			Length (Mean +SD)	Breadth (Mean +SD)	Length (Mean +SD)	Mean Breadth (Mean+SD)		
30-49	Male	8735	18.675 ± 5.865	15.278 ± 3.129	18.675 ± 5.865	15.278 ± 3.129	1:1	38.77**
	Female	1893	18.572 ± 5.276	14.109 ± 4.38	18.572 ± 5.276	14.109 ± 4.38	1:1	
50-69	Male	7080	12.797 ± 3.397	11.532 ± 2.382	12.797 ± 3.397	11.532 ±2.382	1:1	27.37**
	Female	8516	10.533 ± 3.08	10.14 ± 2.14	10.533 ± 3.08	10.14 ± 2.14	1:1	
70-89	Male	3375	8.273 ± 1.325	7.712 ± 1.24	8.273 ± 1.325	7.712 ± 1.24	1:1	13.59**
	Female	2009	6.125 ± 1.314	5.65 ± 1.114	6.125 ± 1.314	5.65 ± 1.114	1:1	
30-89	Male	19190	16.237 ± 5.637	12.59 ± 4.862	16.237 ± 5.637	12.59 ± 4.862	1:1	619.95**
	Female	12418	15.982 ± 5.246	12.537 ± 5.013	15.982 ± 5.246	12.537 ± 5.013	1:1	

Table 4: Age-group and sex-wise comparative dimension of NMSCs.

Recently, Gupta et al., [22] have reported that the sensitivity, specificity, positive and negative predictive values of brush cytology in detecting dysplasia and oral squamous cell carcinoma were 84.21%, 83.33%, 94.12% and 62.5% respectively. Further, they have also suggested that cytologically diagnosed cases of malignancy need not undergo histopathological confirmation and adequate complete excision with a wide safer margin of normal tissue can be done at the first place.

Majority of the carcinomas of the oral cavity are of well differentiated squamous cell type. In such cases, the malignant cells have a characteristic cytologic appearance and are quite easily recognized. Malignant cells are characterized by large nucleus with an increased nuclear/ cytoplasmic ratio, hyper-chromatic nuclei, irregular size and shape of the nuclei, multinucleation, and abnormal shape of the epithelial cells, bizarre shape, tadpole cell, naked nucleus, abnormal mitosis and excessive cornification. In the present study, the most frequently observed NMSCs were poorly differentiated cells, non-keratinized, hyper chromatic with increased N/C ratio- which are the clear indications of the malignancy. All these findings are found to be consistent and corroborates with the earlier findings of many authors [23-25].

Nowadays, with advanced imaging techniques, computerized systems, and the use of quantitative techniques to verify the reliability of cytomorphometric analysis, oral exfoliative cytology is gaining in popularity once again [26]. More recently, the continuing development of automated cytomorphometric methods, DNA content determination, tumour marker detection, and diverse molecular-level analyses has contributed to renewed interest in exfoliative cytology procedures for the diagnosis of oral cancer [27].

Oral exfoliative cytology technique is not intended to replace tissue biopsy, but it is a valuable supplement to biopsy. Particularly, cytomorphometric analysis of cytological atypias in the exfoliated smears of the oral lesions is useful in early detection and even in difficult diagnosis. Measurements of cellular diameter (CD), and nuclear diameter (ND) followed by cellular area (CA), nuclear area (NA) and nuclear-cytoplasmic (N/C) ratio have been used as important parameters for detecting alterations in the oral epithelial tissues [28-30].

Callimeri and Smith [31] found that an increased nuclear to cytoplasmic ratio was one of the consistent findings during progression from benign to a state of malignancy. Cowpe [32] reported that in malignant lesions, an increased nuclear to cytoplasmic ratio was due to a reduction in the mean cytoplasmic area and an elevation in the mean nuclear area; while in case of epithelial dysplasia, an increased nuclear to cytoplasmic ratio was only due to a significant reduction in the mean cell area with little or no change in the mean nuclear area. Ogden

et al., [28] indicated that quantitative techniques based on the parameters such as NA, CA, and NA/CA ratio could increase the sensitivity of exfoliative cytology in the early diagnosis of oral cancer because these techniques were more accurate, objective, and repeatable.

Many factors affect the cytomorphology of the cells collected from the oral mucosa. Some of these factors are systemic diseases, e.g., anaemia [33] and diabetes mellitus [34]; radiotherapy [35]; alcohol consumption [36]; chewing and smoking of tobacco [37]. Tobacco and alcohol contain many carcinogenic substances, mostly DNA-toxic carcinogens. It is well known that these carcinogenic substances cause genetic mutations and chromosomal abnormalities followed by micronuclei formation [37,38].

Hande and Chaudhary [39] reported that the cellular diameter was progressively reduced from normal group, through history of tobacco chewing but without lesion, tobacco-lime lesion and leukoplakia to squamous cell carcinoma. Goregen et al., [40] have analyzed the cytomorphology of buccal mucosa cells of smokers using computerized image analysis based on quantitative parameters such as nuclear area (NA), nuclear perimeter (NP), minimal nuclear diameter (D-min), and maximal nuclear diameter (D-max), as well evaluated the potential dysplastic transformation in epithelial tissues. Their results revealed that the NA, NP, D-min, and D-max values of the buccal mucosa cell nuclei of smokers were higher than those of non-smokers, and the difference was statistically significant in the case of NA, D-min, and D-max values. This increase determined in NA shows smoking-related cellular adaptation and they concluded that this adaptive change in the cell nucleus tends to be a dysplastic change.

Joshi et al., [41] have reported that the cellular area and cellular diameter was highest in normal mucosa, lower in premalignant lesions and lowest in OSCC lesions. The nuclear area and diameter and nuclear to cellular area ratio was lowest in normal mucosa, higher in premalignant lesions and highest in OSCC lesions. However there was no statistically significant difference ($p>0.05$) between premalignant and OSCC lesions in any of the cellular or nuclear parameters.

Analyses of Vanishree et al., [42] have revealed that CD and ND of the control group were found to be in the range of 65.32 µ-75.39 µ and 8.1 µ-9.4 µ respectively. CD values of iron deficiency were 55.05 µ-64.12 µ with the mean CD value of 59.77 µ and ND values were 8.69 µ-11.24 µ with mean ND values of 9.88 µ. It showed positive correlation on correlating the serum ferritin and red cell parameters with the CD and ND values of iron deficiency anaemia. The decrease in cytoplasmic diameter and increase in ND in iron deficiency anaemia and progressive decrease in CD with decrease in serum ferritin levels suggested that iron deficiency causes significant changes in oral exfoliative cells. They have also found that age and sex did not show any significant influence on the CD and ND values of iron deficiency anaemia and control group.

Saranya and Sudha [43] have observed that there was a significant increase in the normal nuclear diameter and nuclear cytoplasmic ratio of the Khaini chewers when compared to the controls ($p<0.05$). A clear proportional increase in ND was shown in those aged 25-50, a decrease in CD among Khaini users and a steady increase in N/C ratio from control individuals to khaini users. In addition to this age dependent increase in abnormal nucleus and cytoplasm ratio was observed in khaini users. Masood et al., [44] have observed that CD and ND were increased in the subjects who used naswar more frequently and have been using it for long duration. N/C ratio was also significantly increased in the group using naswar for longer duration. These findings corroborate with the findings of the study carried out by Saranaya et al., in 2014. These studies suggest that decrease in the mean cytoplasmic diameter of exfoliated buccal mucosal cells could serve as an early indicator of dysplastic change especially in lesions which appear histologically benign.

Detailed report on NMSC has not been reported so far. Morphometrically, the cellular length and breadth of the NMSCs were observed to be less than the NOSCs; whereas the nuclear length and breadth of the NMSC were more and observed to be in increased state than its normal counterpart. Irrespective of age, sex, oral sites and type of addiction (single or mixed) the morphometric parameters of the nucleus and the cell itself are found to be equal and so, the nuclear area and the cytoplasmic area become same in each NMSC. As a result, the N/C ratio of the NMSCs was found to be 1:1 in both sexes which is higher than the NOSCs (1:34.5 in male and 1:34.4 in female). Interestingly, without any alteration in N/C ratios, the quantitative parameters of the NMSC are observed to be significantly ($p \leq 0.01$) decreasing from lower (30-49 years) to higher age group (70-89 years) in both sexes. Furthermore, nuclear hyperchromasia, anisonucleosis and nuclear pleomorphism among the MNSCs observed in both addicted and non-addicted groups of oral cancer patients indicate a true nature of malignancy which needs further research.

Conclusion

Cytomorphometrically, the non-keratinized malignant squamous cells (NMSCs) exhibit decreased cellular parameter and increased nuclear parameter with respect to its normal counterpart. The quantitative parameters of the NMSC were also found to be significantly ($p \leq 0.01$) decreasing with increasing order of age groups. Furthermore, increased nuclear-cytoplasmic (N/C) ratios, nuclear hyperchromasia, anisonucleosis and nuclear pleomorphism were also observed among the NMSCs in both addicted and non-addicted groups of oral cancer patients indicate a true nature of malignancy. Thus, the present study has not only a practical implication in the field of cytopathology but also it proves the reliability and validity of oral exfoliative cytology for the early detection and even in difficult diagnosis of OSCC cases.

Acknowledgement

The authors are thankful to Prof. Gadadhar Parida, M.D, formerly Professor & Head, Department of Oncopathology, Acharya Haihar Regional Cancer Centre (AHRCC), Cuttack, Odisha, for his guidance and supervision during cytopathological analysis. We are also indebted to the Head P.G. Dept. of Zoology, Utkal University, Vani Vihar, Bhubaneshwar, Odisha and to the Director, AHRCC, Cuttack, Odisha, for permitting us to collect samples from oral cancer patients and also for providing library and laboratory facilities. One of us (AM) is grateful to the University Grants Commission (UGC), New Delhi, for awarding UGC Meritorious Research Fellowship to carry out the research work.

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