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Pattern of Keratin Expression and its Impact on Nuclear-Cytoplasmic Ratio in Plump Keratinized Squamous Cells during Oral Carcinogenesis

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Abstract

Background: Cellular alteration is a unique feature during multi-step oral carcinogenesis. Non-keratinized normal oral squamous cells get keratinized in order to protect and preserve their normalcy in both structure and function. But, the pattern of keratinization seriously affects the nuclear-cytoplasmic ratio of the concerned cells. In the present study, the pattern of keratin expression and its impact on nuclear-cytoplasmic ratio in plump keratinized squamous cells during carcinogenesis was vividly analysed.

Materials and methods: Samples in the form of scraped and exfoliated cytosmear were collected from the affected sites of the clinically diagnosed 136 oral cancer patients and were immediately fixed in acetoalcohol (1:3). The wet fixed smears were stained by routine Papanicolaou's staining protocol and Giemsa's solution. Stained tissues were studied under the microscope.

Results: Cytological pleomorphism is a unique feature observed during oral carcinogenesis and pattern of keratin expression in these pleomorphic cell reflect the cellular pathogenecity. Huge keratin expression in the cytoplasm of the plump keratinized squamous cells is presumed be to an important cause of alteration in the architectural regularity of the cell membrane which lead to cytological pleomorphism as well as a significantly ($p<0.01$) increase in nuclear- cytoplasmic (N/C) ratios from precancerous (1:27.9 in male and 1:28.9 in female) to cancerous (1:17.8 in male and 19.6 in female) cases during oral carcinogenesis.

Conclusion: Pattern of keratin expression in well differentiated PKSCs is presumed to be a factor of cytological pleomorphism and increase in N/C ratio from precancerous to cancerous cases. Also, the appearance of PKSCs may be considered as a sign and index of cellular alteration in oral epithelia and a progenitor of cytological pleomorphism during oral carcinogenesis. And thus, the present finding has a practical utility in the field of diagnostic cytopathology in general and early detection of oral cancer during carcinogenesis in particular.

Keywords: Keratins; Cytopathology; Plump keratinized squamous cells; Nuclear-cytoplasmic ratio; Carcinogenesis

Introduction

Keratins are intermediate filament forming proteins with specific physicochemical properties and are expressed in a highly specific pattern related to the epithelial type and stages of cellular differentiation [1]. The different types of keratin and their associated proteins serve as important markers of differentiation thus aiding in diagnosis of various pathological conditions including cancer [2-4]. The keratin proteins have a uniform mode of distribution among the various layers of epithelium which gives an indication of the disease process. Also, various disorders are associated with defects in the keratin and their associated proteins which may manifest in skin or oral cavity or both [5]. The keratins and keratin-associated proteins are useful as differentiation markers because their expression is both region specific and differentiation specific. In the present study, the pattern of keratin expression and its impact on nuclear-cytoplasmic (N/C) ratio in plump keratinized squamous cells (PKSCs) during oral carcinogenesis is analysed.

Materials and Methods

The subjects

Clinically diagnosed 136 oral cancer 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), who have registered at the Out-patient Department (OPD) of Acharya Harihar Regional Cancer Centre (AHRCC), Cuttack, Odisha-(the only Government Hospital for the treatment of cancer patient) during May 2007 to May 2009 were included in this study. Prior to the collection, the 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 in self-prepared questionnaire form 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.

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.

Collection of samples

Samples in the form of scraped and exfoliated cytosmear were collected from the respecyive affected oral sites of the patients. Two smearing slides were prepared from each affected site. Utmost care was taken for uniformly spreading of the smear while smearing. Collected tissues in the form of smears on the slides were immediately fixed in acetoalcohol (1 part of glacial acetic acid: 3 part of ethyl alcohol) fixative.

Staining protocol and scoring

The wet fixed smears were stained by routine Papanicolaou's staining protocol and Giemsa's solution. Photomicrographs of the exfoliated PKSCs were taken out as supporting evidence. The findings were statistically analyzed, interpreted and correlated with age, sex and addiction of the patients.

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 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 analyzed and interpreted with respect to age groups, oral sites, sexes and degree of pathogenicity. Photomicrographs were also taken out as supporting evidence.

Ethical considerations

This study was approved by the Institutional Ethics Committee of Utkal University, Bhubaneswar and necessary permission from the Director, AHRCC, Cuttack, Odisha, India was also obtained for carrying out the research work.

Results

Oral carcinogenesis is a long latency multistep process which proceeds from normal to precancerous to cancerous stage. During study, it was observed that the normal oral squamous cells were appeared to be sky-blue in colour in Papanicolaou's stain (Figure 1a) and faint magenta colour in Giemsa stain (Figure 1b). Interestingly, some of the well differentiated cells were found to be keratinized, either faint or deep orange-yellow in color and exhibited in the form of cellular pleomorphism in the exfoliated cytosmears of oral cancer patients. The shape and size of these cells varied from one to another. The cells were mostly abundant in erythroplakia and carcinoma in situ, but observed to be in plumped or clustered state. It is important to note that these cell are either very few in number and are hypokeratinized or totally absent in malignant cases. Cytoplasm was either of hypo- or hyper-keratinized, where as nuclei of the PKSCs are deeply stained or hyperchromatic (Table 1). The cytoplasm of exfoliated PKSCs was observed to be hypokeratinized in leucoplakia, hyperkeratinized in erythroplakia and more or less moderately hyperkeratinized in benign or carcinoma in situ (CIS) cases (Figure 2a-2c).

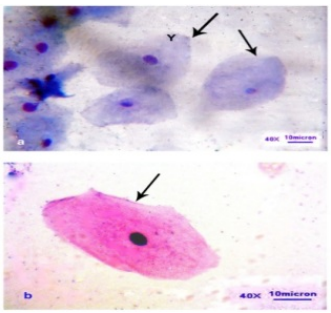

Figure 1: Normal oral squamous cells (a. Papanicolaous stain x 400, b. Giemsa stain x 400).

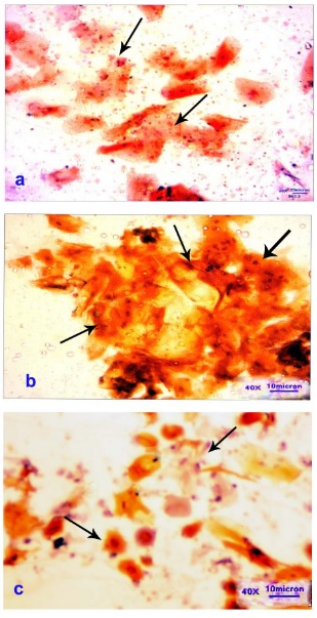

Figure 2: Pattern of keratin expression in PKSCs (a. PKSCs in erythro-leucoplakia, b. Hyper keratinized PKSCs in ulcerative erythroplakia, c. Hypo- and hyperkeratinized PKSCs in CIS).

Considering this pattern of keratin expression and nature of plumping or clustering, these newly found cells are named as plump keratinized squamous cells (PKSCs) and have been reported for the first time in details.

Attributes	Leucoplakia	Erythroplakia	Benign/CIS	Malignant
Number	Less	More	More	Rarely present/Absent
Differentiation	Well differentiated	Well differentiated	Well differentiated	Rarely if present, Well differentiated
Cytoplasm	Hypokeratinized	Hyperkeratinized	Hypo/hyperkeratinized	Rarely if present, Hypokeratinized
Nucleus	Appear to be Normal	Hyperchromasia	Hyperchromasia	Hyperchromasia
N/C Ratio	Appear to be Normal	Increased	Increased	Rarely if present, Increased

Table 1: Diagnostic attributes of PKSCs during multi-step oral carcinogenesis.

On the basis of International Classification of Diseases - 10th Edition, six cancer prone sites (such as lip, tongue, alveolus and gingiva, floor of the mouth, palate and buccal/cheek mucosa) of the oral cavity were taken into consideration. From the numerical point of view (Table 2), more number of PKSCs was scored in precancerous cases (7886 from 32 male and 5183 from 23 female) than the cancerous ones (1488 from 50 male and 519 from 31 female). Thus, a total of 9374 PKSCs from 82 male and 5702 PKSCs from 54 female was scored. With respect to oral sites, the highest relative percentages of the PKSCs (Figure 3) were recorded from buccal mucosa in precancerous (male-49.6% and female-37.5%). Among cancerous cases, the highest relative percentage was recorded from lingual carcinoma in male (21.8%) and from buccal mucosa in female (33.1%). Similarly, the lowest percentages were calculated from the palate in precancerous male (4.5%) and from tongue in female (6.7%). But, in cancerous group, the lowest relative percentages were calculated to be 10.0% in male from palate and 3.3% in female from lip. In addition to that, it has also been observed that, irrespective of age group and gender, the number of PKSCs was found to be in declined trend from precancerous lesions (leucoplakia and erythroplakia) to cancerous cases (Figure 4). It is important to note that, in most of the malignant cases the PKSCs were not found at all.

No	Oral Sites	Age groups in years	No. of samples		Precancerous						Cancerous					
	(ICD-10*)				No. of samples		PKSCs Scored		Total PKSCs		No. of samples		PKSCs Scored		Total PKSCs	
			Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	Lip	30-49	2	Nil	1	Nil	280	Nil	622-7.9	818-15.8	1	Nil	22	Nil	265-17.8	17-3.3
		50-69	2	4	1	3	342	479			1	1	12	17		
		70-89	1	2	Nil	1	Nil	339			1	1	231	Nil		
2		30-49	5	2	4	1	711	101	1182-14.9	357-6.9	1	1	34	9	324-21.8	79-15.3
	Tongue	50-69	5	4	3	2	471	256			2	2	11	8		
		70-89	1	1	Nil	Nil	Nil	Nil			1	1	279	62		
3	Alveolus and gingiva	30-49	4	3	1	2	368	480	1094-13.9	1049-20.2	3	1	72	54	154-10.3	54-10.5
		50-69	8	3	3	2	447	569			5	1	58	Nil		
		70-89	4	Nil	1	Nil	279	Nil			3	Nil	24	Nil		
4	Floor of the mouth	30-49	4	1	2	1	357	123	725-9.2	639-12.3	2	Nil	18	Nil	283-19	140-26.9
		50-69	2	4	1	2	368	516			1	2	Nil	12		
		70-89	1	1	Nil	Nil	Nil	Nil			1	1	265	128		
5	Palate	30-49	4	1	3	Nil	240	116	354-4.5	378-7.3	1	1	25	22	149-10	57-10.9
		50-69	1	2	Nil	1	114	262			1	1	Nil	35		
		70-89	1	Nil	Nil	Nil	Nil	Nil			1	Nil	124	Nil		
6	Buccal mucosa	30-49	14	4	6	1	1478	130	3909-49.6	1942-37.5	8	3	115	32	313-21.1	172-33.1
		50-69	16	19	5	6	1692	1490			11	13	162	125		

		70-89	7	3	1	1	739	322			6	2	36	15		
All sites		30-89	82	54	32	23	7886	5183	7886	5183	50	31	1488	519	1488	519

Table 2: Enumeration and relative percentage of PKSCs with respect to oral sites, age group, gender and degree of pathogenicity. Source: Primary data. Figures in parentheses indicate relative percentage with respect to the total number of PKSCs scored. *ICD-10: International Classification of Diseases-10th Edition.

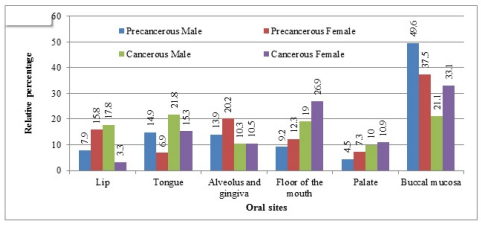

Figure 3: Oral site and sex-wise relative percentage of PKSCs in cancer affected groups.

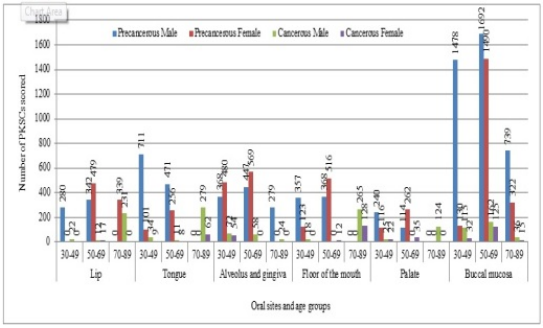

Figure 4: Numerically decreasing trend of PKSCs from precancerous to cancerous cases in different age groups of both sexes.

Considering the age groups and degree of pathogenicity, more than 50% cases were in cancerous group in all those three age groups except in the age group 30-49 years in palate. Almost all the palatal cases were smokers. Probably, due to pyrogenic activity of smoke, the roof of the buccal cavity has been affected in this younger age group, On the other hand, keeping khaini and oral snuff (a hydrated powder form mixture of dried tobacco leaves and lime) at a particular site (on the surface or below the tongue, near the lip, either side of buccal mucosa), chewing of paan/betel, gutkha, betel quids and drinking of alcohol as a modern life style contribute a lot for keratinization in PKSCs during oral carcinogenesis.

Morphometric analysis (Table 3) indicates that the cytoplasmic length, breadth and area of PKSC were found to in decreasing trend from precancerous to cancerous with an increasing trend of nuclear-cytoplasmic (N/C) ratio in both sexes (Figure 5). In precancerous stage, the cytoplasmic mean length, breadth and area of PKSC were measured to be 68.976 µm (±15.238 µm), 62.315 µm (±12.443 µm), 4285.832 µm^2 (±189.606 µm^2).

whereas the mean nuclear length, breadth and area were calculated to be 12.585 µm (±3.201 µm), 12.201 µm (±2.875 µm), 153.525 µm^2 (±3.202 µm^2) respectively with an N/C ratio of 1:27.9 in male. In case of female, the mean cell length, breadth and area were recorded to be 65.887 µm (±14.340 µm), 62.774 µm (±16.135 µm), 3938.329 µm^2 (±231.375 µm^2) followed by the mean nuclear length, breadth and area of PKSC were 12.110 µm (±2.988 µm), 11.245 µm (±3.274 µm), 136.176 µm^2 (±9.782 µm^2) respectively with an N/C ratio of 1:28.9 in female.

Group	Cell type	Sex	Total no. of cells scored	Cytoplasm (C)			Nucleus (N)			N/C Ratio	F-value
				Mean length in µm+SD	Mean breadth in µm+SD	Mean area in µm^2 + SD	Mean length in µm+SD	Mean breadth in µm+SD	Mean area in µm^2 + SD		
Control	NOSC	Male	1000	86.50 ± 17.234	64.23 ± 12.125	5555.89 ± 208.362	12.95 ± 3.368	12.43 ± 1.235	160.968 ± 3.689	1:34.5	
		Female	1000	85.875 ± 16.374	65.012 ± 11.876	5582.905 ± 194.475	12.920 ± 3.544	12.530 ± 3.452	161.887 ± 12.233	1:34.4	
Precancerous	PKSC	Male	7886	68.976 ± 15.238	62.315 ± 12.443	4285.832 ± 189.606	12.585 ± 3.201	12.201 ± 2.875	153.525 ± 3.202	1:27.9	24.36*
		Female	5183	65.887 ± 14.340	62.774 ± 16.135	3938.329 ± 231.375	12.110 ± 2.988	11.245 ± 3.274	136.176 ± 9.782	1:28.9	23.45*

Cancerous	PKSC	Male	1488	52.412 ± 16.963	48.046 ± 17.121	2518.186 ± 290.423	11.905 ± 2.432	11.843 ± 2.245	140.990 ± 5.459	1:17.8	25.12*
		Female	519	52.814 ± 13.275	46.035 ± 11.228	2431.292 ± 149.051	11.303 ± 3.130	10.925 ± 2.385	123.485 ± 7.465	1:19.6	28.33*
Total PKSCs		Male	9374	61.120 ± 17.085	38.947 ± 13.625	2380.440 ± 232.783	12.376 ± 2.012	10.047 ± 2.110	124.341 ± 4.245	1:19.1	52.47*
		Female	5702	60.685 ± 18.535	40.145 ± 13.875	2436.199 ± 257.173	11.487 ± 2.321	10.865 ± 2.245	124.806 ± 5.210	1:19.5	56.33*

Table 3: Morphometric analysis of NOSCs and PKSCs in different groups. Source: Primary data. SD-Standard deviation. *Significantly increased ($p<0.01$) F-values (One-way ANOVA followed by Tukey's pair-wise comparison).

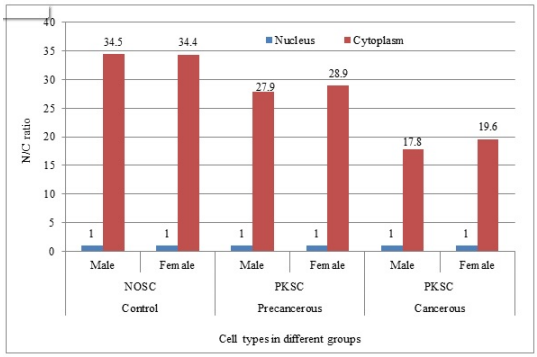

Figure 5: Comparative accounts of Nuclear-Cytoplasmic (N/C) ratio between NOSCs and PKSCs in different groups.

In cancerous groups, the recorded mean cytoplasmic length, breadth and area of PKSC were 52.412 µm (±16.963 µm), 48.046 µm (±17.121 µm), 2518.186 µm^2 (±290.423 µm^2) and mean nuclear length, breadth and area were 11.905 µm (±2.432 µm), 11.843 µm (±2.245 µm), 140.990 µm^2 (±5.459 µm^2) respectively in male. The N/C ratio was calculated to be 1:17.8 in male. Similarly, the mean cytoplasmic length, breadth and area of PKSC in female were recorded to be 52.814 µm (±13.275 µm), 46.035 µm (±11.228 µm), 2431.292 µm^2 (±149.051 µm^2) and mean nuclear length, breadth and area were found to be 11.303 µm (±3.130 µm), 10.925 µm (±2.385 µm), 123.485 µm^2 (±7.465 µm^2) respectively. The N/C ratio was calculated to be 1:19.6 in female.

In toto, the mean cytoplasmic length, breadth and area of PKSC were recorded to be 61.120 µm (±17.085 µm), 38.947 µm (±13.625 µm) and 2380.440 µm^2 (±257.173 µm^2) in where as the mean length, breadth and area of the nucleus were calculated to µm be 12.376 µm (±1.012 µm), 10.047 µm (±1.110 µm) and 124.341 µm^2 (±1.123 µm^2) respectively in males. The N/C ratio in was found to be male 1:19.1. In case of females, the mean length, breadth and area of the PKSCs were measured to be 60.685 µm (±8.535 µm), 40.145 µm (±3.875 µm)and 436.199 µm^2 (±33.073 µm^2), respectively. The mean length, breadth and area of the nucleus were found to be 11.487 µm (±2.321 µm), 10.865 µm (±2.245 µm) and 124.806 µm^2 (±5.210 µm^2) respectively. Thus the N/C ratio, of PKSC in female was calculated to be 1:19.5 in female. Comparatively, the N/C ratios of PKSCs in precancerous and cancerous groups are found to be significantly ($p<0.01$) higher than the normal oral squamous cells (NOSCs) in both sexes (1:34.5 in male and 1:34.4 female).

Discussion

Cytological pleomorphism is well observed during oral carcinogenesis. Not only that keratin expression in such cells has not less importance for pleomorphism and oral carcinogenesis. Mohanta et al. have reported that most of the pleomorphic cells are moderately differentiated and few are either well differentiated or poorly differentiated in oral squamous cell carcinoma (OSCC). Furthermore, they have also reported that during keratinization the water content of the cells decreases dramatically in spite of the fluid environment in the mouth. Keratin in the mouth remains relatively translucent as compared with skin, probably because it is kept moist and because the cornified layers are normally compact [6].

On account of keratin expression in PKSCs, it may be attributed to the carcinogenic effect of tobacco and alcohol. Alcohol along with tobacco acts synergistically on buccal mucosa and alter the nuclear content at the gene level. Basically, tobacco contains a number of carcinogens which trigger gene mutation followed by oral carcinogenesis [7,8]. The mutagenic, clastogenic and carcinogenic properties of areca nut, the major constituent of pan masala, have been extensively studied in a variety of experimental systems [9]. Dave et al., have reported that areca nut contains 5-40% polyphenols and several alkaloids including arecoline, arecaidine, guvacine and guvacoline. Arecoline, the most important areca nut alkaloid, is present at 1% of the dry weight and has been shown to be genotoxic [10]

During carcinogenesis, particularly, the oncogenes are activated and the tumor suppressor genes (TSGs) are inactivated due to genotoxic effect of these carcinogens and mutagens. Numerous report showed that, p16 is absent or rarely expressed where as p53 found to be well expressed in head and neck SCC [11-13]. Babiker et al. have studied the expressions pattern of p16 and CK19 were noticed 40% and 58% in the OSCC respectively. Whereas, expressions pattern were different in control cases for both markers as p16 (70%) and CK19 (20%). There was progressive loss of p16 expression from oral inflammatory lesion to OSCC and this differences were statically significant ($p<0.05$). The positivity of p16 were observed in gender where its expression pattern did not reach statistical significance ($p>0.05$) [14].

Most of the cytokeratins (CK) are found to be over expressed in tumor tissue when compared with the normal. Aberrant expression of K8, K18 is most common change in human oral cancer [15]. K4 and

K13 are expressed in lingual mucosa. Cytokeratins, such as, CK19 and K6hf are expressed significantly higher than the normal oral epithelium. Particularly, the degree of K6hf expression increases from normal to mild epithelial dysplasia to severe epithelial dysplasia (malignant). Schweizer et al. have also reported that the CK19 positive rate in cancerous tissue was 90.9% (30 out of 33) detected using immunohistochemistry, which was significantly higher than that in distant tissue (15.2%, 5 out of 33) which may be correlated with pathologic differentiation grade and prognosis in oral squamous cell carcinoma patients [16]. Findings of Al-Eryani et al. indicated that immature and mature keratin pearls in carcinoma in situ (CIS) and squamous cell carcinoma (SCC) were generated by oxidative stresses derived from erythro-haemophagocytosis, which might mediate HO-1 expression and be regulated by PAR-2. Such haemorrhage from the rupture of blood vessels can be one of the triggers for keratin pearl formation in oral CIS and SCC [17]. Thus, keratin expression in well differentiated PKSCs may be considered as a sign and index of cellular alteration in oral epithelia and a progenitor of cytological pleomorphism during oral carcinogenesis. But, what types of keratins are expressed in these PKSCs demand further investigation.

Earlier workers have shown that cytomorphometry is helpful in determining the malignant changes through estimating the nucleus/cytoplasm ratio in exfoliative cytology [18-21]. Ramaesh, et al. [22] reported the highest cellular diameter in the healthy mucosa and the lowest cellular diameter in the dysplastic lesions and oral SCC, which is consistent with a study by Einstein and Sivapathasundharam who have worked on tobacco users in southern India [23]. Franklin and Smith reported that increased nucleus/cytoplasm ratio might be due to changes in the size of the nucleus relative to the size of the cytoplasm and is possibly a reflection of significant changes in the cell at the morphologic level [24].

From the cytopathological point of view, in this study, it is presumed that the non-keratinized normal oral squamous cells (NOSCs) become keratinized first in response to prevent cellular dehydration and to resist the potentiality of various mutagens and carcinogens present in tobacco and alcohol. As the patients were addicted to various forms of tobacco and alcohol for more than 15 years, the exfoliated mucosal cells become mostly hyperkeratinized. Due to hyperkeratinization, the cytoplasmic content of the PKSCs get condensed followed by reduction in cellular diameter. On the other hand, although the nucleus of the PKSC gets affected due to carcinogenic stress and strain, it tends to maintain its normalcy. Yet, a significant reduction of the cellular and nuclear diameter followed by an increased N/C ratio was observed in the PKSCs, cytomorphometrically.

Conclusion

The pattern of keratin expression in the well differentiated plump keratinized squamous cells (PKSCs) was observed to be either hypokeratinized (in leucoplakia) or hyperkeratinized (in erythroplakia and hypo- or hyperkeratinized in CIS) cytoplasm with distinctly visible hyperchromatic nuclei in the exfoliated cyosmears of precancerous lesion and benign neoplasm. Morphometrically, the cellular and nuclear diameter were reduced considerably than the normal counterpart. But, a significantly ($p<0.01$) increased tendency of N/C ratios of PKSCs from precancerous (1:27.9 in male and 1:28.9 in female) to cancerous groups (1:17.8 in male and 1:19.6 in female). In toto, the N/C ratio in was found to be 1:19.1 in male and 1:19.5 in case of females with respect to the normal oral squamous cells (NOSCs) was observed in both sexes (1:34.5 in male and 1:34.4 female). It indicates that there is a positive correlation between the pattern of keratin expression and N/C ratio of PKSCs which may be considered as a sign and index of cellular alteration in oral epithelia and PKSCs become a progenitor of cytological pleomorphism during oral carcinogenesis. Cytopathologically, mostly ignored, but for the first time reported PKSCs have, thus, a practical utility during diagnoses and in early detection of oral cancer. But, what types of keratins are expressed in these PKSCs in the long run of muli-stage oral carcinogenesis demand further investigation.

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