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Original article

Nuclear pleomorphism-based cytopathological grading in human oral neoplasm

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Abstract: *Objective* — The objective of the present study is record the nuclear pleomorphism in various stages of oral carcinogenesis and to analyse their utility in cytopathological grading for early detection of human oral cancer
Material and Methods — In this hospital based case-control study, oral site, age-group and sex-matched 272 subjects (136 cases and 136 normal healthy individuals) were included. Scraped exfoliated cytosmears were collected from the affected oral site of the subjects and smearing was done in the pre-cleaned-coded glass-slides. Two such slides were prepared from each subject. The cytosmears were immediately fixed in aceto-alcohol (1 part of glacial acetic acid: 3 part of absolute ethyl alcohol) fixative. One set of the slide was stained with Papanicolaou's stain and the other set was counter-stained with Giemsa's Solution for cytopathological analysis. Test of proportion (z-test) was followed and the critical ratio (z-value) was calculated for the test of significance.
Results — Nuclear pleomorphism in the form of round, oval, spindle, elongated fiber as well as irregular shapes were mostly observed in oral squamous cells during different stages of carcinogenesis. Appearance of such nuclear pleomorphism in human oral neoplasm may be considered as a sign of cellular alternation in general and index of oral carcinogenesis in particular. In the present study, the frank malignant cases mimic to be either premalignant lesions or benign/carcinoma in situ were detected on the basis of nuclear pleomorphism-based cytopathological grading and so an increasing trend was observed from precancerous lesions to malignant cases due to shifting of numbers. Diagnostic tests also indicated that the Sensitivity was calculated to be 83.5%, Specificity was 100%, positive predictive value (PPV) was 100%, negative predictive value (NPV) was 30% and the accuracy was found to be 84.6%. Therefore, the nuclear pleomorphism-based cytopathological grading system makes itself an ideal screening test for early detection of human oral cancer.
Conclusion — Pattern of nuclear pleomorphism corresponding to various cytological atypias is a common feature observed during different stages of oral carcinogenesis and thus, it has a practical implication in grading and early detection of oral cancer.

Keywords: nuclear pleomorphism, cytological atypias, cytopathological grading, oral neoplasm

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Introduction

Oral cancer is a long-latency multi-stage pathological event. A progressive gross clinical features in the form of leukoplakia, erythroplakia, benign and malignant tumours are characterized by pleomorphic cytological alteration and a number of nuclear anomalies. Being nucleus is the genetic store-house of the cell [1], any alteration at nuclear level leads to nuclear anomalies in general and the genetic components in particular. Ultimately, the deformity of nucleus has been observed in the cytological atypias during oral carcinogenesis which lead to nuclear pleomorphism. Nuclear pleomorphism coupled with changes in chromatin amount and distribution in the nucleus, remain the basic microscopic criteria for a cytologic diagnosis of cancer. Moreover, in several cancer types, e.g. in breast cancer, cervical cancer nuclear pleomorphism is graded and correlates with clinical aggressiveness and patient outcome [2-4]. Bussolati et al. (2008) have stated that evaluation of nuclear pleomorphism represents a novel parameter of interest in pathological staging and grading [5].

Wang et al. (2015) have pointed out that several nuclear membrane proteins such as emerin, lamin A/C, lamin B, and lamin-associated polypeptide 2 (LAP2) are the architectural components of the nuclear membrane which play an important role in maintaining nuclear structure and coordinating cell activity [6]. Various workers have reported that altered lamin expression or localization and disrupted stoichiometry between A- and B-type lamins can change the elastic properties of the nuclear envelope (NE), which renders it unable to withstand cytoskeleton- and chromosome-based forces. Consequently, these lead to alteration of nuclear morphology followed by nuclear pleomorphism which results in an inheritable disease called laminopathy. Therefore the nuclear lamina alterations might directly account for the cancer-related changes in the nuclear morphology [7-13].

Gadiwan et al. (2014) have reported that nuclear morphology reflects the biological potential and proliferative activity of the cell. They have also confirmed that the nuclear morphometry forms a reliable and reproducible tool that provides an

opportunity to quantify the nuclear changes associated with dysplasia and affords an objective basis for grading dysplasia to predict their malignant potential [14].

Although different histologic grading systems such as Broder's, Anneroth's, Bryne's and Jakobsson's grading systems are used, cytopathology has been used as a primary tool for screening of oral squamous cell carcinoma (OSCC) for many decades. Ignoring invasive punch biopsy for histopathology, the patients usually prefer to non-invasive exfoliative cytopathology for oral cancer detection and diagnosis. Modified version of exfoliative cytopathology like fine needle aspiration cytology (FNAC), brush cytology have also been extensively used for the stated purpose and also used as an adjunct to histopathology [15].

It is important to note that both cytopathology and histopathology are nucleo-centric. On the basis of architectural configuration of the nucleus, the real state of the concerned cell can be determined. Least number of papers has been published on nuclear pleomorphism, to date, so far as human OSCC is concerned. Furthermore, works on nuclear pleomorphism-based grading system are very rare, inadequate and confined to the micronucleus assay only. In this regard, credit goes to Palve and Tupkari (2008) who have found that the percentage of micronuclei was uniformly elevated in all histologic grades of OSCC, suggesting a strong cytogenetic damage of the oral epithelium [16]. Recently, Namala et al. (2016) have studied on micronuclei frequency and reported that there was 60% correlation between the cytological grade and histological grade and the difference between them was found to be insignificant. They have also concluded that cytological grading can be used as an alternative to histological grading in grading of OSCC [17]. Therefore, a humble attempt was undertaken to record a broad spectrum of the nuclear pleomorphism in various stages of oral carcinogenesis, among addicted and non-addicted groups and to analyse their utility in cytopathological grading for early detection of human oral cancer.

Material and Methods

Subjects

In a hospital-based study, out of 136 oral cases, 82 (60.3%) were males and 54 (39.7%) were females registered at the Out-patient Department (OPD) of Acharya Harihar Regional Cancer Centre (AHRCC), Cuttack, Odisha during May 2007 to May 2009 were included in this study. A written consent from the patient was obtained in our self-designed proforma as well as the detailed case-history (including the nature and types of addiction) of each individual was recorded prior to the collection of samples. Considering the nature of addiction, out of 136 cancer-affected individuals, 126 (92.6%) were addicted to different forms of tobacco and alcohol for more than 15 years and 10 (7.4%) were non-addicted.

Out of 82 males, 33 (40.3%) 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 (66.6%) were grouped under 50-69 years and 7 (13.7%) belong to 70-89 years of age group.

Age-group and sex-matched 136 non-addicted healthy individuals were also included in this study as Control group. Thus, a total of 272 subjects were taken into account for this study.

Collection of samples and staining

Prior to the collection of sample, written consent of the respective subject was obtained. Two scalpel -scraped exfoliated cytosmears were collected from the affected site of the oral cavity on the pre cleaned-coded glass-slides. Collected cytosmears were fixed in 1:3 aceto-alcohols (1 part of glacial acetic acid and 3 parts of ethyl alcohol) immediately. A set of smears was stained with Papanicolaou's stain and the other set was counterstained with Giemsa's stain for cytopathological analysis. Photomicrographs were taken out as the supporting evidences by using computer assisted Bio-Catalyst Cat Cam 1.30 microscope camera (Manufacturer: Catalyst Biotech, Maharashtra, India).

Statistical analysis

Out of 1,000 observed cells, nuclear pleomorphism among the various cytological atypias were recorded. Microsoft Excel as well as Software package PAlaentological STatistics (PAST), Version 2.17 were used for statistical analysis. Test of proportion (z-test) was followed and the critical ratios (z-values) were calculated for the test of significance.

Ethical considerations

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

Results

Oral sites

According to International Classification of Diseases-10th Revised Edition (ICD-10), six oral sites (lip, tongue, alveolus and gingiva, floor of the mouth, palate and buccal mucosa) are the cancer-prone sites. On the basis of ICD-10, out of 136 oral cases, highest numbers of cases (45.2% males and 48.2% females) were suffering from cancer at buccal mucosa and the lowest numbers of cases (7.3% males and 35.6% females) were recorded as palatal cancer (*Table* 1).

Degree of pathogenicity

Basing on the degree of gross clinical pathogenicity, 15 (18.3%) males and 14 (25.9%) females were of leukoplakia (21.3%), 17 (20.7%) males and 9(16.7%) females were of erythroplakia, 35 (42.7%) males and 20 (37.0%) females were of benign (40.5%) cases and 15 (18.3%) males and 11 (20.4%) females were of malignant (19.1%) cases (*Figure* 1).

Nuclear pleomorphism

A progressive gross clinical features in the form of leukoplakia, erythroplakia, benign and malignant tumors has been characterized by pleomorphic cytological alteration. Being nucleus is the genetic store-house of the cell, any alteration at nuclear level leads to nuclear anomalies in general and the genetic components in particular. Therefore, nuclear morphology and nature of chromasia provides the cytopathologists with most of information necessary to make a correct diagnosis.

Table 1. Site and sex-wise collected samples

No	Sites	Control group (n=136)			Cancer affected group (n=136)			Grand Total
		Male	Female	Total	Male	Female	Total	
1	Lip	5 (6.1)	6 (11.1)	11 (8.0)	5 (6.1)	6 (11.1)	11 (8.0)	22 (8.0)
2	Tongue	11 (13.4)	7 (12.9)	18 (13.2)	11 (13.4)	7 (12.9)	18 (13.2)	36 (13.2)
3	Alveolus and gingiva	16 (19.5)	6 (11.1)	22 (16.2)	16 (19.5)	6 (11.1)	22 (16.2)	44 (16.2)
4	Floor of the mouth	7 (8.5)	6 (11.1)	23 (9.6)	7 (8.5)	6 (11.1)	13 (9.6)	26 (9.6)
5	Palate	6 (7.3)	3 (5.6)	9 (6.7)	6 (7.3)	3 (5.6)	9 (6.7)	18 (6.7)
6	Buccal mucosa	37 (45.2)	26 (48.2)	63 (46.3)	37 (45.2)	26 (48.2)	63 (46.3)	126 (46.3)
All sites		82	54	136	82	54	136	272

Data presented as number and percentage (from all sites) – no (%).

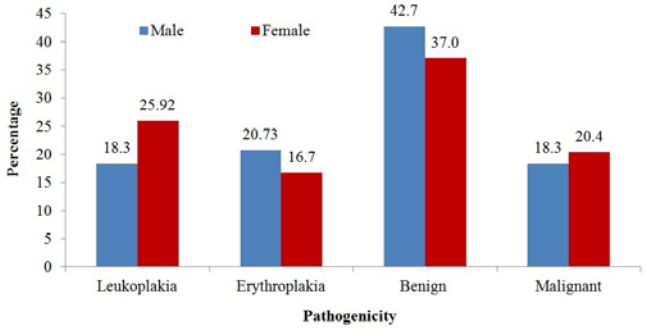

Figure 1. Sex-wise degree of oral pathogenicity

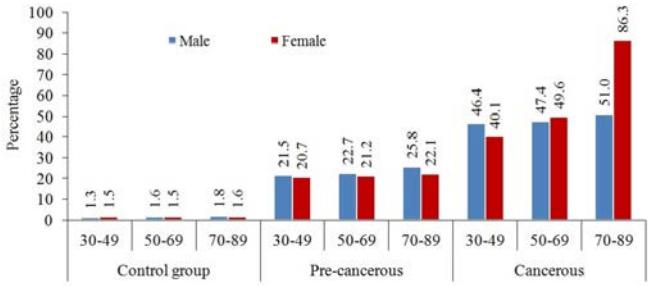

Figure 3. Age and sex-wise percentage of cytological atypias in control and cancer affected groups

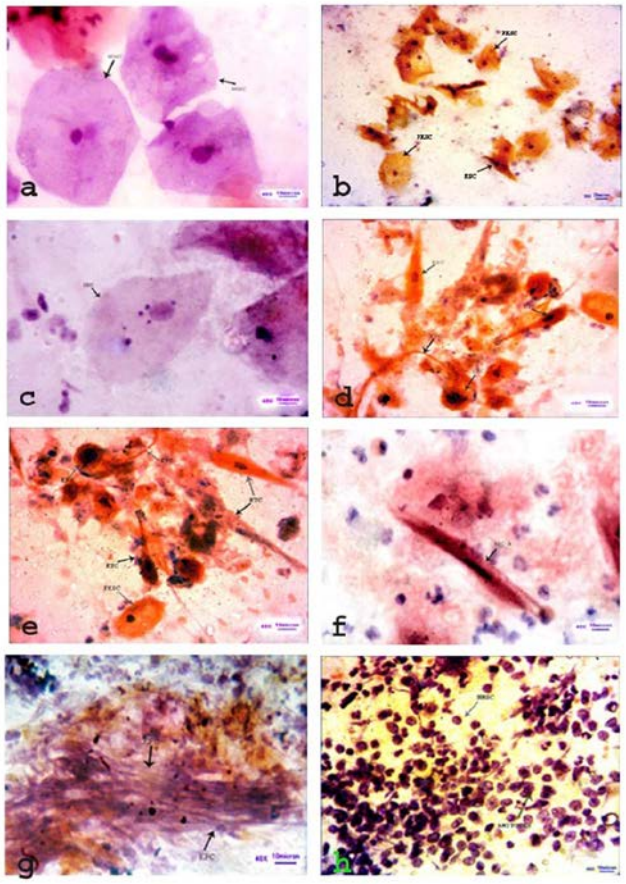

Figure 2. Nuclear pleomorphism in detected oral cytological atypias: a) NOSC; b) PKSCs and KSC; c) an MNC with a number of micronuclei; d) KTCs, KFC and KRC; e) KTCs, KRC and PKSC; f) KSC-A; g) a bundle of KFCs; h) NMSCs with a number of amitoses.

On the basis of their morphological peculiarities and nature of keratinization, a number of pleomorphic cytological atypias were observed in various stages of oral carcinogenesis [18]. In the exfoliated cytosmear of Control group, the cells were mostly normal oral squamous cells (NOSC), few Plump keratinized squamous cells (PKSC) [19] and rarely Micronucleated cells (MNC) were found. The nuclear morphology appears to be either round or oval in shape. In precancerous lesions, as in leukoplakia stage, more numbers of PKSCs and MNCs were scored than the Control group. It has been observed that number of MNCs were observed to be in increasing order with increase in degree of pathogenicity where as the number of PKSCs were found to be in decreasing order gradually [20]. In some of the leukoplakia cases few Keratinized spindle cells (KSC) [21] and Keratinized tadpole cells (KTC) [22] were also observed. Similarly, in erythroplakia stage, few more cytological atypias, like Keratinized tadpole cells (KTC) and Keratinized strap (Anitschkow) cells (KSC-A) [23] were found. In these atypias, the nuclei were observed to be round, oval, elliptical or ribbon like strapped or flattened. In addition to these, Keratinized fibre cells (KFC) [24] and keratinized round cells (KRC) [25] were rarely found among erythro-leukoplakia, but frequently observed in benign / carcinoma in situ (CiS) and malignant neoplasm cases. In KFCs, the nuclei were drastically modified and found to be fibre like elongated. The nuclear-cytoplasmic (N/C) ratios in these cytological atypias were previously reported to be in increased state in comparison to their normal counter-part [18-25].

A unique type of atypia named as Non-keratinized malignant squamous cells (NMSC) was observed in the samples of frank malignant neoplasm cases. These atypias were mostly either round or oval in shape, very small in size and absolutely non-keratinized due to lack of cytoplasm.

Table 2. Age and sex-wise cytological atypias in control and oral cancer affected groups

No	Groups	Age groups, years	Number of samples		Number of atypical cells scored		Percentage of atypical cells		Mean percentage of atypical cells		Critical ratio (z-value)	
			Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	Control	30-49	33	11	445	163	1.35	1.48				
		50-69	34	36	537	552	1.56	1.53	1.52	1.53		
		70-89	15	07	271	110	1.81	1.57				
		30-89	82	54	1,247	825	1.52	1.53	1.52	1.53		
2	Pre-cancerous	30-49	8	5	1,722	1,036	21.53	20.72				
		50-69	18	14	4,080	2,967	22.67	21.19	22.96	21.24	89.73*	71.74*
		70-89	6	4	1,546	883	25.77	22.08				
3	Cancerous	30-49	25	6	11,606	2,406	46.42	40.10				
		50-69	16	22	7,897	10,906	47.36	49.57	48.19	51.29	205.14*	172.34*
		70-89	9	3	4,592	2,588	51.02	86.27				
		30-89	82	54	31,443	20,746	38.35	38.49	38.35	38.49	210.34*	171.18*

* Significant differences (p<0.01) from Control group.

Table 3. Age and sex-wise cytological atypias in control and addicted groups

No	Group	Age group, years	Number of sample screened		Number of atypical cells scored		Percentage of atypical cells		Mean percentage of atypical cells		Critical ratio (z-value)*	
			Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	Control	30-49	33	11	445	163	1.35	1.48				
		50-69	34	36	531	552	1.56	1.53	1.52	1.53		
		70-89	15	07	271	110	1.81	1.57				
		30-89	82	54	1,247	825	1.52	1.53	1.52	1.53		
2	Chewers	30-49	11	06	3,823	2,406	34.75	40.40				
		50-69	07	11	2,530	5,124	36.14	46.58	36.86	46.83	110.11*	126.98*
		70-89	05	03	2,127	1,836	42.54	61.20				
3	Smokers	30-49	02	Nil	401	Nil	20.05	Nil				
		50-69	05	02	1,110	464	22.20	23.20	23.43	23.20	48.09*	22.93*
		70-89	02	Nil	598	Nil	29.90	Nil				
4	Alcoholics	30-49	04	04	836	829	20.90	20.73				
		50-69	05	12	1,070	2,850	21.40	23.75	23.94	24.18	57.21*	70.03*
		70-89	03	02	967	673	32.23	33.65				
5	Chewers-Smokers	30-49	09	Nil	4,438	Nil	49.31	Nil				
		50-69	12	03	4,814	1,467	40.11	43.06	44.44	49.88	132.6*	61.03*
		70-89	03	01	1,415	528	47.16	52.23				
6	Chewers smokers alcoholics	30-49	04	Nil	2,566	Nil	64.15	Nil				
		50-69	03	06	1,415	3,409	51.60	56.81	58.11	56.81	102.28*	86.16*
		70-89	01	Nil	535	Nil	53.50	Nil				
7	Non-addicted cancers	30-49	03	01	1,264	207	42.13	20.70				
		50-69	02	02	905	559	45.25	27.95	44.41	30.00	66.72*	39.19*
		70-89	01	01	496	434	49.60	43.40				
Total (2-7)		30-89	82	54	31,443	20,786	38.35	38.49	38.35	38.49	210.34*	171.18*

* Significant differences (p<0.01) from Control group.

Mohanta et al. (2016) have reported that the small, round or oval nucleus of the NMSC become enlarged and touched the cell boundary (plasma membrane) thereby the nuclear-cytoplasmic (N/C) ratio was found to be 1:1 [26]. They have also reported that except the NOSC and NMSCs, all these atypias get keratinized and appear to be either orange or yellow or orange-red in color due to acidic (eosinophilic) cytoplasm with Papanicolaou's stain. Frequent amitoses were observed in these cytological atypias (*Figure* 2).

The cytoplasm in various atypias appears to be dense, and keratinized. Tremendous reactive changes were observed during different stages of oral carcinogenesis. From the numerical point of view, the percentages of atypical cells were calculated to be 1.35, 1.56, 1.81 in males and 1.48, 1.53, 1.57 in females in 30-49, 50-69 and 70-89 years in Control group. Thus, the mean percentages of atypical cells were recorded to be 1.52 in male and 1.527 in female. In precancerous group, the percentage of atypical cells were found to be 21.53, 22.67 and 25.77 in male, whereas in female, these were 20.72, 21.19 and 22.08 in 30-49, 50-69 and 70-89 years of age groups, respectively. The mean percentages of atypical cells were found to be 22.96 in male and 21.24 in female in precancerous group. In cancerous group, the percentages of atypia were 46.424 in male and 40.10 in female respectively in the age group 30-49 years, 49.36 in male and 49.57 in female in the group of 50-69 years and 51.02 in male and 86.27 in female in 70-89 year of age group (*Figure* 3). The mean percentages of atypical cells were calculated to be 48.19 in male and 51.29 in female in cancerous group. In both precancerous and cancerous groups, the z-values in males and females were observed to be significantly ($p<0.01$) higher than the normal observed value, where $z = 2.58$ (*Table* 2).

Table 4. Nuclear pleomorphism-based cytopathological grading in multi-stage oral carcinogenesis

No	*Groups*	*Principal Atypias*	*Nuclear pleomorphism*	*Number of cases, no. (%)*			*CPG*
				Male	Female	Total	
1	Control	NOSC, PKSC, MNC	Round, Oval, Regular	82 (100)	54 (100)	136 (100)	Grade 0
2	Leukoplakia	PKSC, MNC, KSC	Round, Oval, Elliptical, Spindle	5 (6.1)	4 (7.4)	9 (6.6)	Grade I
3	Erythroplakia	PKSC, MNC, KSC, KSC-A, KTC,	Round, Oval, Elliptical, Spindle, Elongated flat	12 (14.6)	9 (16.7)	21 (15.4)	Grade II
4	Benign	PKSC, KSC, MNC, KSC-A, KTC, KRC, KFC	Round, Oval, Elliptical, Spindle, Elongated flat/ Fibre	26 (31.7)	18 (33.3)	44 (32.4)	Grade III
5	Malignant	PKSC, KSC, MNC, KSC-A, KTC, KRC, KFC, NMSC	Round, Oval, Elliptical, Spindle, Elongated flat/ Fiber, Regular	39 (47.6)	23 (42.6)	62 (45.6)	Grade IV
Total (2-5)				82 (100)	54 (100)	136 (100)	

Data in parentheses indicate relative percentage with respect to number of cases. CPG, cyto-pathological grading.

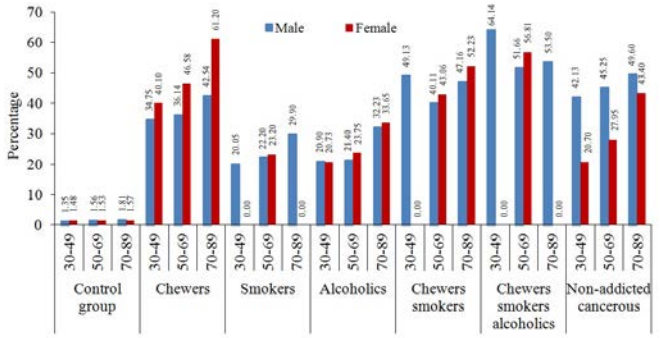

Figure 4. Age and sex-wise frequency of cytological atypias in control, addicted and non-addicted cancer affected groups

Among the addicted groups, the lowest percentages of atypical cells were recorded to be 20.90, 21.40 and 32.23 in males and 20.73, 23.75 and 33.65 in females in alcoholic group in the age group of 30-49 and 70-89 years, respectively. The percentages of cytological atypias in chewers-smokers-alcoholics group were recorded to be the highest and were found to be 2-4 folds to that of alcoholic group. In chewers-smokers-alcoholics group, the percentages of atypical cells are recorded to be 64.15, 51.6 and 53.50 in males in 30-49, 50-69 and 70-89 years of age groups, respectively. No female patients were recorded in the age group of 30-49 and 70-89 years. However, in 50-69 years, the percentage was calculated to be 56.81. In non-addicted cancerous group, the percentages of cytological atypias was recorded to be 42.13 in male and 20.70 in females, in the age group of 30-49 years, 45.20 in males and 27.95 in females in the age group of 50-69 years, 49.60 in male and 43.40 in females in 70-89 years, respectively (*Figure* 4). The lowest mean percentage of cytological atypias were recorded to be 23.43 in male and 23.20 in female in Smokers group and the highest were found to be 58.11in male and 56.81 in female in multi-addicted Chewer-smoker-alcoholics group. The test of proportions also clearly indicates that the z-values in each group are significantly ($p<0.01$) higher than the tabulated values, where $z=2.576$ (*Table* 3).

It was also observed that there was an increase in the percentage of atypias from lower to higher age group in both sexes in Control and cancer affected groups. On the other hand, a higher percentage of cytological atypias were marked in 30-49 years followed by 70-89 and 50-69 years in addicted groups. This was probably due to the multi-carcinogenic effect of tobacco and alcohol on the buccal mucosa of younger generation.

In this case-control study, the number of cytological atypias followed by nuclear pleomorphism was found to be in gradual increasing order from Control/Normal group to malignant group. The frequency of atypias and nuclear anomalies were also observed to be in increased state from lower (30-49 years) to higher age (60-79 years) groups with increase in degree of pathogenicity. It has also been found that more number of nuclear pleomorphism in multi-addicted (chewer-smoker-alcoholic, chewer-smoker) groups than the single addicted (chewers, smokers, alcoholics) groups. Interestingly, least number of pleomorphism was recorded among smoker and alcoholic groups. It is important to note that the frequencies of cytological atypias were recorded to be more in non-addicted cancerous group than the single-addicted smoker- and alcoholic groups which demands further investigation.

Cytopathological grading

Based on nuclear pleomorphism, cytopathological grading was done. Cytological atypias such as PKSCs and MNCs with round or oval nuclei were observed to be well differentiated and morphologically resembled with the NOSCs. Therefore, both PKSCs and MNCs were kept under Grade 0. Moderately differentiated atypias including more MNCs and KSCs if found in the exfoliated cytosmears were of Grade I and more atypias, particularly KTCs and KSC-A if observed, were kept under Grade II. Similarly, cytosmears with KFCs and KRCs along with other atypias were assigned to Grade III and atypia with more NMSCs in cytosmear if found were considered as Grade IV (*Table* 4). Cytological atypias, such as PKSC and MNC are well differentiated; KSC, KSC-A, KTC, KFC and KRC are moderately differentiated whereas NMSCs are absolutely poorly differentiated cytological atypias. As a result, this novel cytopathological grading, partially corroborates with the Broder's grading system of cytological differentiation [27].

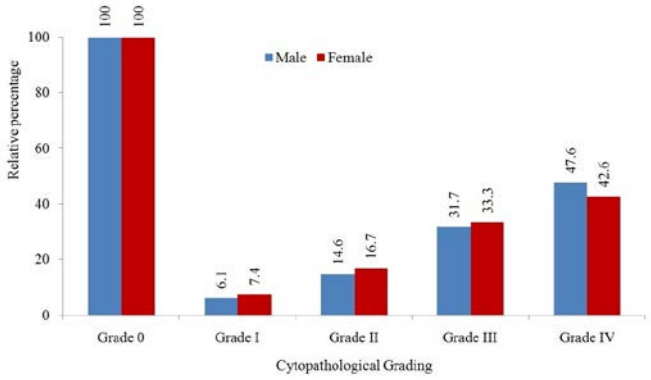

Figure 5. Age and sex-wise relative percentage with respect to cytopathological grading

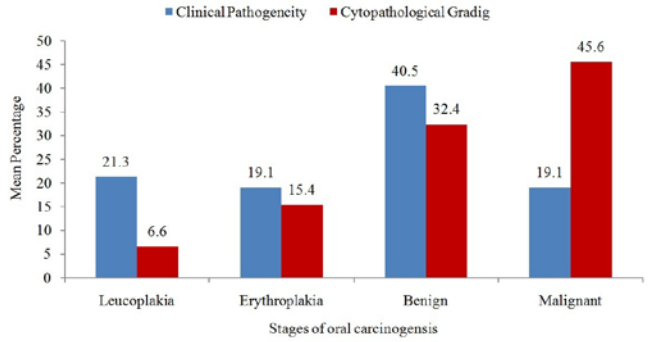

Figure 6. A comparative account of mean percentages between gross clinical pathogenicity and cytopathological grading

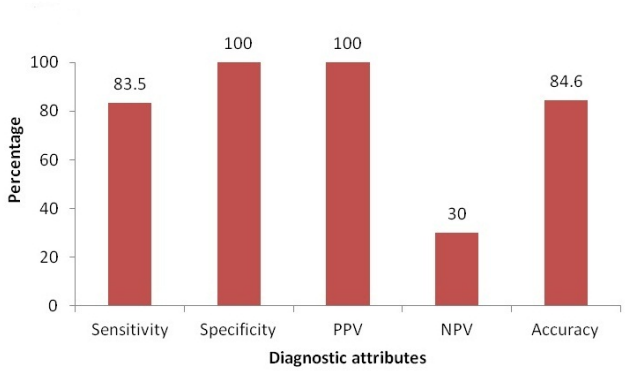

Figure 7. Outcome (in percentage) of diagnostic test for cytopathological grading

On the basis of such cytopathological grading, all the normal healthy individuals (82 male and 54 female) were included in Grade 0. But, drastic alteration in number of cases with respect to degree of pathogenicity was observed due to nuclear pleomorphism-based cytopathological grading. More number of cases were included in malignant group/ Grade IV (45.6%) who were earlier included in the premalignant lesion groups (Leukoplakia/ Grade I-6.6% and Erythroplakia/ Grade II-15.4%) and benign group/Grade III (32.4%) respectively (*Figure* 5). In other words, cases in lower groups with higher malignant potentiality were shifted to the malignant group leading to increase the mean percentage from 19.1% to 45.6% (*Figure* 6).

Table 5. Status of oral malignancy potential with respect to cytopathological grading

Disease status	*Malignant*	*Non-malignant*	*Row Total*
Malignant	TP = 106	FP = 0	TP+FP = 106
Non-malignant	FN = 21	TN = 9	FN+TN = 30
Column Total	TP+FN = 127	FP+TN = 9	TP+FN+FP+TN = 136

TP, true positive cases; FN, false negative cases; FP, false positive cases; TN, true negative cases.

Table 6. Diagnostic tests for cytopathological grading

Attributes	*Calculating formula*	*Outcome*
Sensitivity, %	TP/ (TP+FN)	83.5
Specificity, %	TN/ (FP+TN)	100
PPV, cu	TP/ (TP+FP)	100
NPV, cu	TN/ (FN+TN)	30.0
Accuracy	(TN + TP)/ (TN+TP+FN+FP)	84.6

TP, true positive cases; FN, false negative cases; FP, false positive cases; TN, true negative cases; PPV, positive predictive value; NPV, negative predictive value.

Sensitivity, specificity and accuracy of the diagnostic test

Considering the malignancy potential, 106 cases were found to be true positive (TP) and 9 cases were of true negative (TN). No cases were recorded to be false positive (FP), whereas, false negative (FN) cases were recorded to be 21 in number (*Table* 5). Therefore, the Sensitivity was calculated to be 83.5%, Specificity was 100%, Positive Predictive Value (PPV) was 100% and Negative Predictive Value (NPV) was 30%. Ultimately, the accuracy of our diagnostic test was calculated to be 84.6% (*Table* 6, *Figure* 7).

Discussion

Oral cancers are malignant neoplasms that affect the tissues of the mouth. Eventually, the multi-stage transformation of normal oral mucosal cell to a malignant one has been attributed to the consumption of tobacco and alcohol, some of the biological agents (oral lichen planus, Epstein-Barr virus, human papilloma virus), poor oral hygiene, age, gender, familial pre-disposition, changing food pattern, diet and modern life style [29].

The biology of OSCC has been evaluated and descriptively categorized as highly, moderately and poorly differentiated. Broder primarily developed this quantitative grading of cancer in 1920. Lack of correlation between Broders' grades and the prognosis of OSCC has been explained by the fact that SCC's usually exhibit a heterogeneous cell population with probable differences in invasiveness and metastasis behaviour [30]. Subsequently, multi-factorial malignancy grading systems were developed by Jakobsson et al. (1973) and further improved by Anneroth and Hansen (1986) for application to SCCs in the tongue and the floor of the mouth [31,32]. Histological grading based on Broder's classification has been a traditional pathologic tool with documented prognostic value, but has not been incorporated into standard therapeutic planning strategies. This is mainly due to the subjectivity of the current grading system and the lack of consensus regarding its prognostic value [33]. Extensive scoring methods have been designed in an attempt to increase the objectivity of these parameters [34-37]. Other ancillary parameters, such as mitotic index, DNA content, Ki-67, proliferation cell nuclear antigen, and bromodeoxyuridine labelling index, may be used in an attempt to identify new prognostic indicators [38, 39]. But, due to financial constraints, availability of advanced parameters along with advanced technology for carrying

out research in the developing countries, like us, are the real limitations. Therefore, cost, benefit and effectiveness oriented exfoliative cytology can be utilized as a mobile on-site-lab- tool for mass screening of oral cancer in the poor and developing world.

Cytological pleomorphism has been well documented with respect to human oral carcinoma and nuclear pleomorphism has long been known as a typical feature of cancer cells and still retains a fundamental role in routine cytopathological and-histopathological diagnoses. Nuclear pleomorphism includes variation in shape, size, color and texture of the respective cytological atypias in the oral neoplasm. Earlier workers have reported that chromatism (staining pattern) as well as chromatin pattern in tumour cell nuclei in histopathologic preparations often varies from normal nuclei, and from each other [40-42]. Abnormal mitoses are also well observed in tumour cells [43]. These features are sufficiently unique to form much of the basis of the diagnosis of tumours in histopathology and cytopathology. More severe degrees of these nuclear abnormalities often correlate loosely with greater clinical aggressiveness of tumours [40-42].

Generally, irregularity of nuclear membrane was observed in neoplastic nuclei, while its normal counterpart was found to be regular. Irregularity of the nuclear membrane might be due to adhesion of abnormal chromatin clumps to the inner surface. Fuhrman et al. (1982) have reported that cellular degeneration promotes irregularity of nuclear membrane in neoplastic nuclei and has been observed to increase with increasing gradations of carcinoma [44]. In the present study, various nuclear pleomorphism which ranges from round/oval (in PKSC, MNC, KRC) to strapped/flattened (in KSC-A) and from spindle (in KSC) to fibre (in KFC) were observed. Except KFC, in all other atypias, the nuclear membranes were found to be deviated from the normal counterpart, but regular and distinctly visible which contradicts with the facts of Fuhrman et al. However, Zink et al. (2004) were of opinion that there are characteristic differences in the nuclear architectures of cancer cells, compared with normal cells, and some anticancer treatments restore normal nuclear structure and function [45]. In the present study, all the patients were referral patients and none of them were undergone any type of treatment and therefore, it warrants further investigation.

Nuclear pleomorphism is a distinctive feature of malignancy. Lower the degree of nuclear variation, lower is the degree of pathogenicity and vice-versa. In a nut-shell, nuclear pleomorphism is directly proportional to the degree of oral pathogenicity. In the present study, it has been observed that there was a hell and heaven differences in number of cases when compared with gross clinical study on degree of pathogenicity and nuclear pleomor phism-based cytopayhological grading. Cytopathologically, more number of patients were found to be in advanced stage and were mimic to be in premalignant and vice versa. As a result of cytopathological grading, the real picture came into existence and so an increasing trend was observed from precancerous lesions to malignant cases due to shifting of numbers. The cytological findings of Sousa et al. (2014) have demonstrated that the 86.79% of the cases were found to have malignant potential [46]. Thus, the present finding corroborates the earlier reports of Mc Kinley and Sousa et al.

Francois et al. (1997) have reported that chromatins were very finely granular and almost invisible in normal oral squamous cells, while these chromatins form well defined irregular clumps with variable size, shapes and sharp pointed projections in neoplastic cells. Furthermore, they have reported that lower grades of OSCC showed delicate chromatin strands and homogenous chromatin pattern while higher grades exhibited coarse, clumped and heterogeneous chromatin pattern. Normal inconspicuous, regular nucleoli in normal cells turned into very prominent, enlarged, irregular and more in number, with occasional sharp pointed projections in malignant cells which may be attributed to increased protein synthesis [47]. Abnormal mitoses were also frequently noticed and found to be more in higher grades of carcinoma than the lower grades which corroborates with the findings of Francois et al.

Bignold (2003) has reported that genetic instability is an early and essential part of tumour development. This instability provides for substantially random cell-to-cell genomic variation (genomic heterogeneity) to arise among cells of individual tumours. Genetically unstable cells then produce 'successful' clones of cells with the necessary mutations for malignant transformation [48].

Chewing and smoking of tobacco as well as drinking of alcohol have been reported as the risk factors for oral cancer. Chewing areca quid generates reactive oxygen species (ROS) that might cause oxidative DNA damage to surrounding tissues in the oral cavity [49]. The production and release of ROS occurs under alkaline condition, during the auto-oxidation of areca-nut polyphenols in the saliva of betel-quid-chewers [50]. Prokopczyk et al. (1987) have opined that areca-nut-specific-nitrosamines (ASNA) along with tobacco-specific-nitrosamines (TSNA) are found to be mutagenic, genotoxic, and carcinogenic in nature and are capable of inducing tumors in the oral cavity [51]. The areca nut extract can also impairs actin organization that causes fibroblastoid morphologic changes of oral keratinocytes [52]. The genetic susceptibility to such environmental carcinogens and the resulting altered molecular expressions might be potential markers for a diagnosis and prognosis of OSCC [53]. Mohanta et al. (2013) have reported that there has been a detrimental genotoxic effect of tobacco and alcohol on oral mucosa. They have found that chewing and smoking of tobacco as well as drinking of alcohol enhance the rate of formation of micronuclei in MNC along with other cytological atypias followed by OSCC [54]. The combined effects of tobacco abuse and alcohol consumption are found to be multiplicative. Compared with persons who neither drink nor smoke, the risk of developing OSCC is increased 80 fold in persons with the highest level of smoking and alcohol consumption [55].

To be addicted is now become a fashion, a part of modern life style. In our study, out of 136 cancer affected individuals, 126 (92.6%) were addicted to different forms of tobacco and alcohol for more than 15 years and 10 (7.4%) were non-addicted. The frequency of cytological atypias along with nuclear pleomorphism was recorded to be more in multi-addicted (chewer-smoker-alcoholic, chewer-smoker) groups than the single addicted (chewer, smoker, alcoholic) groups. Interestingly, least number of pleomorphism was recorded among smoker and alcoholic groups. Really, the combined effects of tobacco and alcohol trigger genotoxicity followed by nuclear pleomorphism in oral squamous cells. Ultimately, cellular alteration takes place in the respective oral site which accelerates the multi-stage mechanism of oral carcinogenesis.

Different diagnostic methods such as routine exfoliative cytopathology, fine needle aspiration cytology (FNAC), histopathology, and immunohistochemistry are available today. Out of these, exfoliative cytopathology is, particularly, valuable for

mass screening of oral carcinoma. Evaluating the quality of cytology as a diagnostic method for OSCC, Fonte et al. (2013) have reported that the sensitivity was 83.1%, the specificity was 100%, the positive predictive value (PPV) was 100%, the negative predictive value (NPV) was 49% and the accuracy was 85.5% [56]. Recently, compared with cytopathological and histopathological diagnoses, Hafez et al. (2014) have also found that the sensitivity was 93.5%; specificity was 96.2%; PPV was 97.7%; NPV was 89.3% with a diagnostic accuracy of 94.4%. According to them, FNAC was found to be highly accurate in the diagnosis of oral lesions. Detailed cytomorphologic examination coupled with clinical data and appropriate immuno-cytochemical study, in some cases, can lead to an accurate diagnosis of the oral cavity lesions [57]. In the present study, the sensitivity was calculated to be 83.46%, specificity was 100%, PPV was 100%, NPV was 30% and the accuracy of the diagnostic test was found to be 84.55% which corroborates with the findings of Fonte et al. and Hafez et al.

The presence of typically atypical cells may be correlated with tumour progression. The infiltrating macrophage count was correlated with the progression of OSCC and is a prognostic marker [58, 59]. Cytomorphological alterations indicate the pathological status of the concerned cell. Alizadeh et al. (2016) have reported that shape differences are sufficient to enable a neural network to classify cells accurately as belonging to the highly invasive or the less invasive phenotype. The patterns of shape changes were also reproducible for repetitions of the experiment. They have also strongly suggested that cell-shape may provide a means to read out the phenotypic state of some cell types, and shape analysis can be usefully performed using a Zernike moment representation [60].

In the present study, the broad spectra of detected cytological atypias not only exhibit cytological pleomorphism but also nuclear pleomorphism in different stages of oral carcinogenesis. Thus, nuclear pleomorphism may be considered as a sign of cellular alternation and index of oral carcinogenesis.

Early detection of oral cancer is not an easy task. Most of the malignant neoplasms mimic to be either precancerous lesions or benign ones and vice versa leading to delay in diagnosis and treatment. It is the nucleus that expresses the genotypic alterations caused in the process of malignancy and exfoliative cytology is a method that gives better insight of the nuclear changes in individual cells. Our present findings clearly depict the importance of nuclear pleomorphism in the respective cytological atypias in grading of human oral neoplasm and thereby drawing out a simple solution for such difficult diagnostic dilemma in general and early detection of oral cancer in particular.

Conclusion

Nucleus reflects biological potential and general activity of the cell. Being the genetic store house of the cell, any alternation either at gross level or at molecular level, the nucleus and its constituents become reactive and exhibit pleomorphism. Alteration may be due to the induction of mutagenic, genotoxic or carcinogenic agents, nuclear pleomorphism is a real fact. Appearance of nuclear pleomorphism in human oral neoplasm may be considered as a sign of cellular alternation in general and index of oral carcinogenesis in particular. In the present study, the frank malignant cases mimic to be either premalignant lesions or benign/carcinoma in situ were detected on the basis of nuclear pleomorphism-based cytopathological grading and so an increasing trend was observed from precancerous lesions to malignant cases due to shifting of numbers. Diagnostic tests also indicated that the Sensitivity was calculated to be 83.46%, Specificity was 100%, PPV was 100%, NPV was 30% and the accuracy was found to be 84.55%. Therefore, the nuclear pleomorphism based cytopathological grading system makes itself an ideal screening test for early detection of oral cancer.

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References

1. McKinley ET. General cytologic principles. In: Atlas of diagnostic cytopathology, 2nd Edition. BF Atkinsons, Ed. Saunder, Elsevier Inc, Philadelphia, 2004: 4-25.
2. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991; 19(5): 403–410. https://doi.org/10.1111/j.1365-2559.1991.tb00229.x.
3. Volpi A, Bacci F, Paradiso A, Saragoni L, Scarpi E, Ricci M, et al. Prognostic relevance of histological grade and its components in node-negative breast cancer patients. *Mod Pathol* 2004; 17(9):1038–1044. https://doi.org/10.1038/modpathol.3800161.
4. Tang JR, Mat Isa NA, Ch'ng ES. Evaluating nuclear membrane irregularity for the classification of cervical squamous epithelial cells. *PLoS ONE* 2016; 11(10): e0164389. https://doi.org/10.1371/journal.pone.0164389.
5. Bussolati G. Proper detection of the nuclear shape: ways and significance. *Rom J Morphol Embryol* 2008; 49(4): 435–439. https://www.ncbi.nlm.nih.gov/pubmed/19050790.
6. Wang J, Kondo T, Yamane T, Nakazawa T, Oish N, Mochizuki K, Katoh R. Expression of nuclear membrane proteins in normal, hyperplastic, and neoplastic thyroid epithelial cells. *Virchows Arch* 2015; 467: 427. https://doi.org/10.1007/s00428-015-1816-6.
7. Swift J, Ivanovska IL, Buxboim A, Harada T, Dingal PC, Pinter J, et al. Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed differentiation. *Science* 2013; 341: 1240104. http://dx.doi.org/10.1126/science.1240104.
8. Coffinier C, Jung HJ, Nobumori C, Chang S, Tu Y, Barnes RH 2nd, et al. Deficiencies in lamin B1 and lamin B2 cause neurodevelopmental defects and distinct nuclear shape abnormalities in neurons. *Mol Biol Cell* 2011; 22: 4683-4693. http://dx.doi.org/10.1091/mbc.E11-06-0504.
9. Fukuda R, Hayashi A, Utsunomiya A, Nukada Y, Fukui R, Itoh K, et al. Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). *Proc Natl Acad Sci USA* 2005; 102: 15213-15218. http://dx.doi.org/10.1073/pnas.0507184102.
10. Chuang CH, Carpenter AE, Fuchsova B, Johnson T, de Lanerolle P, Belmont AS. Long-range directional movement of an interphase chromosome site. *Curr Biol* 2006; 16: 825-831. http://dx.doi.org/10.1016/j.cub.2006.03.059.

11. Weber SC, Spakowitz AJ, Theriot JA. Nonthermal ATP-dependent fluctuations contribute to the in vivo motion of chromosomal loci. *Proc Natl Acad Sci USA* 2012; 109: 7338-7343. http://dx.doi.org/10.1073/pnas.1119505109.
12. Maraldi NM, Capanni C, Cenni V, Fini M, Lattanzi G. Laminopathies and lamin-associated signaling pathways. *J Cell Biochem* 2011; 112: 979-992. http://dx.doi.org/10.1002/jcb.22992.
13. Capell BC, Collins FS. Human laminopathies: nuclei gone genetically awry. *Nat Rev Genet* 2006; 7: 940-952. http://dx.doi.org/10.1038/nrg1906.
14. Gadiwan M, Madhushankari GS, Mandana DD, Praveen SB, Selvamani MS, Pradeep DS. Nuclear features in different grades of epithelial dysplasia in leukoplakia: A computer assisted microscopic study. *J Oral Maxillofac Pathol* 2014; 18(2): 194-200. http://dx.doi.org/10.4103/0973-029X.140747.
15. Rickles NH. Oral exfoliative cytology: an adjunct to biopsy. *CA Cancer J Clin* 1972; 22(3): 163–171. http://dx.doi.org/10.3322/canjclin.22.3.163.
16. Palve DH, Tupkari JV. Clinico-pathological correlation of micronuclei in oral squamous cell carcinoma by exfoliative cytology. *J Oral Maxillo Fac Pathol* 2008; 12(1): 2-7. http://dx.doi.org/10.4103/0973-029X.42189.
17. Namala S, Guduru VS, Ananthaneni A, Devi S, Kuberappa PH, Udayashankar U. Cytological grading: an alternative to histological grading in oral squamous cell carcinoma. *J Cytol* 2016; 33:130-4. http://dx.doi.org/10.4103/0970-9371.188048.
18. Mohanta A, Mohanty PK, Parida G. Pattern of keratinization in oral squamous cells during carcinogenesis. *IOSR J Dent Med Sci* 2014; 13(7.IV): 83-91. http://www.iosrjournals.org/iosr-jdms/papers/Vol13-issue7/Version-4/S013748391.pdf.
19. Mohanta A, Mohanty PK. Pattern of keratin expression and its impact on nuclear-cytoplasmic ratio in plump keratinized squamous cells during oral carcinogenesis. *J Med Diagn Meth* 2016; 5(1): 1-7. http://dx.doi.org/10.4172/2168-9784.1000200.
20. Mohanta A, Mohanty PK, Parida G. An in vivo Cytogenetic analysis of human oral squamous cell carcinoma. *South Asian J Cancer* 2015; 4(3): 123-126. http://dx.doi.org/10.4103/2278-330X.173178.
21. Mohanta A, Mohanty PK, Parida G. Keratinized Spindle cell: A diagnostic cytological atypia in oral neoplasm. *IOSR J Dent Med Sci* 2014; 13(8.IV): 72-80. http://dx.doi.org/10.9790/0853-13847280.
22. Mohanta A, Mohanty PK, Parida G. Keratinized tadpole cells in human oral neoplasm: A cytodiagnostic approach. *IOSR J Dent Med Sci* 2014; 13(9.VI): 110-119. http://dx.doi.org/10.9790/0853-1396110119.
23. Mohanta A, Mohanty PK, Parida G. Keratinized strap cells: a rare cytological atypia resembles Anitschkow cells, in human oral neoplasm. *Int J Clin Oncol* 2016; 21: 59–67. http://dx.doi.org/10.1007/s10147-015-0865-9.
24. Mohanta A, Mohanty PK, Parida G. Differential cytomorphometric analysis of Keratinized fiber cells in human oral carcinoma. *IOSR J Dent Med Sci* 2014; 13(12.I): 48-57. http://dx.doi.org/10.9790/0853-131214857.
25. Mohanta A, Mohanty PK, Parida G. Cytomorphometric analysis of keratinized round cells in human oral carcinoma. *J Cytol* 2015; 32(2): 107-112. http://dx.doi.org/10.4103/0970-9371.160561.
26. Mohanta A, Mohanty PK. Cytomorphometric analysis of non-keratinized malignant squamous cells in exfoliated cytosmears of human oral neoplasm. *J Carcinog Mutagene* 2016; 7(1): 1-8. http://dx.doi.org/10.4172/2157-2518.1000247.
27. Broders AC. Carcinoma: grading and practical application. *Arch Pathol Lab Med* 1926; 2: 376. http://www.translationalres.com/article/S0022-2143(32)90498-3/pdf.
28. Mohanta A, Mohanty PK, Parida G. Aetio-pathology of oral mucosal cells during carcinogenesis. *Pranikee Journal of Zoological Society of Orissa* 2013; XXVI: 44-64.
29. Anneroth G, Batsakis J, Luna M. Review of the literature and a recommended system of malignancy grading in oral squamous cell carcinomas. *Scand J Dent Res* 1987; 95: 229-249. https://www.ncbi.nlm.nih.gov/pubmed/3299675.
30. Anneroth G, Hansen LS. A methodologic study of histologic classification and grading of malignancy in oral squamous cell carcinoma. *Scand J Dent Res* 1984; 92: 448-468. https://www.ncbi.nlm.nih.gov/pubmed/6593810.
31. Roland NJ, Caslin AW, Nash J, Stell PM. Value of grading squamous cell carcinoma of the head and neck. *Head Neck* 1992; 14:224-9. http://dx.doi.org/10.1002/hed.2880140310.
32. Ensley JF, Crissman J, Kish J, Jacobs J, Weaver A, Kinzie J, et al. The impact of conventional morphologic analysis on response rates and survival in patients with advanced head and neck cancers treated initially with cisplatin-containing combination chemotherapy. *Cancer* 1986; 57: 711-717. http://dx.doi.org/10.1002/1097-0142(19860215)57:4<711::AID-CNCR2820570405>3.0.CO;2-C.
33. Jakobsson PA. Histological grading of malignancy and prognosis in glottic carcinoma of the larynx. *Can J Otolaryngol* 1975; 4(5): 885-892. https://www.ncbi.nlm.nih.gov/pubmed/1203794.
34. Crissman JD, Lin WY, Gluckman JL, Cummings G. Prognostic value and histopathologic parameters in squamous cell carcinoma of the oropharynx. *Cancer* 1984; 54: 2995-3001. http://dx.doi.org/10.1002/1097-0142(19841215)54:12<2995::AID-CNCR2820541230>3.0.CO;2-R.
35. Anneroth G, Hansen LS, Silverman S. Malignancy grading is oral squamous cell carcinoma: I, Squamous cell carcinoma of the tongue and floor of the mouth: Histologic grading in the clinical evaluation. *J Oral Pathol* 1986; 15: 162-168. http://dx.doi.org/10.1111/j.1600-0714.1986.tb00599.x.
36. Zatterstrom UK, Wennerberg J, Ewers SB, Willen R. Attewell R. Prognostic factors in head and neck cancer: histologic grading, DNA ploidy, and nodal status. *Head Neck* 1991; 13: 477-487. http://dx.doi.org/10.1002/hed.2880130603.
37. Barona de Guzman R, Martorell NA, Basterra J, Armengot M, Alvarez-Valdes R, Garin L. Prognostic value of histopathological parameter in 51 supraglottic squamous cell carcinomas. *Laryngoscope* 1993; 103: 538-540. http://dx.doi.org/10.1288/00005537-199305000-00011.
38. Klijanienko J, Janot F, De Braud F, Carlu C, Micheau C, Armand JP, et al. Correlation of tumor vascularization, mitotic index and Ki-67 expression in fresh biopsies from squamous cell carcinoma of the head and neck: New evaluation techniques and application of classic markers. In: Third International Head and Neck Research Conference, Las Vegas. Ann Arbor, MI: The University of Michigan Medical Centre, 1990: 26-28.
39. Kearslay JH, Furlong KL, Cooke RA, Waters MJ. An immunohistochemical assessment of cellular proliferation markers in head and neck squamous cell cancers. *Br J Cancer* 1990; 61: 821-827. https://www.ncbi.nlm.nih.gov/pubmed/2372483.
40. Walter JB, Talbot IC. General Pathology. Churchill Livingstone, Edinburgh, 1996: 425-470.
41. Frost JK. Pathologic Processes affecting cells from inflammation to cancer. In: Comprehensive Cytopathology. 2nd Edition. M Bibbo, Ed. Saunders, Philadelphia, 1997: 75-89.
42. Pathology. 3rd Edition. Rubin E, Farber JL, Eds. Lippincott, Philadelphia, 1999: 154-211.
43. Nandini DB, Subramanyam RV. Nuclear features in oral squamous cell carcinoma: A computer assisted microscopic study. *J Oral Maxillofac Pathol* 2011; 15(2): 177–181. https://dx.doi.org/10.4103%2F0973-029X.84488.
44. Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* 1982; 6: 655-663. https://www.ncbi.nlm.nih.gov/pubmed/7180965.
45. Zink D, Fischer AH, Nickerson JA. Nuclear structure in cancer cells. *Nature Reviews Cancer* 2004; 4: 677-687. https://dx.doi.org/10.1038/nrc1430.
46. Sousa MC, Alves MGO, Souza LA, Brandão AAH, Almeida JD, Cabral LAG. Correlation of clinical, cytological and histological findings in oral

squamous cell carcinomas. *Oncology Letters* 2014; 8: 799-802. https://dx.doi.org/10.3892%2Fol.2014.2212.

47. Francois C, Decaestecker C, Petein M, Van ham P, Peltier A, Pastees JL et al. Classification strategies for the grading of renal cell carcinomas, based on nuclear morphometry and densitometry. *J Pathol* 1997; 83: 141-150. https://doi.org/10.1002/(SICI)1096-9896(199710)183:2%3C141::AID-PATH916%3E3.0.CO;2-0.
48. Bignold LP. The mutator phenotype theory of carcinogenesis and the complex histopathology of tumours: support for the theory from the independent occurrence of nuclear abnormality, loss of specialisation and invasiveness among occasional neoplastic lesions. *Cell Mol Life Sci* 2003; 60(5): 883-891. https://doi.org/10.1007/s00018-003-2226-5.
49. Chen CL, Chi CW,Liu TY. Hydroxyl radical formation and oxidative DNA damage induced by areca quid in vivo. *J Toxicol Environ Health* 2002; 65: 327−336. https://doi.org/10.1080/15287390252800909.
50. Nair U, Obe G, Nair J, Maru GB, Bhide SV, Pieper R, Bartsch H. Evaluation of frequency of micronucleated oral mucosa cells as a marker for genotoxic damage in chewers of betel quid with or without tobacco. *Mutat Res* 1991; 261(3): 163-168. http://dx.doi.org/10.1016/0165-1218(91)90063-R.
51. Prokopczyk B, Brunnemann KD, Bertinato P, Hoffmann D. The role of N(nitrosomethylamino)-propionitrile in betel-quid carcinogenesis. *IARC Sci Publ* 1987; 84: 470-473. https://www.ncbi.nlm.nih.gov/pubmed/3679425.
52. Yang SC, Lin SC, Chiang WF, Yen CY, Lin CH, Liu SY. Areca nut extract treatment elicits the fibroblastoid morphological changes, actin re-organization and signaling activation in oral keratinocytes. *J Oral Pathol Med* 2003; 32: 600−605. http://dx.doi.org/10.1034/j.1600-0714.2003.00199.x.
53. Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, et al. Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis. *Cancer Sci* 2008; 99: 1507−1514. https://doi.org/10.1111/j.1349-7006.2008.00863.x.
54. Mohanta A, Mohanty PK, Parida G. Genotoxicity of tobacco and alcohol on human oral mucosal cells. Euro J Exp Biol 2013; 3(2): 503-14.
55. Scully C. Oral cancer aetiopathogenesis: past, present and future aspects. *Med Oral Patol Oral Cir Bucal* 2011; 16(3): 306-311. http://dx.doi.org/doi:10.4317/medoral.16.e306.
56. Fontes KBFC, Cunha KSG, Rodrigues FR, Silva LE, Dias EP. Concordance between cytopathology and incisional biopsy in the diagnosis of oral squamous cell carcinoma. *Braz Oral Res (São Paulo)* 2013; 27(2): 122-127. http://dx.doi.org/10.1590/S1806-83242013000100018.
57. Hafez NH, Fahim MI. Diagnostic accuracy and pitfalls of fine needle aspiration cytology and scrape cytology in oral cavity lesions. *Russian Open Medical Journal* 2014; 3: 0405. http://dx.doi.org/10.15275/rusomj.2014.0405.
58. Liu SY, Chang LC, Pan LF, Hung YJ, Lee CH, Shieh YS. Clinicopathologic significance of tumor cell-lined vessel and microenvironment in oral squamous cell carcinoma. *Oral Oncol* 2008; 44: 277−285. https://doi.org/10.1016/j.oraloncology.2007.02.007.
59. Lu CF, Huang CS, Tjiu JW, Chiang CP. Infiltrating macrophage count: a significant predictor for the progression and prognosis of oral squamous cell carcinomas in Taiwan. *Head Neck* 2010; 32: 18−25. https://doi.org/10.1002/hed.21138.
60. Alizadeh E, Lyons SM, Castle JM, Prasad A. Measuring systematic changes in invasive cancer cell shape using Zernike moments. *Integr Biol* 2016; 8: 1183-1193. https://doi.org/10.1039/c6ib00100a.

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