International Journal of Academic Health and Medical Research (IJAHMR)
ISSN: 2643-9824
Vol. 3 Issue 10, October – 2019, Pages: 1-5

Assessment of Cytological Atypia, AgNOR Count and p53 Protein in Epithelial Oral Mucosa Exposed to *Toombak* Among Sudanese Snuffers

Abdalraheem Ali Babiker[1], Ahmed Elhaj[2], Khalid Abdelsamea Mohamedahmed[3*], Elhadi Abdella Ahmed[4]

[1] Department of Histopathology and cytology, Faculty of Medical Laboratory Sciences, University of Gezira, Wad Medani, Sudan.
[2] Department of Oncology, National Cancer Institute, University of Gezira, Wed Medani, Sudan.
[3] Department of Hematology and Immunology, Faculty of Medical Laboratory Sciences, University of Gezira, Wad Medani, Sudan.
[4] Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, University of Gezira, Wad Medani, Sudan.

Abstract: Background: *Toombak is known to be contain a high concentration of tobacco specific nitrose amine (TSNA), which is a potent carcinogen. Therefore, the high frequency of patients affected with oral cancer, who were mostly toombak dippers reflect the importance of the subject. In recent two decades, there are dramatic switch from histopathological to cytological and molecular methods of disease diagnosis and evaluation, and have gained importance as rapid and simple methods.* ***Objective:*** *The study aimed to assess the cytological atypia, argentaffin nucleolar organizer reason (AgNOR) and p53 protein in epithelial oral mucosa exposed to Toombak among Sudanese users.* ***Methods:*** *Descriptive prospective cross-control study was conducted on 100 individuals of toombak users. Oral mucosal smear samples were prepared and stained by PAP stain, AgNOR stain and immunocytochemistry (ICC) application use p53 as primary antibody.* ***Results:*** *The age of participants was ranged between 18 – 75 years with the mean of 40.94 years. Epithelial atypia was observed in 6% (6/100) of examined smears by PAP stain while the AgNOR increased in number with 31% (31/100). Furthermore, the p53 protein was positive in 3% (3/100) of participants. The controlled group showed no cellular changes. AgNOR had significant correlation within age (P value = 0.000), Toombak chewing duration /year (P value = 0.000), and Toombak chewing frequency /day. Also there was an association between cellular changes and position of Toombak dipper, mainly increased in lower lip.* ***Conclusion:*** *Oral exfoliative cytology is a reliable, simple non – invasive procedure that can be implemented for comprehensive oral screening program. AgNOR was more sensitive for detection of cytological changes.*

Keywords: *Toombak, Cytological atypia, AgNOR, p53 protein, Pap stain, Sudanese.*
[*]**Corresponding Author:** Khalid Abdelsamea Mohamedahmed, E-mail: Khalid.abdelsamea@hotmail.com

Introduction:

A significant proportion of oral squamous cell carcinomas (OSCCs) develop from premalignant lesions such as leucoplakia and oral sub mucous fibrosis. Histological examination of tissue remains the gold standard for diagnosis and identification of malignant oral lesions. Biopsy is an invasive technique with surgical implications, technique limitations for professionals and psychological implications for most patients. In recent decades, we have seen dramatic switch from histopathological to cytological and molecular methods of disease diagnosis and have gained importance as rapid and simple methods. Changes occur at the cellular and molecular level before they are seen within the tissue and before clinical changes occur [1]. The cellular changes that characterize cancers are initiated by various degrees of interaction between host factors and exogenous agents. Many factors have been involved in the initiation of cancerous changes. These factors may be direct or indirect intact to the individual life-style. Principal environmental factors implicated in the genesis of oral cancer are tobacco and alcohol [2]. Identification of high-risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys to reducing the mortality, morbidity and cost of treatment associated with oral cancer. In addition, certain patients are known to be at high risk for head and neck cancer, specifically those who use tobacco or alcohol and those over 45 years of age [3]. There are two types of smokeless tobacco, snuff (Toombak in Sudan) and chewing tobacco. Toombak is the native name for tobacco species *Nicotina rustica*. It is a smokeless tobacco product that has been used in Sudan for centuries and is widely spread in the North, East and Central Sudan [4]. Currently, the most effective way to control oral cancer is to combine early diagnosis with determination of an appropriate treatment. Oral exfoliative cytology may serves as an important to early detection of premalignant lesions; particularly if employed value in the diagnosis for screening at risk population [5]. Nucleolar organizer regions (NORs) represent loops of DNA activity transcribing to ribosomal RNA and thus to rihosomes and ultimately to protein. The NORs are associated with acidic, argyrophilic non histonic proteins that are visualized with the use of a silver staining technique, the AgNOR technique [6]. p53 has been described as the guardian of the genome, referring to its role in conserving stability by preventing genome mutation. It plays an important role in cell cycle control and apoptosis.

Defective p53 could allow abnormal cells to proliferate, resulting in cancer. As many as 50% of all human tumors contain p53 mutants [7]. If the p53 gene is damaged, tumor suppression is severely reduced people who inherit only one functional copy of p53 must likely develop tumors in early adulthood, a disease known as Li – fraumeni syndrome. The p53 can also be damaged in cells by mutagens. Some agents like Chemicals, radiation or viruses, increase the likelihood that cell wells begin uncontrolled division. More than 50 percent of human tumors contain a mutation or deletion of the p53 gene [8].

Methodology:

Study design:

Descriptive prospective cross-control study carried out among Sudanese toombak users. The study was conducted in Faculty of Medical Laboratory Sciences, University of Gezira, Wad Madani, Sudan.

Sample collection and preparation:

Three smears was obtained from 100 participants. Tongue depressor was used to collect smears from tongue, lip and buccal mucosa. All samples were fixed using absolute methanol and transferred to Cytopathology Laboratory and stored at room temperature until used.

Ethical consideration:

The study was approved by the Scientific Research Committees of Faculty of Medical Laboratory Sciences – Gezira University – Wad Medani – Sudan. All participants were asked to sign a written consent before taking the specimen.

Papanicolaou stain protocol:

Wet smears were hydrated, and stained in Harris hematoxylin for 5 minutes. After rinsed in water smears were differentiated in 0.5% of HCl for 10sec. Bluing was done after second washing, smears were dehydrated in 70%, 95%, and 95% of alcohols. Then smears were stained by OG6 for two minutes. After rinsing in 95% alcohol for two time smears were stained by EA50 for 3 minutes. Rising in 95% alcohol for two minutes were done before dehydration in two changes of absolute alcohol. Finally smears were cleared in two changes of xylene and amounted with DPX.

Detection of AgNOR by Silver nitrate protocol:

Wet smears were fixed immediately in 95% ethyl alcohol for 15 minutes hydrated in 70% alcohol for 2 minutes and rinsed in distilled water then incubated in freshly prepared working solution for 45 minutes at room temperature then washed in an industrial water, dehydrated in tow change of absolute alcohol finally the smears was cleared in tow change of xylene and amounted with DPX medium.

Detection of p53 protein using Immunocytochemistry protocol (ICC):

For protein blocking the slides were dried by tissue paper. Smears was covered with non immune protein blocking serum, and then slide was incubated in humid chamber for 30 minutes. Ready to used primary antibody (p53) was implicated by covering the smear area; about two to three drops were added and incubated in humidity chamber for 60 minutes. Primary antibody was then washed by Phosphate buffer saline (PBS) and ready to used secondary antibody was then added; two to three drops were added and incubated in humidity chamber 30 minutes. Secondary antibody was washed by PBS and ready to used supper enhancer was added; two to three drops were added and incubated in humidity chamber 45 minutes. Then diluted DAB chromogen was added (one drop of DAB chromogen with 1ml of DAB buffer), one drop was added and incubated in humidity chamber about 3 minutes. Mayer haematxyline used as counter stain. Finally stained smears were rinsed in xylene and mounted using DPX. Positive and negative controls were processed with each run.

Results interpretation:

All quality control measures were adopted during specimen collection and processing, all stain smears were fair quality smears and were examined by light microscope for the assessment of cytological atypia using PAP stain, AgNOR and p53 protein.

Data analysis:

The SPSS (statistic package of social science) computer program (V 16.0) were used for data analysis.

Results:

Ages of participants were ranged between 18 to 75 years, with a mean of 40.94 years. The majority of participants 33% (33/100) were at age group between 36 – 45 years. Otherwise 80% of participant have 1 – 20 years toombak chewing duration. While most participants (62%) have 6 – 10 toombak chewing/day and 51% were put toombak in Lower upper lip (Table 1).

Table 1. Demographics characteristics of study cases.

		Frequency	Percent
Age groups			
	15 – 25 years	12	12
	26 – 35 years	24	24

	36 – 45years	33	33
	46 – 55 years	18	18
	More than 55 years	13	13
Toombak chewing duration/year			
	1 – 10 years	44	44
	11 – 20 years	36	36
	21 – 30 years	15	15
	31 – 40 years	3	3
	Above 40 years	2	2
Toombak chewing frequency/day			
	1 – 5	27	27
	6 – 10	62	62
	11 – 15	7	7
	More than 15	4	4
Location of Toombak			
	Lower lip	15	15
	Upper lip	32	32
	Lower upper lip	51	51
	Others	2	2

Study results were showed the epithelial atypia was observed in 6% (6/100) of examined smears by PAP stain, while the AgNOR increased in number with 31% (31/100). Furthermore, the p53 protein was positive in 3% (3/100) of participants (Table 2).

Table 2. Results of Papstain, AgNOR and p53 protein for study cases (100 Tombak snuffers).

		Frequency	**Percent**
Pap stain			
	Cytological changes	6	6
	No cytological changes	94	94
	Total	100	100
AgNOR			
	Increase in number	31	31
	Normal number	69	69
	Total	100	100
p53 protein			
	Positive	3	3
	Negative	97	97
	Total	100	100

AgNOR had significant correlation within age (*P value* = 0.000), Toombak chewing duration /year (*P value* = 0.000), and Toombak chewing frequency /day (*P value* = 0.026) (Table 3).

Table 3. Distribution of AgNOR according to age group, Toombak chewing duration and frequency.

Age group	**Increased AgNOR**
15 – 25 years	0
26 – 35 years	0
36 – 45 years	0
46 – 55 years	18
More than 55 years	13
Total	31
Duration/year	
1 – 10	2

11 – 20	9
21 – 30	15
31 – 40	3
More than 40	2
Total	31
Frequency/day	
1 – 5	5
6 – 10	15
11 – 15	7
More than 15	4
Total	31

Discussion:

Toombak is known to be contain a high concentration of tobacco specific nitrose amine (TSNA), which is a potent carcinogen. Therefore, the high frequency of patients affected with oral cancer, who were mostly toombak dippers reflect the importance of the subject. In Sudan the habit of taken Toombak is socially not for female, hence female users are rare. Toombak dippers develop a clinically and histologically characteristic lesions at the site of dipping. This study assessed the oral cytological changes in lips, buccal mucosa and lateral ventral tongue among Sudanese Toombak users. To assess the cytological changes among Toombak users three indictors were studied; cytological atybia by Pap stain, AgNOR by silver stain and p53 protein by ICC.

Cytological atypia in the present study was recorded with 6% (6/100) and this is higher than that of non-users (Zero/50), and same finding was reported by Ahmed in Sudan [5]. This changes may be due to the common compounds in Toombak; specially the TSNAs which may stimulate the epithelial cells to undergo squamous differentiation or cellular morphological changes that may lead to malignancy as suggested by others authors [4].

Five of the 6 Toombak users that showed cytological a tybia were used Toombak for more than 20 years, while only one user for less than 10 years. Therefore 10 years of Toombak using enough to develop atypia, and this also was the finding by Idris *et al.*, [9]. This may be because of the fact that the mechanical and chemical effects need more time to increase their effect on the oral mucosa which in agreement with the finding by Winn et al., [10].

Increased in AgNOR may indicate that abnormalities response increases by Toombak using, these findings supported by other study [11]. There is an association between increased AgNOR and age, duration, frequency with the significant values. The increase in AgNOR number among Toombak users is supported by Akhtar *et al.*, [12]. AgNOR counts have been of great value for the assessment of cellular proliferated activity that is frequently encountered in pre-malignant and malignant changes. A number of studies had pointed out that the AgNOR count is a rapid and an easily reproducible method which permits a clear distinction between malignant and benign cells, and there are many previous AgNOR studies of oral mucosa with benign, pre-malignant and malignant lesions, and only a few studies have been conducted on exfoliative cells obtained from oral mucosa exposed to cigarette smoking or alcohol [13 – 15].

From our knowledge, p53 was not studied in depth among Toombak users, in spite of that p53 was less sensitive in detection of cytological changes when compared with Pap stain and AgNOR.

Conclusion:

Toombak may regarded as a risk factor for developing abnormal oral epithelial cells. So, Toombak users should undergo continuous screening programs. AgNOR was more sensitive for detection of cytological changes among Toombak users. Therefore, this finding suggest AgNOR as predictive, diagnostic and prognostic biomarker for oral lesions and cancer.

Acknowledgments:

The Faculty of Medical Laboratory Sciences are thanked for their Molecular laboratory.

References:

1. Mehrotra, R., Gupta, A., Singh, M., Ibrahim, R. Application of cytology and molecular biology in diagnosing premalignant or malignant oral lesions. *Mol Cancer.* 2006; **5**:11.

2. AgarwaI, S. P., Raamamoorthy, K. Manual for health professionals. National cancer control programme. Ministry of health and family welfare, Government of India. November 2005.

3. Win, S. S., NIshioka, M., Myint, B. A., Takagi, M. Assessment of p53 protein expression in leukoplakia, oral submucous fibrosis and oral lichen planus in Myanmar patients. *Oral Med Pathol.* 2005; **10**:15-21.

4. Idris, A. M., Prokopczyk, B., Hoffmann, D. Toombak: a major risk factor for cancer of the oral cavity in Sudan. *Prev Med.* 1994.

5. Ahmed, H. G-E., Idris, M. A., Ibrahim, O. S. Study of oral epithelial atypia among Sudanese tobacco users by exfoliative cytology. *Anticancer research*. 2003; **23:**1943-1945.
6. Crocker, J. N. P. Nucleolar organiler regions in lymphomas. *Pathol*. 1987; **151** (2): 111-118.
7. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., Vousden, K. H. p14ARF links the tumour suppressors RB and p53. *Nature*. 1998; **395**:124-12.
8. Blagosklonny M. V. p53:An ubiquitous target of anticancer drugs. *International Journal of Cancer*. 2002; **98**:161-166.
9. Idris, A. M., Warnakulasuriya, K. A., Ibrahim, Y. E., Nielsen, R., Cooper, D., Johnson, N. W. Toombak-associated oral mucosal lesions in Sudanese show a low prevalence of epithelial dysplasia. *J Oral Pathol Med.* 1996; **25** (5):239-44.
10. Win, S. S., NIshioka, M., Myint, B. A., Takagi, M. Assessment of p53 protein expression in leukoplakia, oral submucous fibrosis and oral lichen planus in Myanmar patients. *Oral Med Pathol*. 2005; **10**:15-21.
11. Warnakulasuriya, K. A., Ralhan, R. Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco. *J Oral Pathol Med*. 2007.
12. Akhtar, G. N., Chaudrhy, N. A., Tayyab, M. A., Khan, S. A. AgNOR Staining in Malignant and Benign Effusions. *Pakistan J Medical Science*. 2004; **20** :29-32.
13. Recardo, P., Manoel, S. F., Paula, L. B., Isabel, L. AgNOR quantification in cells of normal oral mucosa exposed to smoking and alcohol: A cytopathologic study. *Analytical and quantitative cytology and histology / the International Academy of Cytology [and] American Society of Cytology*. 2004; **26** (3):175-80
14. Ahmed, H. G-E., Babike, A. A. Assessment of cytological atypia, AgNOR and nuclear area in epithelial cells of normal oral mucosa exposed to toombak and smoking. *Rare Tumors*. 2009; **22** (1): e18.
15. Patrícia, F., Gustavo, C., Jaqueline, I., Adriana, B., Janete, A. Head and Neck Pathology. Vol. 2. *Humana Press*. 2008: pp.157-162.