Main Article

Brush Cytology on Pre-Malignant and Malignant Oral Lesions with Histopathological Correlation

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ABSTRACT

Introduction

Oral cancer is the sixth most common malignancy worldwide and accounts for 30% of all cancers in India, with 5-year survival rate, except when diagnosed in the early stages. Hence, early diagnosis of oral cancer is very much essential for the sake of the patient. However its burden on the economy for providing healthcare is substantial and with the increasing incidence of oral cancer in developing countries like India and the other South-East-Asian countries, the role of screening methodologies for early detection of pre–cancerous and cancerous lesions of oral cavity are becoming more vital.

Materials and Methods

An observational cross-sectional study conducted in the departments of Otolaryngology & head neck surgery in close association with department of Pathology in a tertiary based teaching institute in North Bengal, India, during April 2021 to March 2022. All the patients aged above 18 years, who visited the outpatient department of Otolaryngology & Head Neck Surgery, and admitted in the ward of the same, having oral lesions which are clinically suspected as pre- malignant and malignant lesions were included in this study.

Results

The study population comprised of total 69 cases. Among them 47 cases (~68%) were malignant lesions, 13 (~19%) cases were pre-malignant and 9 (~13%) cases were diagnosed as benign lesions considering Histopathology result. 30 (63.8%) out of 47 malignant cases show class-5 cytological grading in brush cytology smear, stained with Pap stain. 25.5% of the malignant cases were in class-4 and 10.6% cases were in class-3 whereas, in premalignant cases (n=13), 3 cases were in class-2 and 7 cases were in class-3 and 3 were in class-1. Maximum value of AgNOR counts for benign, pre malignant and malignant lesions were 3.54, 4.16, 7.28 respectively.

Conclusion

The brush cytology with PAP grading and AgNOR analysis in clinically suspected oral lesions can be used as an early diagnostic tool for diagnosing oral squamous cell carcinoma especially for lower socio-economic status people who present with late stages.

Keywords

Oral Cancers; Pap Stain; Brush cytology; Histopathology

Oral cancer is the sixth most common malignancy worldwide and accounts for 30% of all cancers in India, with 5-year survival rate, except when diagnosed in the early stages.[1, 2] In India there is a delay in diagnosis which increases the morbidity and mortality[1] and thus early diagnosis is the need of the hour.[3] Biopsy has been the primary method for its diagnosis and is carried out only when the lesions become symptomatic, i.e. in the late/advanced stages.[4] Exfoliative cytology is one of the valuable aids for screening of malignant and potentially malignant oral lesions.[5] The most commonly

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followed technique for staining exfoliative cytology smears is the Papanicolaou (Pap) technique. Though exfoliative cytology is an easy, reliable technique, this comes with a high false-negative rate (range, 0–31%).[6] One of the most common failures of exfoliative cytology are faulty techniques of smear collection which result in insufficient quantity of cells.[7] Cytobrush can be one such tool where this faulty technique can be rectified. By using a cytobrush, cells can be uniformly spread over a slide thus allowing an easier interpretation.[8] Silver staining of nucleolar organizer regions-associated proteins (AgNORs) has become a frequently used method in tumour pathology mainly for assessing the prognosis of malignant tumors. NORs represent loops of DNA actively transcribing to ribosomal RNA and hence to ribosomes and ultimately to proteins.[9] NORs are associated with acidic, argyrophilic, non-histone proteins that are visualized using a silver staining technique.[10] Recent studies show a positive correlation between the number and/or the size of the argyrophilic NORs (AgNORs) and cellular proliferation.[11] Cytological study of oral cells is a non-aggressive technique that is well accepted by the patient, and is therefore an attractive option for the early diagnosis of oral cancer, including epithelial atypia, dysplasia and squamous cell carcinoma. However its usage has been limited so far due to poor sensitivity and specificity in diagnosing oral malignancies.

The purpose of this study is to determine the diagnostic accuracy of PAP staining and AgNOR staining in brush cytology specimens of clinically suspected oral lesions (Pre-malignant & malignant) & correlating with the histopathological diagnosis from the punch biopsy sample of the same. This study will also shed light to the occurrence, etiological factors, types of oral cancers among the population of the northern region of West Bengal.

Materials and Methods

This is an observational cross-sectional study conducted in the departments of Otolaryngology & head neck surgery in close association with department of Pathology in a tertiary based teaching institute in North Bengal, India, during April 2021 to March 2022. All the patients aged above 18 years, who visited the outpatient department of Otolaryngology & Head Neck Surgery, and admitted in the ward of the same, having oral lesions which are clinically suspected as pre- malignant (oral leukoplakia, oral erythroplakia, oral lichen planus, tobacco pouch keratosis and oral submucous fibrosis etc.) and malignant lesions were included in this study. Patients who refused to give consent or those who have received previous treatment or radiation for lesions, and those with recent onset of any local trauma or infection, were excluded from this study.

After rinsing the oral cavity thoroughly with water and mouth wash, the lesion has been visualized under adequate illumination. A commercially available cytobrush available in the Pap smear kit is being used to obtain a complete trans-epithelial sampling with minimal discomfort. Using moderate pressure, the cytobrush was repeatedly brushed and rotated in one direction over the entire lesion many times until pinpoint bleeding was obtained, signaling entry into lamina propria and thus obtaining epithelial cells through the full thickness of the epithelium. The material from the brush was spread on the middle third of 4 clean, dried glass slides. The smears were fixed immediately with 100% ethanol or by an alcohol-based spray fixative for staining with the modified Papanicolaou's method i.e., PAP stain (2 slide) and with AgNORs staining (2 slides). Finally, Punch biopsies were taken from the lesions and sent for histopathological examination (HPE). The diagnosis of HPE was considered final and later was corroborated with the findings of PAP and AgNOR smears.

The results which will be obtained in the counting procedure will be analyzed statistically by using the Student's t-test and one way analysis of variance (ANOVA test) for inter group comparisons.

The final grand chart was prepared compiling multiple tabulation sheets using Windows Excel software (Microsoft Corporation; Redmond, Washington, USA). Data entered were analyzed and presented in tabular and pictorial forms through relevant statistical methods

(proportions, percentages, etc.) using SPSS (Statistical Package for Social Sciences) software version 22 (IBM Corporation; Armonk, New York, USA). P values of less than 0.05 were considered statistically significant. The study was approved by the Institutional Ethical Committee. Informed consent in writing was obtained from each patient prior to his/her inclusion in the study. Investigations and interventions were strictly according to the principles stated in the declaration of Helsinki 1964 and its subsequent amendments.

Results

The study population comprised of total 69 cases having clinically suspected oral lesions (pre-malignant and malignant). Among them 47 cases (~68%) were diagnosed as malignant lesions, 13 (~19%) cases were pre-malignant and 9 (~13%) cases were diagnosed as benign lesions considering Histopathology result (Fig. 1).

The age distribution in the malignant cases, ranged from 31years to more than 71 years where majority of the cases were in the fifth decade (31.9%), followed by sixth decade (23.4%). In pre-malignant cases, age ranged from 31 to 70 years; maximum cases were in fifth decade (38.5%), followed by fourth decade (30.8%). Similarly benign cases were found from 4th to 6th decade with equal proportion in 4th and 5th decade (Fig. 2).

Out of 69 cases, 42 were males and 27 were females. The Male: Female ratio was 1.56 : 1. In malignant cases, Male: Female ratio was 1.04 : 1 & in pre-malignant cases, the Male: Female ratio was 5.5 : 1 (Fig. 3). All the patients (cases) in this study belonged to mid and low socio-economic status with a Mid: Low ratio of 1.16 : 1.

Among the 69 cases, 13 (18.8%) cases gave history of smoking with tobacco consumption and 13 (18.8%) cases gave history of all the three abuses (smoking, tobacco & betel nut consumption) together.12 cases gave history of tobacco & betel nut consumption, followed by 11 cases with history of only smoking. All the 13 cases having history of three abuses had been diagnosed having malignant lesions. 11 out of 13 cases with the habit of smoking & tobacco consumption were also diagnosed having malignant lesions. Out of 4 cases with no habit abuse, 1 was diagnosed malignant. Details of other similar risk factors findings are summarized in (Fig. 4).

17(~36.2 %) out of 47 malignant cases have cervical lymph-node involvement. Patients having pre-malignant and benign oral lesions had no regional lymph node involvement (Fig. 5).

The most common site of involvement was buccal mucosa (53.6%), followed by tongue (17.4%) (Fig. 6).

49.3% lesions appeared as ulcero-proliferative growth (49.3%) followed by ulcerative lesions (Fig. 7).

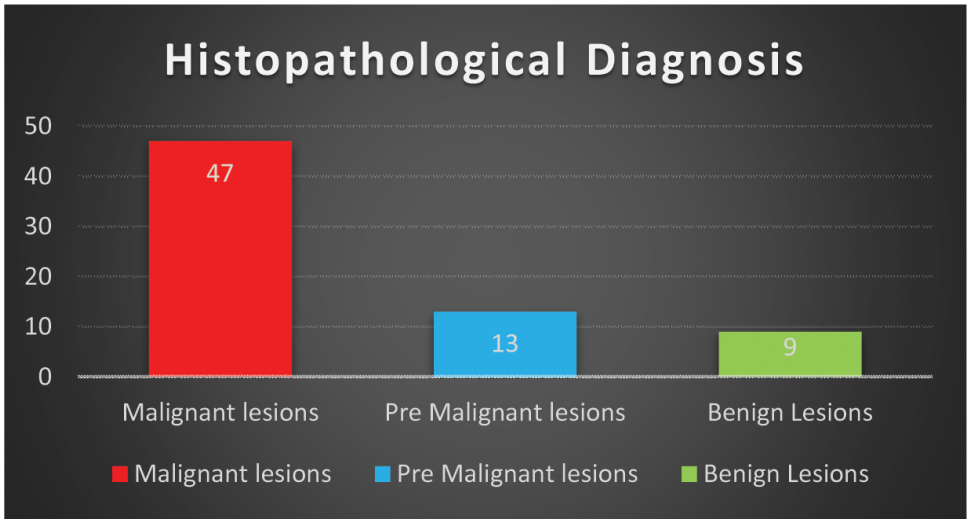

Fig. 1. Histopathological diagnosis of oral lesions in 69 cases

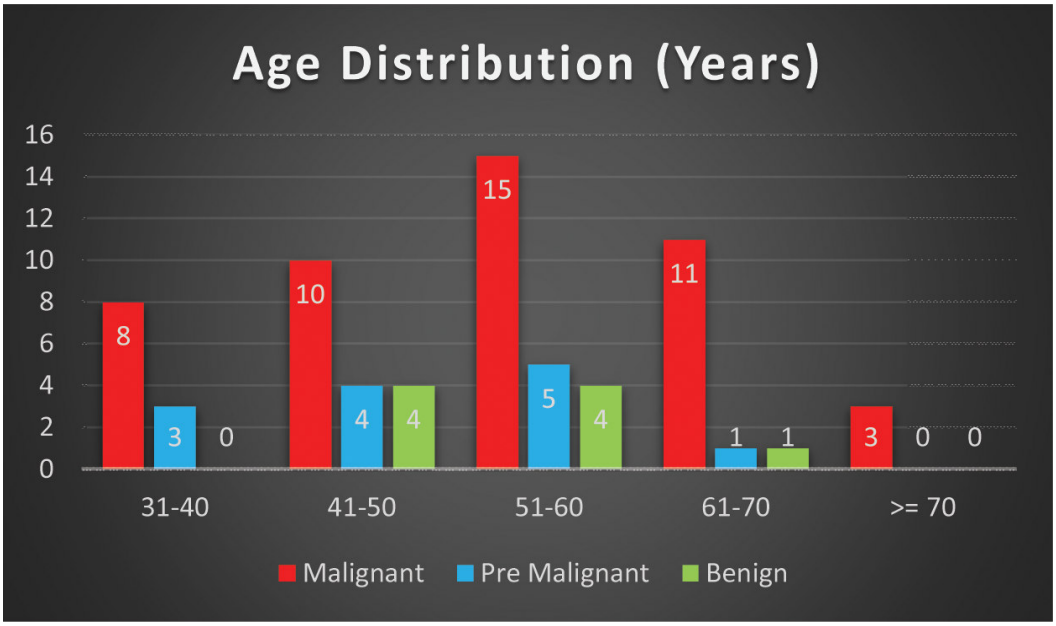

Fig. 2. Age distribution in years

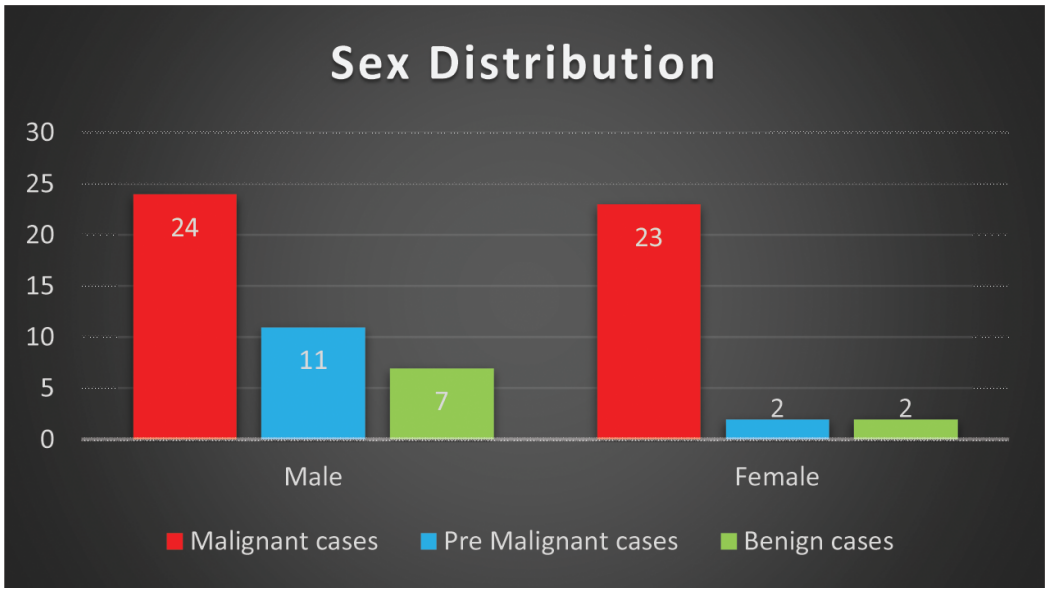

Fig. 3. Sex Distribution

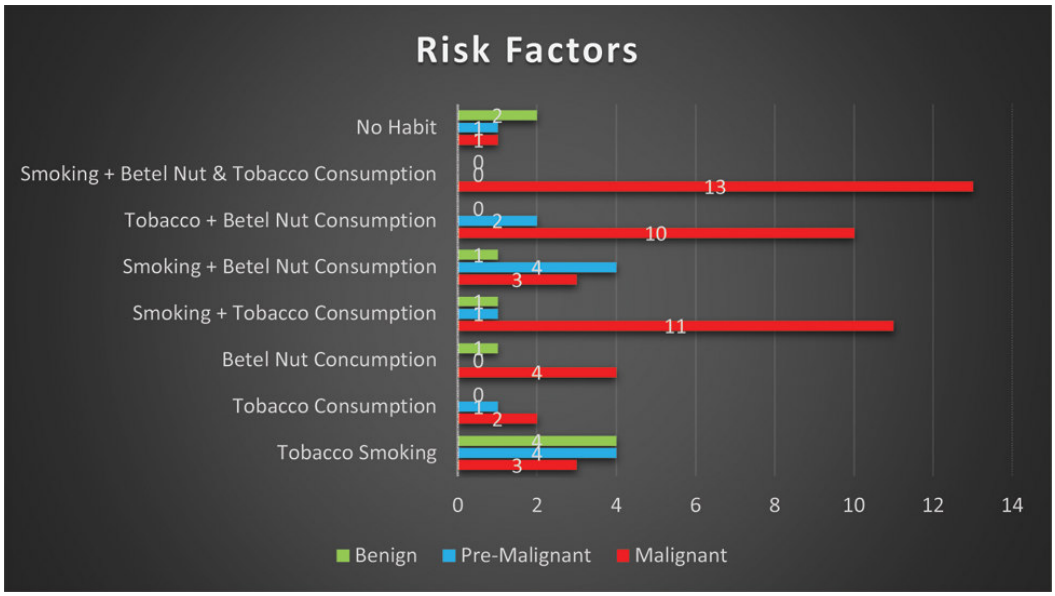

Fig. 4. Risk factors

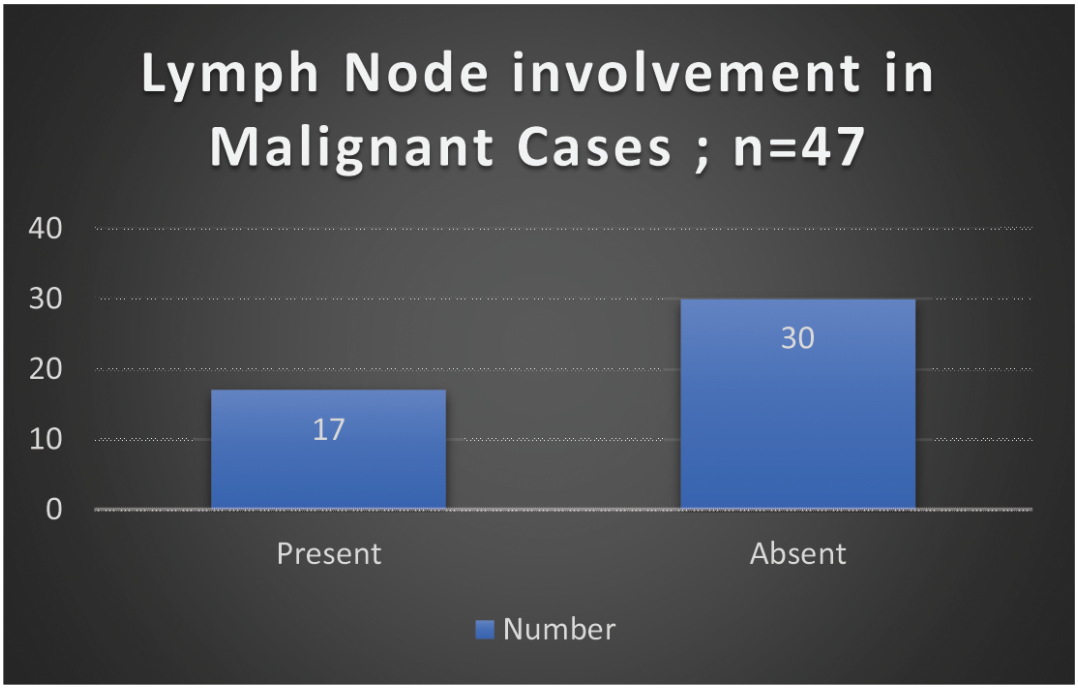

Fig. 5. Lymph Node Involvement

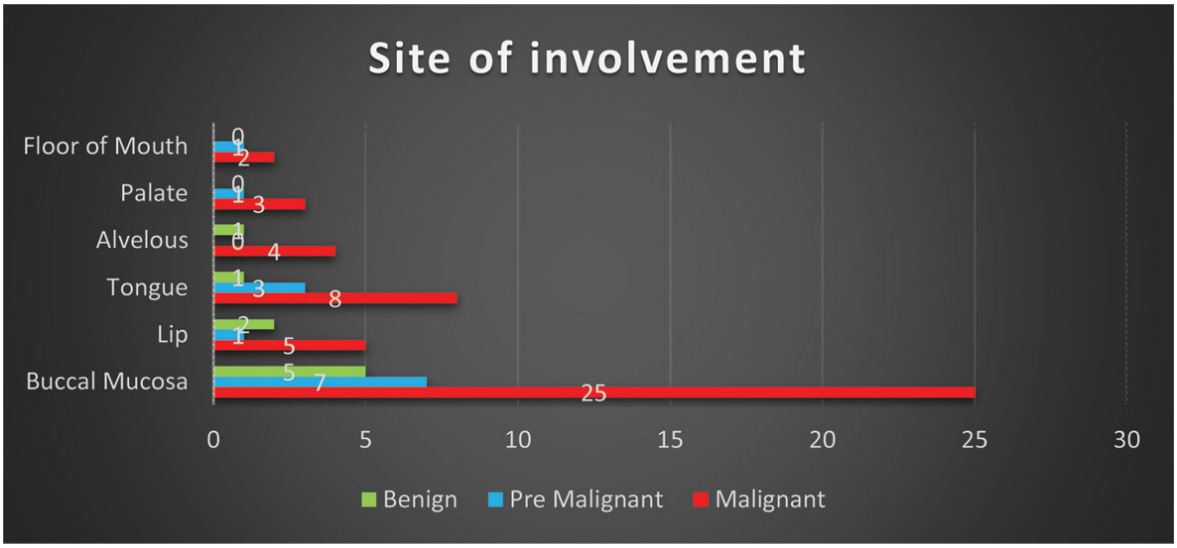

Fig. 6. Site of Involvement

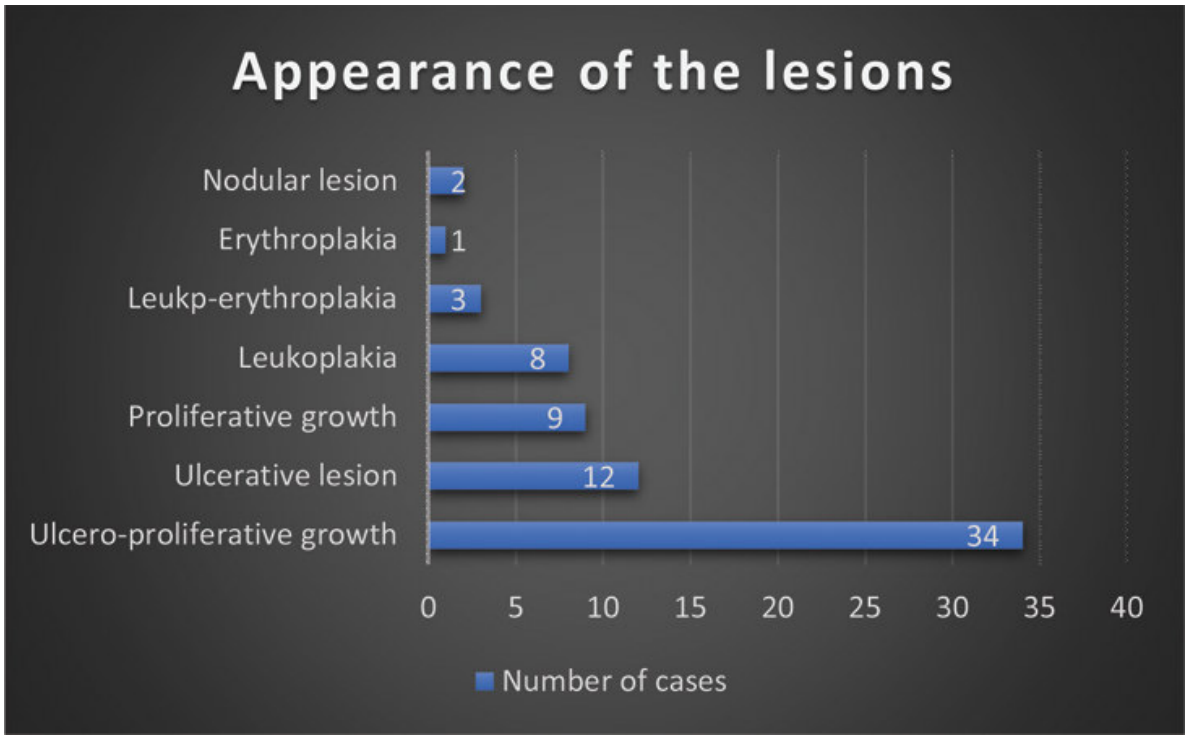

Fig. 7. Appearance of the lessions

Out of 69 cases, 13 cases were diagnosed as pre-malignant; of which 46.1% showed moderate dysplasia, 30.8% showed severe dysplasia, 15.4% showed mild dysplasia and 7.7% showed squamous hyperplasia in histopathology (Table I).

Out of 47 malignant cases, 91.5% were squamous

Table I: Histopathological findings of pre-malignant lesions and Histological grading of malignant lesions

HISTOPATHOLOGICAL FINDINGS OF PRE-MALIGNANT LESIONS	NUMBER OF CASES	PERCENTAGE
Squamous hyperplasia	01	7.7%
Mild dysplasia	02	15.4%
Moderate dysplasia	06	46.1%
Severe dysplasia	04	30.8%
Total	13	100%
HISTOLOGICAL GRADING OF MALIGNANT CASES	**NUMBER OF CASES**	**PERCENTAGE**
Well differentiated SCC	24	51.1%
Moderately differentiated SCC	19	40.4%
Poorly differentiated SCC	04	8.5%
Total	47	100%

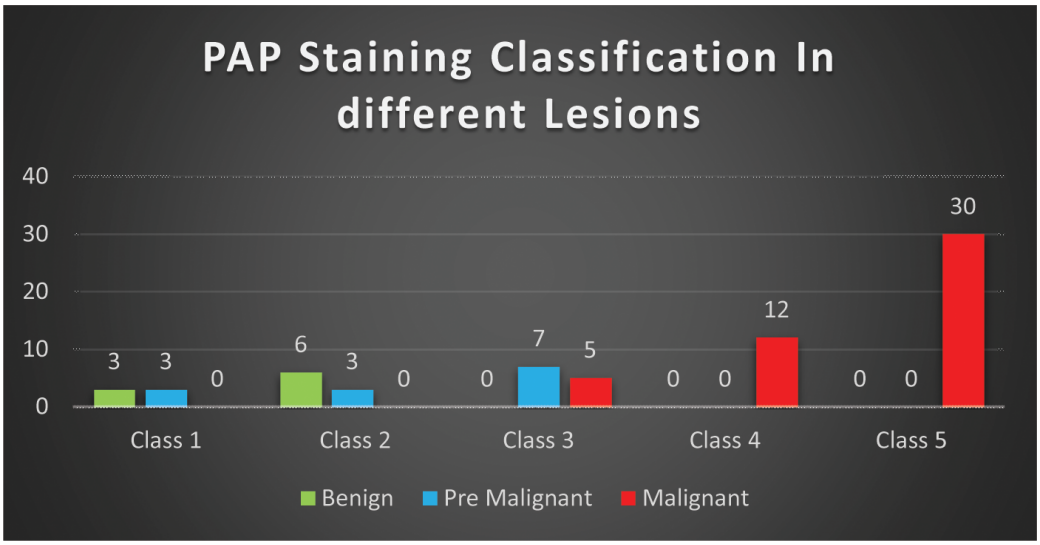

Fig. 8. Pap Staining classification in different lesions

cell carcinoma (SCC), rest were special variants of squamous cell carcinoma – 4.3% were verrucous carcinoma, basaloid and sarcomatoid squamous cell carcinoma were 2.1% each. Among these 47 malignant cases, 51.1% were well differentiated, 40.4% were moderately differentiated and 8.5% were poorly differentiated. (Table I). In our study, 30 (63.8%) out of 47 malignant cases show class-5 cytological grading in brush cytology smear, stained with Pap stain. 25.5% of the malignant cases were in class-4 and 10.6% cases were in class-3 whereas, in premalignant cases (n = 13), 3 cases were in class-2 and 7 cases were in class-3 and 3 were in class-1. In benign cases (n = 9), 3 cases were in class-1, 6 cases were in class-2. In Pap class-5 and class-4 cases were all malignant (Fig. 8).

There was an overlapping in class-3 (5 cases a malignant and 7 cases pre-malignant), in class-2 (3 cases pre-malignant and 6 cases benign) and also in class-1 (3 cases benign and 3 cases pre-malignant). Thus, Sensitivity and specificity of Pap grading in brush cytology using PAP stain for distinguishing malignant + pre-malignant cases (Positive malignant potential) from benign cases are

Sensitivity of Pap grading – (54/60) x 100 = 90%

Specificity of Pap grading – (9/9) x 100 = 100%

The mAgNOR count status with standard deviation of malignant, pre-malignant and benign cases is given in Table II and Mean value of Clusters, Satellites, Total AgNOR count and mAgNOR in different types of cases in mentioned in Table III.

Table II: Mean AgNOR count status.

	MEAN OF mAgNOR COUNTS	MINIMUM VALUE OF mAgNOR COUNTS	MAXIMUM VALUE OF mAgNOR COUNTS
Malignant cases	**6.0551(SD–0.89)**	**4.66**	**7.28**
Pre-malignant cases	**3.8492(SD–0.24)**	**3.34**	**4.16**
Benign cases	**3.0156(SD–0.34)**	**2.60**	**3.54**

Table III: Mean value of Clusters, Satellite, Total AgNOR count and mAgNOR in different type of cases

	AgNOR IN CLUSTERS IN 100 CELLS	AgNOR SATELLITE DOTS IN 100 CELLS	TOTAL AGNOR COUNT IN 100 CELLS	mAgNOR COUNT
Mean of Malignant cases	**114.9574**	**490.5532**	**605.5106**	**6.0551**
Mean of Pre-malignant cases	**144.9231**	**240.0000**	**384.9231**	**3.8492**
Mean of Benign cases	**157.3333**	**144.2222**	**301.5556**	**3.0156**

Table IV: Statistical analysis table for AgNOR analysis of brush cytology vs Histopathological findings

AgNOR ANALYSIS (mAgNOR COUNT)	HISTOLOGY POSITIVE FORM ALIGNANCY (MALIGNANT)	HISTOLOGY NEGATIVE FOR MALIGNANCY (BENIGN AND PRE-MALIGNANT)
> = 4.8	**44**	**00**
< 4.8	**03**	**22**
Total	**47**	**22**

The best cut-off value of the mean number of AgNORs per nucleus distinguishing benign and pre-malignant from malignant cells was 4.8. Statistical analysis for AgNOR analysis of brush cytology vs histopathological findings is mentioned in Table IV. This makes the sensitivity and specificity of AgNOR analysis in brush cytology of oral lesions for assessing malignant lesions vs non- malignant (benign and pre-malignant) lesions to be:

Sensitivity of AgNOR = (44/47) x 100 = 93.6%

Specificity of AgNOR = (22/22) x 100 = 100%

Discussion

Maximum pre-malignant cases belonged to fifth decade (38.5%), followed by fourth decade (30.8%) & majority of the malignant cases were also in fifth decade (31.9%), followed by sixth decade (23.4%) and fourth decade (21.3%). Studies done by Ara N et al[12] had mean age of 54.23 years (For Pre malignant lesions), Talole et al[13] had majority of cases between 45-55 years (For Pre malignant lesions). Our study in malignant cases showed, maximum cases were in 4th, 5th & 6th decade (Majority between 41-70 yrs). 61% of patients in our study were

male & 39% were female (Male: Female = 1.56:1). In malignant cases, Male: Female ratio was 1.04:1 & in pre-malignant cases it was 5.5: 1. Similar findings were observed by Ara N et al[12] from Pakistan where out of 60 patients, males were more than females (1.5:1) and by Iype EM et al[14] from Trivandrum who found 70% males in their study.

All the patients belonged to middle and lower socio-economic status with a ratio of Mid to Low of 1.16:1. 13 (18.8%) cases gave history of smoking with tobacco consumption and 13 (18.8%) cases gave history of all the three abuses (Smoking, tobacco & betel nut consumption) together. Along with other findings observed in our study, we found similarities in other studies done by Khandekar S P et al[15] where 71.3% of the patients were chewing tobacco, 63.3% were smoking tobacco in the form of cigarettes or bidis; Patel S M et al[16] who demonstrated, 28 out of 30 (93.33%) cases of SCC showed positive history of tobacco chewing. 55.55% of dysplastic lesions were associated with tobacco chewing; Durazzo M D et al[17] who noted tobacco smoking was identified in 80.8% patients.

The most common site of the lesion was in buccal mucosa (53.6%), followed by tongue (17.4%). The most prevalent site for malignant lesions (53.2%) and for pre malignant lesions (53.8%) was also buccal mucosa. This was in concordance with studies done by Patel MM et al[18], conducted at Surat, Gujrat, where anterior 2/3rd of the tongue was the commonest site (23.02%) followed by posterior 1/3rd of tongue (19.64%), alveolus, lips and cheeks. In various studies, anatomically more anterior parts (buccal mucosa, anterior 2/3rd of the tongue, alveolus and lip) were frequently involved sites in oral malignancies. This could be due to long duration of contact with the carcinogens in tobacco, betel nut, alcohol. Majority of the lesions appeared as ulcero-proliferative growth (49.3%), followed by ulcerative lesions (17.4%). Similar findings were found in the previous studies i.e., Durazzo et al[17], Patel M M et al[18]. 13 cases were diagnosed as pre-malignant; of which 46.1% had moderate dysplasia, 30.8% had severe dysplasia, 15.4% had mild dysplasia and 7.7% had squamous hyperplasia. Similar findings were noted in study done by Maheswari V et al[19] where out of 25 premalignant cases, 10 cases each were diagnosed as mild and moderate dysplasia (15.38%), rest of the cases are severe dysplasia (7.69%). Our result, though corroborates with Maheswari V et al who showed moderate dysplasia was most prevalent but severe dysplasia was the second most common type of pre-malignant lesions which is an exceptional finding in our study. 91.5% of malignant lesions had squamous cell carcinoma (SCC). Our result findings are similar to a study of Patel MM et al[18], where all the 504 patients i.e., 100% had squamous cell carcinoma. Mehrotra[20] and colleagues also found squamous cell carcinoma as the commonest histological variety, comprising of 85.12% of oral and 97.5% of oropharyngeal malignancies. Durazzo M D et al[17] from Brazil also found squamous cell carcinoma was the most frequent histological type and was present in 90.3% of patients included in their study. Glandular carcinoma was found in 4% of them. 51.1% of tumors were well differentiated, 40.4% were moderately differentiated and 8.5% were poorly differentiated in our study. This was again similar to studies by Khandekar S P et al[15] who found well differentiated squamous cell carcinoma in 33.75%, moderately differentiated in 20% and poorly differentiated in 18.75% cases.

The brush cytology finding using PAP staining showed that among the malignant cases, 63.8% cases give class-5 and 25.5% cases gave class-4 cytological grading. 54 out of the 60 pre malignant and malignant cases were found to have atypical squamous cells in brush cytology smear (can be put under Pap classification-3,4,5). Correlation between exfoliative cytology finding using PAP stain and histopathological finding (Gold standard) from punch biopsy specimen is statistically significant (P value < 0.005 by ANOVA test). The mean AgNOR value was significantly higher in case of malignant lesions than the benign and pre-malignant lesions (P value is < 0.005). The mean value was significantly different in all groups. Mao et al[21] reported the mean AgNOR counts per nucleus in exfoliated cells of the cancer group at 4.69 ± 0.72 and 2.44 ± 0.37 for normal mucosa. His AgNOR counts showed that the mean value for cancerous lesions were significantly higher than those of the normal mucosa

(*P*<0.005). He found no overlap between the two groups. Also, in a study by Rajput D V et al[22], the mAgNOR count was 2.568 (± 0.3178) in the benign group; 4.223 (± 0.1902) in verrucous carcinoma and was 5.384 (+/- 0.3444) in the oral squamous cell carcinoma group. The mAgNOR counts were significantly different in all groups, the *P*-value being <0.005. The mAgNOR counts in were slightly higher than those reported by Mao, which may be related to the advanced grades of lesions and/or due to racial variations. Also, the Sensitivity of the PAP analysis in oral smears for the detection of oral cancer was 91.176%, while specificity for the detection of non-neoplastic cells was 100%. Remmerbach T W et al[23] showed in a study that, AgNORs were strictly located only within nuclei and were clearly visible as distinct black or dark-brown dots. Normal epithelial cells revealed one to two clusters (mean 0.03; SD 0.01) with one to six dots in each cluster (mean 2.28; SD 1.7). Some cells also contained satellites, even up to ten satellites were found lying outside the clusters. The silver reaction in neoplastic cells generally showed more dots as satellites and clusters. The number of dots lying within clusters was 2–6 for SCC (mean 2.28; SD 1.95). The total AgNOR count, including the number of dots lying within clusters and as dots (satellites). The mean number of all AgNOR dots per nucleus was 3.39 (SD 0.41 in inflammatory lesions, 3.88 (SD 0.59) in oral leucoplakias and 8.99 (SD 2.64) in oral squamous cell carcinoma. We have considered this value (mAgNOR-4.8) as a cut off for malignant and non-malignant oral lesions (premalignant and benign) because in above study done by Remmerbach et al[23], it was found that the best cut-off value of the mean number of AgNORs per nucleus distinguishing benign and pre-malignant from malignant cells was 4.8. Following the cut off value (mAgNOR-4.8), the sensitivity and specificity of AgNOR analysis in brush cytology of oral lesion for assessing malignant lesions vs non- malignant (benign and pre-malignant) lesions was 93.6 % & 100% respectively.

Conclusion

Brush cytology (exfoliative cytology) of oral lesions is a simple, rapid, outdoor based, non-invasive procedure & well accepted by the patients in clinically suspected oral lesions for detection, early diagnosis (even at the pre-cancerous stage), follow up of oral malignancy. Brush cytology using cytobrush cell collector has been found to be an effective exfoliative cytology technique because all the smears in the study prepared using cytobrush have adequate numbers of cells for cytological examination.

Brush cytology finding using PAP stain (PAP grade) was statistically significant with histological diagnosis in case of oral lesions. This cytological finding was sensitive and specific for differentiating pre-malignant and malignant lesions from benign lesions.

The mAgNOR count is a reliable marker for diagnosing malignant squamous cells in oral brush cytology smear. AgNOR analysis in brush cytology smear is highly sensitive and specific for differentiating malignant and non-malignant lesions of oral cavity.

This study also helps to reflect the occurrence, etiological, social factors and types of pre-malignant and malignant lesions of oral cavity among the population of northern region of Bengal. Till date, there is no comprehensive study documented regarding the epidemiology and screening of oral cancer in the northern region of West Bengal.

Thus, the brush cytology with PAP grading and AgNOR analysis in clinically suspected oral lesions can be used as an early diagnostic tool for diagnosing oral squamous cell carcinoma especially for lower socio-economic status people who present with late stages.

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