

# Comparative Assessment of Nuclear and Nucleolar Cytochemical Parameters of Oral Epithelial Cells in Smokers and Non-Smokers by Methyl Green-Pyronin Staining

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## Abstract

**Introduction:** A strong relationship exists between cigarette smoking and the development of oral squamous cell carcinoma. Smoking can significantly increase cellular proliferation. Nevertheless, there is little reference in literature to the cytological assessment of oral mucosa in this respect. **Methods:** Changes in nuclear and nucleolar cytomorphometric parameters such as diameter, surface, number and color intensity, in cytologic smears which were collected from normal buccal mucosa of 30 cigarette smokers and 30 non smokers, using methyl green-pyronin staining were studied. **Results:** Our findings attested to smoking as significant inductive factor in cytochemistry as well as morphologic changes. **Conclusion:** This technique is a valuable tool.

**Key Words:** Cytochemistry, methyl green-pyronine, nucleolus, oral mucosa, smoking.

## Introduction

Head and neck squamous cell carcinoma is the sixth most prevalent cancer the world over. Unfortunately, no significant improvement was seen in its survival rate despite diagnostic as well as therapeutic breakthroughs (1). As survival to a large extent, depends on the stage at which cancer is detected or prophylactic treatment plan prior to any further spread (2), a reliable diagnostic index must be routinely applied since no clinical evidence on visual examination could assist in this respect.

Nucleus as the center of cellular proliferation control is subjected to mutation, particularly by environmental factors as the main trigger. Mitogens primarily result in an increase in DNA content of the cell during the resting phase. Genomic instability can well be detected prior to any evidence of morphological changes throughout the development of a malignancy. Also, a gradual increase in quantitative DNA aberrations has been found to correlate with increasing degree of dysplasia in oral mucous membranes (3).

To detect DNA and RNA content of oral epithelial cells exposed to tobacco-derived carcinogens, we need a practical, easy-to-apply and cost-effective method.

Applying prevailing methods, Schulte et al. (4) concluded that methyl green-pyronin, a specific method for selective staining of nucleic acids is the best for routine use.

Methyl green tends to bind specifically to DNA, staining the nuclei green whereas pyronin is specific for RNA, staining nucleoli red. Therefore, this method has the potential to differentiate DNA and RNA (5).

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The aim of this study was to determine the two most important cellular chemical components (DNA and RNA) and their morphometric parameters in the oral squamous epithelium of smoker and non-smoker individuals based on cytochemical technique (smear preparation and methyle green pyronine staining), in order to determine it's suitability to become a useful method for demonstrating the proliferative states of cells in clinically normal oral tissue sections and may possibly be applied to premalignant and malignant lesions.

## Materials and Methods

A total of 60 smokers and non-smokers with an age range of 30-40, were selected for the study. These were patients of the Faculty of Dentistry of the Mashhad University of Medical Sciences, for routine dental checkups. The smokers had been using a minimum of 20 cigarettes a day for at least 10 years. Neither the smokers nor non-smokers had any oral lesions, systemic disease or even any histopathological dysplasia in microscopic evaluation. The smears were taken from clinically normal buccal mucosa.

First, the patients used a mouthwash with 0.9% sodium chloride solution for 15 minutes, and the area to be smeared was wiped of excessive saliva and surface debris. The smears were obtained with a wooden tongue spatula scraped firmly across the mucosa and the cells were scattered on a dry glass slide, and then fixed in 95% ethanol for 12 hours.

After keeping slides in an incubator (48°C) for 24 hours, methyl green-pyronin staining (Merck, Darmstadt, Germany) was performed. Methyl green (1.5g) and pyronin (2.5g) were dissolved in 200 ml of distilled water. After which 60 ml of distilled water and 20 ml phosphate buffer (M/10), PH5, were mixed and added to the above solution. Excess stain was blotted from the slides. Slides were placed in an alcoholic solution (25% ethanol and 75% butanol) for 2 min, dipped in xylene for a further 2 min, and were finally mounted with Entellan (Merck). The PH of the solution was reconfirmed after each slide was stained.

In this method, pyronin stains RNA red, while methyl green stains DNA green. All sections were examined at a magnification of x 1,000 under a light microscope (Leica Galen III Microscope; Buffalo, NY, USA) equipped with a digital camera (Sony ExWaveHAD, Model No. SSC-DC58AP; Tokyo, Japan) and an eyepiece micrometer.

For each section, 100 epithelial cells were selected randomly in different fields. An ocular micrometer mounted in one of the eyepieces was used to measure the diameters of nuclei as well as nucleoli. Also, the

formula of an ellipse was used to compute the area of nuclei and nucleoli. The numbers of nucleoli were counted in 10 high-power fields. In addition, mean color intensity was determined using a quantifying grading system of + for mild, ++ for moderate and +++ for intense positivity. The slides were assessed by two independent observers blinded for data.

Minitab and Excel software were used to analyze the data. One-way analysis of variance (ANOVA) was used to compare the above parameters in the studied groups.

## Results

As shown in Table 1, ANOVA revealed a considerable difference in mean large diameter of nucleus, with smokers being 7.98 and non-smokers 7.32 ( $P<0.001$ ). The mean small diameter of nucleus, likewise, showed significant disparity, with a mean of 6.13 and 5.47 for the former and the latter group, respectively ( $P<0.001$ ). The area of nucleus was depicted considerably higher in smokers, with the mean of 39.2 compared to 32.04 in non-smokers ( $P<0.001$ ). Nevertheless ANOVA did not illustrate any substantial gap regarding the diameter, number and area of the nucleoli in the both groups.

As to nuclear color intensity, weak positivity was shown to be more frequent in smokers. In contrast, non-smokers showed a greater propensity for moderate and intensive positivity, with the highest proportion in the mild positivity group. This correlation was established in Chi-Square test, whereby a significant correlation ( $P<0.001$ ) was demonstrated between color intensity and smoking (Table 2).

**Table 1.** ANOVA findings regarding nuclear morphometric parameters

Histochemical parameters		Statistical measure		
		Mean	Standard deviation (SD)	Range
Large diameter	Non-smokers	7.32	0.26	6.22 – 8.13
	Smokers	7.98	0.29	7.1 – 9.15
Small diameter	Non-smokers	5.47	0.20	4.68 – 6.25
	Smokers	6.13	0.22	5.38 – 7.23
Area	Non-smokers	32.04	20.62	24.18 – 40.31
	Smokers	39.2	30.58	30.5 – 52.91

**Table 2.** Chi-square test results to establish correlation between nuclear color intensity and smoking

Color intensity	Number of nuclei			
	Incidence	Non-smokers	Smokers	Total
Mild (+)	Observed	68	123	191
	Incidence			
	Expected	95.5	95.5	
Moderate (+ +)	Observed	1194	1171	2365
	Incidence			
	Expected	1182.5	1182.5	
Intense (+ + +)	Observed	138	106	244
	Incidence			
	Expected	122	122	
	Total	1400	1400	2800

## Discussion

Despite considerable breakthroughs in diagnostic as well as therapeutic procedures, head and neck squamous cell carcinoma is still taking its toll worldwide (2).

Early oral cancers and precancerous lesions are often subtle and asymptomatic (6). In addition, histopathological changes may be present in areas in which there is no clinical evidence of an oral lesion on visual examination alone (7). Therefore, it is important for the clinician to maintain a high index of suspicion, especially if risk factors such as tobacco use or alcohol abuse is present (6).

Globally, there is a rapid increase in consumption of smoking tobacco products (1). Also, the strong association between cancers of the oral cavity and pharynx with the use of tobacco is well established. Epidemiological studies show that the risk of

developing oral cancer is five to nine times greater for smokers than for non-smokers (8).

There are many evidences demonstrate that cigarette smoking significantly increases cellular proliferation (9-11). Tobacco contains carcinogens that influence the DNA repair, cell cycle control and may produce chromosomal aberrations (12). In one study (13) nicotine discovered as a mutagenic agent, because nicotine-stimulated DNA synthesis resulted in cellular proliferation. In another survey, Schwartz et al. (14), showed that smoking Increases DNA content, aneuploidy, percentage of cells in synthesis (S) and G2+Mitosis (M) phases. In 2007, Salehinejad et al. (11) compared normal buccal mucosa in cigarette smokers and non-smokers and found significant increasing in cellular proliferation in smokers even before presence of any clinical symptoms.

Recently, several staining methods have been used to evaluate cellular proliferation activity, such as AgNORs, Pyronine Y, Toluidine Blue, feulgen and methyl green. These specific staining techniques are used for detection of the nucleus or nucleolus compartments (15).

The argyrophilic nucleolar organizer regions (AgNORs) are loops of DNA in the nucleolus which code for ribosomal RNA and thus are of vital significance in the synthesis of proteins. They are demonstrable as black dots in the silver stained tissue section. The proliferative activity has also been studied by AgNOR technique which has been extensively used in different malignancies. With increasing degree of malignancy, the number and size of the nucleoli are increased and the positions of the nucleoli are shifted to the periphery of nucleus (16).

Pyronine Y, an iron-based method, selectively stains sulfated mucins in addition to aiding detection of other tissue parameters, including RNA, elastic fibers, and mast cell granules (17). Pyronin Y was selected as RNA stain on the reports of its efficacy as an absorption stain for RNA (18).

Tolonium chloride, more commonly referred to as Toluidine Blue, has been used for more than 40 years to aid in detection of mucosal abnormalities of the cervix and the oral cavity. Toluidine Blue is a metachromatic vital dye that may bind preferentially to tissues undergoing rapid cell division (such as inflammatory, regenerative and neoplastic tissue), to sites of DNA change associated with oral premalignant lesions or both. There is evidence that Toluidine Blue is effective as a diagnostic adjunct for use in high-risk populations and suspicious mucosal lesions (19).

Feulgen staining represents a staining method to quantitatively document the DNA content of a nucleus. Thus, it is an excellent and straightforward method to reflect the irregular increase in DNA content of a malignant cell as a sign of genetic instability (20).

Methyl green staining is also utilized for nuclear staining. This method is useful for staining chromatin DNA (7).

As mentioned above AgNOR and pyronin Y staining methods are specific for nucleolar proteins and RNA, while Toluidine Blue, feulgen and methyl green are used to identify DNA.

A staining method that labels multiple tissue elements in a single section would save time and resources and enable assessment of possible topographic relationship between these elements. Pyronin Y has long been used, in combination with other dyes such as methyl green, as a differential stain for nucleic acids in paraffin tissue sections (21). Methyl green-pyronin is superior to other methods, covering both DNA and RNA components.

The aim of this study was to evaluate association between morphometric parameters of the nuclei and nucleoli of methyl green-pyronin stained cells of clinically normal oral epithelium, with smoking. The findings demonstrated a progressive increase in nuclear color intensity, diameter and area in smokers than non-smoker.

## Conclusion

This technique can be used to measure exposure to tobacco carcinogens, and possibly establish a link to premalignant and malignant transformation before a lesion is noted. The simplicity of this technique makes it a valuable tool even for daily routine.

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## References

1. Hunter KD, Parkinson EK, Harrison PR. Profiling early head and neck cancer. *Nat Rev Cancer* 2005; 5: 127-35.
2. Mashberg A, Boffetta P, Winkelman R, Garfinkel L. Tobacco smoking, alcohol drinking, and cancer of the oral cavity and oropharynx among U.S. veterans. *Cancer* 1993; 72: 1369-75.
3. Sudbo J, Ried T, Bryne M, Kildal W, Danielsen H, Reith A. Abnormal DNA content predicts the occurrence of carcinomas in non-dysplastic oral white patches. *Oral Oncol* 2001; 37: 558-65.
4. Schulte EK, Lyon HO, Hoyer PE. Simultaneous quantification of DNA and RNA in tissue sections. A comparative analysis of the methyl green-pyronin technique with the gallocyanin chromalum and Feulgen procedures using image cytometry. *Histochem J* 1992; 24: 305-10.
5. Preto P, Lyon HO. Methyl green-pyronin Y staining of nucleic acids: studies on the effects of staining time, dye composition and diffusion rates. *Biotech Histochem* 2003; 78: 27-33.
6. Silverman SJR. Demographics and occurrence of oral and pharyngeal cancers. The outcomes, the trends, the challenge. *J Am Dent Assoc* 2001; 132:7S-11S.

7. Ehrig T, Abdulkadir SA, Dintzis SM, Milbrandt J, Watson MA. Quantitative amplification of genomic DNA from histological tissue sections after staining with nuclear dyes and laser capture microdissection. *J Mol Diagn* 2001; 3: 22-5.
8. Neville BW, Day TA. Oral cancer and precancerous lesions. *CA Cancer J Clin* 2002; 52: 195-215.
9. Tan D, Goerlitz DS, Dumitrescu RG, Han D, Seillier-Moiseiwitsch F, Spornak SM, Orden RA, Chen J, Goldman R, Shields PG. Associations between cigarette smoking and mitochondrial DNA abnormalities in buccal cells. *Carcinogenesis* 2008; 29: 1170-7.
10. Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer Cell* 2010; 17: 89-97.
11. Salehinejad J, Kalantari MR, Omid AA, Zare R. Evaluation of AgNOR Staining in exfoliative cytology of normal oral (buccal) mucosa: effect of smoking. *J Mash Dent Sch* 2007; 3: 22-24.
12. Souto GR, Caliar MV, Lins CE, de Aguiar MC, de Abreu MH, Mesquita RA. Tobacco use increase the number of aneuploid nuclei in the clinically healthy oral epithelium. *J Oral Pathol Med* 2010; 39: 605-10.
13. Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Appl Physiol* 1998; 84: 2089-98.
14. Schwartz JL, Muscat JE, Baker V, et al. Oral cytology assessment by flow cytometry of DNA adducts, aneuploidy, proliferation and apoptosis shows differences between smokers and non-smokers. *Oral Oncol* 2003; 39: 842-54.
15. Mohtasham N, Mahdavi-Shahri N, Salehinejad J, Ejtehadi H, Torabi-Parizi M, Ghazi N. Detection of nucleoproteins in squamous cell carcinoma, and dysplastic and normal mucosa in the oral cavity by methyl green-pyronin staining. *J Oral Sci* 2010; 52: 239-43.
16. Khan AH, Khan SA, Chaudhry NA, Tayyab M. Comparison of argyrophilic nucleolar organiser regions (AgNORS) with proliferating cell nuclear antigen (PCNA) in different grades of transitional cell carcinoma of urinary bladder. *Annals of King Edward Medical University* 2007; 13: 186-90.
17. Meyerholz DK, Rodgers J, Castilow EM, Varga SM. Alcian Blue and Pyronine Y histochemical stains permit assessment of multiple parameters in pulmonary disease models. *Vet Pathol* 2009; 46: 325-8.
18. Shapiro HM. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and pyronin Y. *Cytometry* 1981; 2: 143-50.
19. Patton LL, Epstein JB, Kerr AR. Adjunctive techniques for oral cancer examination and lesion diagnosis: a systematic review of the literature. *J Am Dent Assoc* 2008; 139: 896-905.
20. Knüchel R, Koufou SV, Speicher M, Schwamborn K, Zaak D, Stöhr R. Precancerous lesions of the urothelium. From Feulgen staining to single cell CGH. *Pathologie* 2008; 29: 364-70.
21. Li B, Wu Y, Gao XM. Pyronin Y as a fluorescent stain for paraffin sections. *Histochem J* 2002, 34: 299-303.

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