RESEARCH ARTICLE

EVALUATION OF DIAGNOSTIC ACCURACY OF PAP (PAPANICOLAOU) AND AgNOR (ARGYROPHILIC NUCLEOLAR ORGANIZER REGIONS) STAINING IN EXFOLIATIVE CYTOLOGY OF ORAL MUCOSA IN SMOKERS AND NON-SMOKERS

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ABSTRACT

Background: A strong causal relationship exists between cigarette smoking and the development of oral cancer. Most of the cases of oral cancer are diagnosed at advanced stage that results in an unfavorable prognosis and high mortality rate. In such cases certain noninvasive procedures such as Exfoliative Cytology plays an important role in the detection and monitoring of initial alteration and for the establishment of adequate treatment. The purpose of this study is to compare exfoliative cytology from oral mucosa of smokers and non smokers, with evaluation of proliferative activity by PAP (Papanicolaou) and AgNOR.

Materials and method: The study was conducted in the department of Oral Pathology and Microbiology between 2011-2013 in Eastern Uttar Pradesh, India. The detailed information of each patient was noted in a pretested Performa. Exfoliative cytology specimens were obtained from clinical normal mucosa from the lateral border of the tongue in 60 nonsmokers and 60 smokers ranging from 30 to 50 years of age using cigarettes for at least 10 years. The cytologic specimens were evaluated by Papanicolaou staining and AgNOR quantification.The data collected was analyzed using IBM SPSS statistics version 22 (Armonk,NY:IBM corp).P value <0.05 was considered as statistically significant. Statistical analysis of chi- square (χ2) test, Student t-test and One way ANOVA by running post hoc test were applied.

Result and observation: Statistically significant increases in mean number of AgNOR /nucleus were found in smoker and non smoker patient according to cytologic evaluation. Cytologic specimens evaluated by PAP also showed the presence of inflammation, dysplasia, keratinisation and higher proliferative activity in smokers than non smokers.

Conclusion: AgNOR quantification increases sensitivity and specificity by reduces the chances of false negative or positive and can be used as an adjunct diagnostic tool over routine PAP staining in cytopathology to assess cellular chnages in oral mucosa.

INTRODUCTION

Smoking is currently the most inevitable cause of diseases and death worldwide and is one of the main risk factors for the development of oral cancer. Cigarette smoking in development of oral malignancy has drawn increasingly interest. Therefore, it is mandatory to monitor the smoking patients carefully in view of the succession of alterations that smoking can cause (Fontes, 2008). Exfoliative Cytology is one of the interesting tool by which one can screen the status of oral health in habit associated patients. Nevertheless, there is little reference in the literature to a cytological assessment of the effects of smoking upon normal oral Mucosa (Salehinezhad, 2007). According to the World Health Organization, cancers of the mouth and oropharynx are the most frequent head and neck tumors, with

about 390,000 new cases per year worldwide, corresponding to 4% of all cancer cases (Fontes, 2008), Cancer is considered to be a major public health problem throughout the world. It is currently the second cause of death in most countries (de, 2013). Oral cancer affects as many as 274,000 people worldwide annually, and the frequency of oral cancer around the world is often indicative of the patterns of use of tobacco products. It has been established that there is a dose-response relationship between the amount of tobacco product used and the development of oral cancer. All parts of the oral cavity are susceptible to cancer from tobacco smoking or chewing, including the lip, tongue, palate, gum, and cheek (Proia, 2006). The prevalence of oral SCC in cigarette smokers is 4–7 times higher than in non-smokers, and when alcohol or chewing tobacco habits are also present, the disease prevalence increases by 19- and 123-fold, respectively (Nozad-Mojaver, 2009). Considering the possible influence of smoking on the occurrence of oral cancer and of precursor lesions, exfoliative cytology might be a useful tool for the detection and monitoring of initial alterations and for the establishment of adequate treatment in smokers.

It is a complementary diagnostic method which presents several advantages such as rapid and easy execution, low cost, diagnostic safety, efficacy and non-invasiveness, and can be repeated several times (Fontes, 2007; Paiva, 2004 and Remmerbach, 2003 and Sethi, 2003). Papanicolaou(PAP) staining is used as a routine method for the analysis of cytological aspects and permits the identification of basic inflammatory, dysplastic or malignant alterations. Ever since PAP described exfoliative cytology technique, which is non-painful, non-invasive procedure, it has become a valuable tool for cancer screening (Ahmed, 2009). Argyrophilic Nucleolar organizer regions (AgNORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded. Nucleolar organiser regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods. After silver-staining, the NORs can be easily identified as black dots exclusively localised throughout the nucleolar area, and are called "AgNORs". The NORs' argyrophilia is due to a group of nucleolar proteins, which have a high affinity for silver (AgNOR proteins) (Trere, 2002). NORs are proteins that are associated with the fibrillar centers and dense fibrils of the cell nucleus during interphase and are responsible for the replication of RNA. Thus, the larger the number of NORs, the higher the replication rate of ribosomes and cells. This technique has therefore been used for the quantification of cell proliferation in different tissues and lesions (Cancado, 2001). In the present study, we examined exfoliative cytology from the lateral border of the tongue which is a site associated with a high incidence of oral cancer, in both smokers and nonsmokers. The objective of this study was to determine the influence of smoking habit on oral mucosa with the help of proliferative markers and to determine whether a correlation exists between the results obtained by Papanicolaou staining and by histochemical AgNOR quantification to analyze the accuracy of AgNOR over wide usage of PAP for cytopathology.

MATERIAL AND METHODS

The present prospective study was conducted in the Department of Oral pathology and Microbiology of a dental college in Eastern Uttar Pradesh, India between 2011-2013.

Verbal informed consent was obtained from every individual participating in study and the study was approved by ethical committee of the institution. The subjects who were addicted to alcohol, with any oral lesion, and with any prior or present history of benign or malignant oral neoplasm were excluded from the study. Two groups were analyzed: smokers and non-smokers. Non-smokers were defined as people who have never smoked. Exfoliative cytology specimens were collected from the lateral border of the tongue of 60 nonsmokers and 60 smokers ranging from 30 to 50 years of age using at least 10 cigarettes/day over 10 years. Cytological smear were obtained by using a standard wooden tongue spatula moistened with normal saline. The material collected was smeared on two slides and immediately fixed in 95% ethyl alcohol for 15 minutes.

The spray-fixed smears were stained by a commercially available RAPID-PAP Papanicolaou stain and evaluation of Papanicolaou the-stained smears was carried out according to the standardized procedure (Paiva, 2004). The cells suspected to be abnormal were evaluated at higher magnifications and marked accordingly. AgNOR staining was performed according to the one-step method of Ploton et al and Linder (Ploton, 1986 and Lindner, 1993). The cytologic specimens which were evaluated by Papanicolaou staining were graded as class I-V on the basis of the presence of cytological alterations suggestive of inflammation, dysplasia, keratinization, and proliferative activity of epithelial cells (Figure 1). NORs were directly counted under a light microscope according to the parameters established by Crocker's method (Crocker, 1989 and Ahmed, 2003). NORs were visible as dark brown to black "dots" of varying size in the brown-stained nucleus on a pale yellow background of the cells, overlapping or fused black dots being considered a single structure (Figure 2). The results were reported as mean ± standard deviation and were submitted to statistical analysis. The chi- square ($\chi^{2)}$ test was used to compare the differences in categorical variables between the groups. Relationships between variables were analyzed using One way ANOVA by running hoc test. Level of significance was analysed by "p" value (p>0.05 =Non Significant (NS), while p <0.05 was considered to be statistically significant. Specificity and sensitivity values for each stain method and the curve under ROC area were estimated.

RESULTS

A total of 120 exfoliative cytology smears (60 smokers & 60 nonsmokers) were obtained from lateral border of tongue with age ranging from 30-50 years. The mean age of population for the study group was 37.81± 6.18.Demographic variables showed no significant correlation among two groups (Table 1). The mean duration of smoking was approximately 21.27 ±7.14 years and the mean number of cigarettes smoked per day was approximately 28.38 ±9.35. The proliferation activity was assessed by AgNOR count and PAP staining method in 60 smokers and 60 non smokers.

Assesment of agnor count amongst two group: On assessing the AgNOR count amongst two groups (Group-1 as smokers and Group-2 as non-smokers) the comparison of the mean number of AgNORs showed a significant difference amongst smokers and non smokers. On evaluating the proliferation activity amongst smokers, the maximum number of AgNOR count calculated around 7-8 AgNOR/Nu (>3.5AgNOR/Nu) in

Table 1. Demographic variables – comparison between groups

		Group		Total	Chi square test	
		1(smokers)	2(non-smokers)		Chi square value (df)	p-value
Sex	Male	56(93.3%)	50(83.3%)	106(88.3%)	2.91(1)	0.09(NS)
	Female	4(6.7%)	10(16.7%)	14(11.7%)		
	Group	N	Mean	SD	Mean difference (95%CI)	t	df	p-value
AGE	1	60	38.82	6.245	2.02(-0.20, 4.23)	1.80	118	0.07(NS)
	2	60	36.80	6.005				

Independent sample t test
*p<0.05 statistically significant, p>0.05 Non significant, NS

**Table 2. Comparasion of mean number of AgNOR in smokers and non smokers**

Habit	>3.5AgNOR/ NU (Cut off value AgNOR 3.5)	≤ 3.5AgNOR /NU.	p -Value
Smokers	56(93.3%)	4(6.6%)	<0.05*
Non-smokers	3(5%)	57(95%)	

χ^2 = 33.149, d.f= 1, *p<0.05 statistically significant, p>0.05 Non significant, NS

Table 3. Distribution of pap class in smokers and non smokers

Pap class	Smokers	Non-smokers	p - Value
I	09(15%)	55(91.7%)	
II	51(85%)	05(8.3%)	<0.05*
TOTAL	60	60	

χ^2 = 70.848, d.f= 1, *p<0.05 statistically significant, p>0.05 Non significant, NS
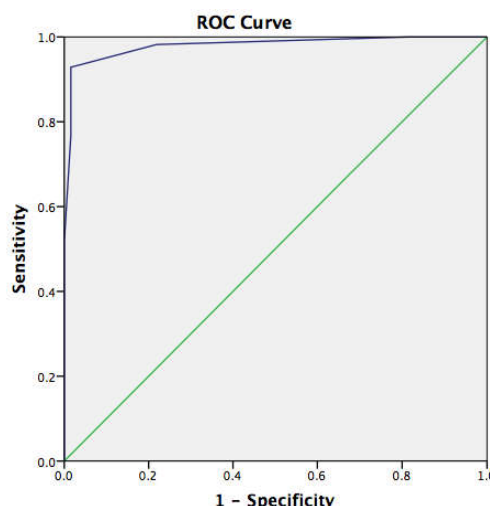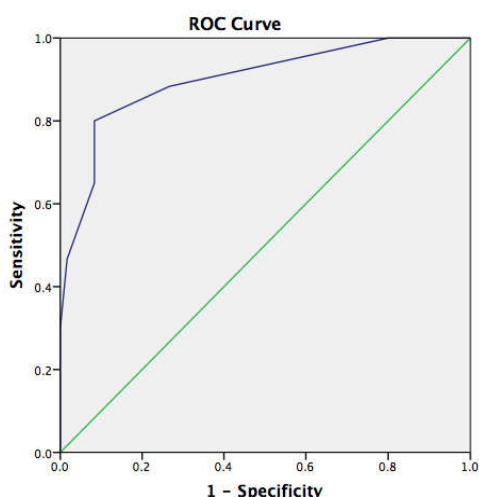
93.3% of cases while in non-smoking group maximum calculation was around 3 AgNOR/Nu (<3.5AgNOR/Nu) in 95% of cases, whereas 1-2 AgNOR/Nu in 5%of cases (Table 2, Figure 1).

Assesment of pap class amongst two groups: Similarly on assessing PAP class in two groups, 51(85%) of all samples were classified as PAP class II whereas 9(15%) were characterized as class I (Figure 2). Amongst non -smoking group 91.7% cases were categorized under PAP class I, while only 8.3% classified in class II (Table 3).

No. of AGNOR /NU in two groups according to pap class: The number of AgNOR was determined in two groups according to cytological evaluation. A significant difference was found in the proliferation activity of smokers and non smokers classified as PAP class II (Table 4).

Influence of frequency of smoking habit on number of AgNOR: To evaluate the influence of the smoking habit on the number of AgNORs, smoking patients were divided into three groups according to the number of cigarettes smoked per day:

group A consumed 10-20 cigarettes per day; group B consumed 21-30 cigarettes per day; and group C, more than 30 cigrattes per day. The mean AgNOR number and respective standard deviation were determined in each group. A significant difference in the mean number of AgNOR per nucleus was observed between three groups. The mean difference is significant at the level of 0.05 on multiple comparasion .One way anova test is done to assess the mean and standard deviation between three groups by running a post hoc test which was found to be stastically significant (Table 5).

Influence of duration of smoking habit on number of AgNOR

The influence of the duration of smoking on proliferation activity was evaluated by dividing smoking patient in three groups; group I: 10-20 years; group II : 21-30 years ; group III: more than 30 years. The mean AgNOR number and respective standard deviation were determined in each group via one way anova test by running post hoc test which was found to be stastically significant (Table 6).

Table 4. Number of AgNOR /nucleus in smoker and non smoker patient according to cytologic evaluation

Habit	Class I (mean ±s.d)	Class II (mean ±s.d)	Student T test	d.f	P-value
Smokers	09 (2.22±0.441)	51 (5.92±1.742)	12.99	50.928	<0.001
Non-smokers	55 (2.04±0.769)	05 (4.6±1.517)	6.518	58	<0.001
		PAP		Total	
		2	1		
AgNOR	>3.5	52(92.9%)	1(1.6%)	53(44.2%)	
	<3.5	4(7.1%)	63(98.4%)	67(55.8%)	
Sensitivity		92.86%(83.02, 97.19)			
Specificity		98.44%(91.67, 99.72)			
Positive Predictive Value		98.11%(90.06, 99.67)			
Negative Predictive Value		94.03%(85.63, 97.65)			
Diagnostic Accuracy		95.83%(90.62, 98.21)			

Table 5. Mean of AgNOR/nucleus according to the frequency of smoking the frequency of smoking

Frequency	N	Mean	SD	ANOVA	
				F - value	p-value
10 – 20	14	3.21	1.31	31.07	<0.001*
21 – 30	18	4.72	1.41		
>31	28	6.86	1.58		

Levene statistic = 0.15, p=0.86(NS)
*p<0.05 statistically significant, p>0.05 Non significant, NS

(I) Frequency	(J) Frequency	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1.51	0.52	0.02*	-2.77	-0.25
	3	-3.64	0.48	<0.001*	-4.80	-2.48
2	3	-2.14	0.45	<0.001*	-3.20	-1.07

Tukey post hoc test *p<0.05 statistically significant, p>0.05 Non significant, NS

Table 6. Mean of AgNOR /nucleus in the group of 60 smokers divided according to the duration of smoking

Duration	N	Mean	SD	ANOVA	
				F - value	p-value
10 – 20	32	4.81	2.09	7.79	0.001*
21 – 30	20	5.30	1.56		
>31	8	7.75	1.75		

Levene statistic = 1.31, p=0.33(NS)
*p<0.05 statistically significant, p>0.05 Non significant, NS

(I) Duration	(J) Duration	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-0.49	0.54	0.64(NS)	-1.78	0.81
	3	-2.94	0.75	0.001*	-4.73	-1.14
2	3	-2.45	0.79	0.008*	-4.35	-0.55

Tukey post hoc test *p<0.05 statistically significant, p>0.05 Non significant, NS
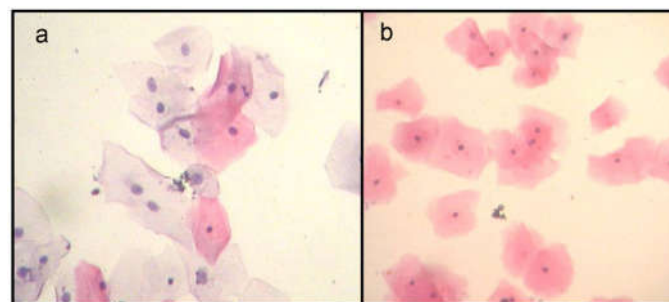

Figure 1. Histochemical AgNOR quantification (a) Smear obtained from smoker showing cells with upto 9 AgNORs /nucleus(x100) (b) smear obtained from a non smoker, black arrows showing cells with upto 3 AgNORs /nucleus (x100)
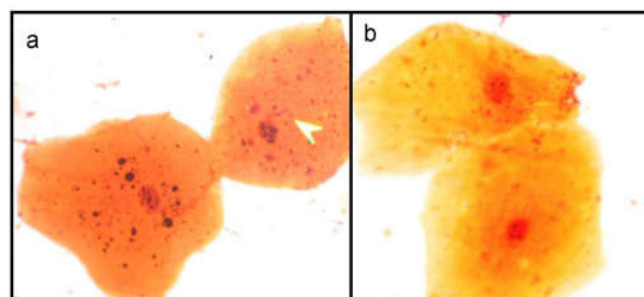

Figure 2. Papanicolaou staining (a) PAP class II, showing superficial and intermediate squamous cells with anisocytosis and anisonucleosis (x100) (b) PAP class I, showing superficial cells with no evidence of cellular atypia

DISCUSSION

Early detection of oral cancer has been the most helpful approach to decrease morbidity and mortality of cancer patients. If a lesion is considered to be suspicious on clinical examination, an easily viable, non-invasive, painless, safe, and accurate screening method for detection of the dysplastic changes need to be done (Rao, 2017). Exfoliative cytology is one of the diagnositic techinque which is based on the observation of cells that are constantly exfoliating from the epithelium (Fontes, 2007). It is an easy, non invasive, reliable technique that could be useful for the early detection of oral cancer. Cytological diagnosis of oral cancer cells are difficult and need much experience which requires highly specialized cytopathologists for the appropriate interpretation of the smear. However, recently, the various molecular markers, image analysis systems and proliferation markers have been introduced to eradicate the above error (Fontes, 2008). Numerous studies have used different markers of cell proliferation as an auxiliary tool in the diagnosis of oral cancer. These cell proliferation markers acts appreciatively in the identification alteration in cells at cellular as well as at nuclear level. Most recent reports have suggested that the number of AgNORs per nucleus is related to cellular proliferation and differentiation.

These findings suggest that AgNORs can be used as an aid in early diagnosis of malignancy such as OSCC. However, there is a big lacuna in the studies which analyze cell proliferation markers by exfoliative cytology (Fontes, 2008; Rajput, 2010 and Cancado, 2004). In the present prospective study a total of 120 subjects (60 smokers and 60 non-smokers) were included with age ranging from 30-50 years. The site showing highest incidence of oral cancer in lateral border of the tongue which was analyzed with both PAP and histochemical AgNOR quantification (Eslami, 2003). In the present study, AgNORs were visualized as well defined black dots inside the nucleoli (1, 21-22). The histochemical AgNOR quantification revealed a higher proliferative activity in smokers as compared to non-smokers. A significant difference in the mean percentage of AgNOR per nucleus was seen between two groups. The percentage of cell with >3.5AgNOR/NU was higher in smokers 48(80%) than non-smokers 5 (8.3%), while the cells showing <3.5AgNOR /NU in smoking & non-smoking group was 30% and 55% respectively which showed high proliferation activity amongst smokers (Ogden, 1990). On assessing the mean number of AgNOR in two groups according to PAP class, a significant difference was observed in the proliferation activity of smokers and non-smokers (5.92±1.742 versus 4.6± 1.517) in PAP Class II.

These values are close to those reported by Sethi and Shah and Orellana-Bustos *et al.*, but differ from those obtained by Cancado *et al*. who observed a mean number of 1.94 ± 0.13 in smoking patients (Fontes, 2008; Sethi, 2003; Cancado, 2001; Sapp, 1997). Sensitivity ans specificity of AgNOR was found to be 92.86% and 98.44% respectively with a diagnostic accuracy of 95.83%.On analysing mean of AgNOR/Nucleus according to frequency of smoking a significant difference was found between three groups according to frequency of smoking. The mean number was higher in group C (6.86) which consumed more number of cigarette (>30 cigarette/day). The number of cigarette consumed per day is directly proportional to number of AgNOR /nucleus which concludes that the proliferation activity increases as the number of

cigarette cosumption increases. Similarly duration of smoking revealed significant difference amongst three groups, which is not concomitant with Fontes *et al* and Concado *et al* (Fontes, 2008 and Cancado, 2001). It was also seen that cytological atypia like anisocytosis, anisonucleosis, binucleation was higher in smokers when compared with non-smokers. The similar findings were also evaluated by Ahmed *et al* in there study which suggests that toombak dipping and cigarette smoking are associated with a risk for occurrence of oral epithelial atypia, which can be detected by use of simple cytological methods. The findings further suggested that tobacco components, specially the TSNAs, may stimulate the epithelial cells to undergo squamous differentiation/ or cellular morphological changes that might lead to malignancy (Fontes, 2008; Ahmed, 2009; Ogden, 1990). Pavanello *et al*. Also reported a larger number of inflammatory alterations and an increased rate of cell maturation in smokers (Chattopadhyay, 2002). Tobacco has been considered to be an initiating factor in the process of oral carcinogenesis, which is frequently associated with alcohol as a promoting factor.

In addition, the results suggest that the oral mucosa is susceptible to the effects caused by cigarette smoking responding with an increase in cell proliferation (Fontes, 2008; Trere, 2000 and Pavanello, 2006). It was found that tobacco smoking produces cellular alterations in clinically normal oral mucosa when analysed by exfoliative cytology. Because this method of using a brush to obtain deeper cells is a non-invasive technique and an easy procedure, the AgNORs count can be an auxiliary method to control risk groups for the prevention of oral cancer (Sapp, 1997 and Orellana-Bustos, 2004). Although PAP method of cytological evaluation is well defined and widely used method. However, this technique is marred with a high false-negative rate (range, 0–31%). One of the most common failures of exfoliative cytology in previous studies was the faulty techniques of smear collection, often yielding insufficient quantity of cells as required for microscopic examination. Another common flaw of exfoliative cytology is subjectivity in interpretation of the given sample when stained by the routine PAP method (Rajput, 2010). However, we found that AgNOR quantification set up to be diagnostically accurate in assessing the oral status in smokers in terms of proliferation and atypia at cellular and nuclear level. Comparison of the mean number of AgNORs per nucleus in inflammatory smears showed a significant difference between smokers and non-smokers.

Conclusion

In conclusion, both PAP and AgNOR were found to be diagnostically accurate as a preliminary diagnostic tool to rule out any early changes in oral cavity. However, because of ease of demonstration and good specificity AgNOR can be considered as a reliable cytopathological marker over routine PAP method in assesing the proliferation activity of cells which can be directly correlated with neoplastic squamous cells in oral smear. All those involved in these abusive habits should undergo a continuous screening program to reduce the risk of oral cancer.

Limitations: Althoough there seems to be a generally positive correlation between PAP and AgNOR on oral smears associated with smoking habit, further investigations with more number of study samples will be required in this perspective.

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