

Detection of nucleoproteins in squamous cell carcinoma, and dysplastic and normal mucosa in the oral cavity by methyl green-pyronin staining

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Abstract: Squamous cell carcinoma (SCC) is the most malignant neoplasm of the oral cavity. The mortality rate associated with head and neck cancers remains high, and thus detection techniques are important. In the present study, methyl green-pyronin staining was applied to 5 normal mucosa specimens, 10 cases of dysplastic mucosa, 12 cases of SCC (grade I), 10 cases of SCC (grade II) and 5 cases of SCC (grade III) in the oral cavity. This is the first study in which nuclear and nucleolar cytomorphometric parameters (diameter, area and number) in such oral lesions were evaluated using this method. As methyl green-pyronin selectively stains nucleic acids, it can differentiate between DNA (green) and RNA (red). The results reveal that the proposed method can be used to evaluate changes in the nucleus and nucleolus in premalignant lesions in the oral cavity. (*J Oral Sci* 52, 239-243, 2010)

Keywords: squamous cell carcinoma; methyl green-pyronin staining; nucleus; nucleolus.

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Introduction

The cell division cycle consists of four phases: G₁ (first gap), S (DNA synthesis), G₂ (second gap) and M (mitosis). The molecules that are most often affected in tumors are those involved in the control of the G₁-S transition of the cell cycle. Genetic alterations, if unrepaired in the G₁-phase, may be carried into S-phase and perpetuated in subsequent cell divisions. The regulatory pathways controlling the cell cycle phases include several oncogenes and tumor suppressor genes, and alterations in different families of cell cycle regulators will cooperate in tumor development. Increases in ribonuclease activity, DNA and RNA synthesis, and anaerobic glycolysis are some of the differences between normal and tumor cells (1,2). The distinction between malignant and benign tumors is commonly based on clinical and histopathological criteria using hematoxylin and eosin (H&E) staining. In difficult cases, the histochemical and immunohistochemical methods may also assist in making the diagnosis.

Squamous cell carcinoma (SCC) is the most prevalent malignant neoplasm of the oral cavity. The histopathologic grade of SCC is dependent on its differentiation (3), which is commonly evaluated by H&E staining. However, in some cases, pathologists may disagree regarding the histopathologic grading of SCC.

Methyl green-pyronin staining is a specific method for selectively staining nucleic acids. Methyl green binds specifically to deoxyribonucleic acid (DNA), staining

nuclei green, while pyronin is specific for ribonucleic acid (RNA), staining nucleoli red. Therefore, this method has the potential to differentiate DNA and RNA (4).

In the present study, cytomorphometric changes in the nucleus and nucleolus in SCC of different grades, and dysplastic and normal mucosa in the oral cavity, were evaluated by methyl green-pyronin staining in order to determine its suitability for detecting premalignant lesions and carcinogenesis.

Materials and Methods

Forty-two formalin-fixed paraffin-embedded tissue blocks were retrieved from the files of the Oral and Maxillofacial Pathology Department of Mashhad Dental School, Iran. The samples comprised 5 normal mucosa, 10 dysplastic mucosa, 12 cases of SCC (grade I), 10 cases of SCC (grade II) and 5 cases of SCC (grade III). Sections (4 μ m) were cut and placed on glass slides, after which they were deparaffinized and hydrated in distilled water. After keeping slides in an incubator (48°C) for 24 h, methyl green-pyronin staining (Merck, Darmstadt, Germany) was performed. Methyl green (1.5 g) and pyronin (2.5 g) were dissolved in 200 ml of distilled water, after which 60 ml of distilled water and 20 ml phosphate buffer (M/10), pH 5, 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 $\times 1,000$ 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 and the following parameters were evaluated:

- 1) Diameters of nuclei and nucleoli: an ocular micrometer mounted in one of the eyepieces was used to measure diameters.
- 2) Nuclear area: the formula for an ellipse was used to compute the area of nuclei.
- 3) Number of nucleoli: the number of nucleoli was counted in 10 high-power fields.

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

Results

ANOVA gave the following results:

- 1) The mean large diameter of the nucleus and related statistical measures in the studied groups are shown in Table 1. The differences in mean nuclear large diameter were statistically significant ($P < 0.002$).
- 2) The mean small diameter of the nucleus and related statistical measures are summarized in Table 2.

Table 1 Statistical data on large-diameter nuclei in the studied groups

Group	Statistical measure			
	Mean	Median	Standard deviation (SD)	Range
Normal mucosa	4.43	4.4	0.138	4.26-4.58
Dysplastic mucosa	4.62	4.71	0.253	4.28-5.08
SCCI	4.92	5.01	0.333	4.36-5.36
SCCII	5.01	4.93	0.366	4.47-5.62
SCCIII	5.15	5.2	0.283	4.86-5.46

Table 2 Statistical data of small-diameter nuclei in the studied groups

Group	Statistical measure			
	Mean	Median	Standard deviation (SD)	Range
Normal mucosa	3.44	3.38	0.085	3.32-3.54
Dysplastic mucosa	3.70	3.75	0.178	3.36-3.96
SCCI	3.74	3.69	0.251	3.42-4.16
SCCII	3.83	3.84	0.355	3.37-4.55
SCCIII	3.99	4.14	0.395	3.54-4.38

ANOVA confirmed a significant difference between the mean nuclear small diameters ($P < 0.04$). The small and large diameters of the nucleus in the different groups were ranked as follows: SCC III > SCC II > SCC I > dysplastic mucosa.

- 3) As shown in Fig. 1, differences in mean nuclear area in the studied groups were statistically significant ($P < 0.04$).
- 4) The mean number of nucleoli is shown in Fig. 2. The data showed significant differences when assessed by one-way ANOVA ($P < 0.00$). Thus, a progressive increase in mean nuclear area and number of nucleoli is seen from normal mucosa through SCC III. Nuclei and nucleoli in SCC I and SCC III are shown in Figs. 3 and 4, respectively.
- 5) Correlation between the mean diameters of the nucleolus and type of studied mucosa was evaluated by analysis of variance. Thus, a significant difference was not observed ($P > 0.05$).

Discussion

SCC is the most malignant neoplasm in the oral cavity. The mortality rate associated with head and neck cancers remains high because the disease is often diagnosed at an advanced stage (3,5). Therefore, the early detection of oral SCC is of critical importance. Multiple detection techniques have thus been developed to address this problem.

Evaluation of the histopathologic grade of oral SCC is dependent on differentiation, which is based on criteria such as nuclear pleomorphism, intercellular bridges, changes in nucleocytoplasmic ratio, number of keratin pearls and mitosis. Well-differentiated SCC is considered to be grade I, whereas a poorly differentiated SCC is denoted as grade III (3). The different grades of SCC are routinely evaluated by H&E staining, but in some cases, controversy can exist among pathologists.

There are several differences between normal and tumor cells. Proliferative activity of the nucleus and nucleolus progressively increases from normal to dysplastic mucosa and SCC. AgNOR, toluidine blue O, azure B, pyronin Y, methyl green and feulgen are some of the specific staining techniques used for detection of the nucleus or nucleolus.

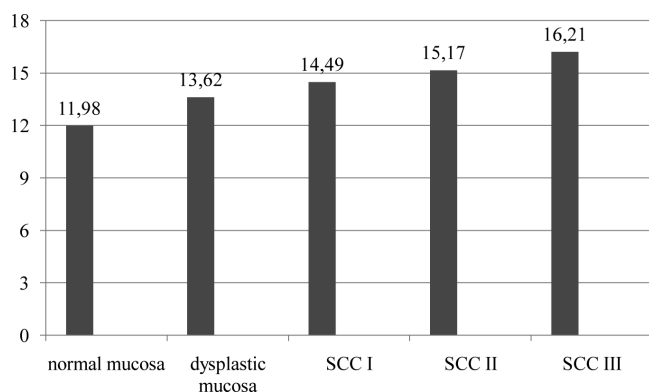


Fig. 1 Mean nuclear area in the studied groups.

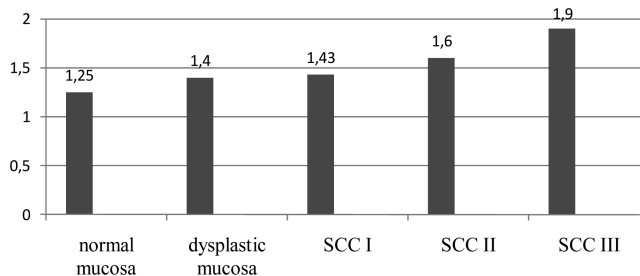


Fig. 2 Mean number of nucleoli in the studied groups.

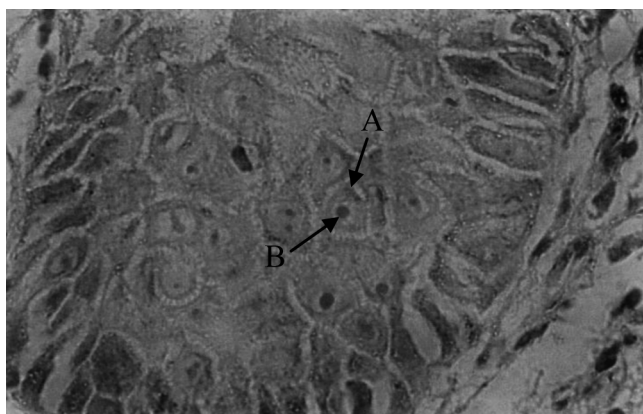


Fig. 3 Nuclei (A) and nucleoli (B) in SCC I (methyl green-pyronin, original magnification $\times 1,000$).

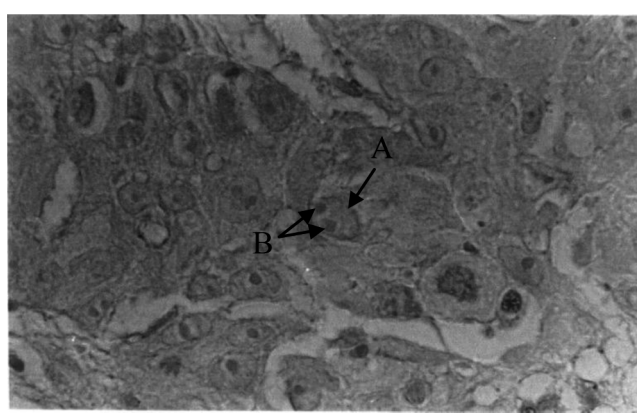


Fig. 4 Nuclei (A) and nucleoli (B) in SCC III (methyl green-pyronin, original magnification $\times 1,000$).

AgNOR staining can give an indication of cell proliferation. AgNOR proteins are a set of argyrophilic nucleolar proteins that accumulate in highly proliferating cells, whereas their expression is very low in non-proliferating cells. Some of these proteins remain associated with nucleolar organizer regions (NORs) during mitosis. NORs are selectively stained by silver-staining, after which NORs can be identified as black dots exclusively localized throughout the nucleolar area, and are called "AgNORs". The intensity of staining depends on the transcriptional activity of the cells. Expression of AgNOR proteins is measured globally by quantification of silver-staining levels using morphometry and image analysis (6,7). It has been reported that the number of AgNOR dots helps to differentiate hyperplastic, premalignant and malignant tissues (8). In one study (7), oral SCC was compared to the surrounding dysplastic and normal tissues by the AgNOR method. The findings of the current study agree with those of previous reports.

The problem with AgNOR staining is that doublets or clusters are impossible to discern by light microscopy. Stain deposition also may be misinterpreted as proliferate activity. AgNOR scores reported by different authors for the same types of tumor also sometimes differ (9). Using methyl green-pyronin staining, the above shortcomings can be alleviated.

Toluidine blue O, azure B, and methyl green (active ingredient, ethyl green) are essentially one-component nuclear dyes. Because of their cationic nature, these dyes are believed to bind to negatively charged DNA in the nuclei. The mechanism of interaction between methyl green and DNA has been investigated in detail and is believed to involve not only electrostatic interaction, but also nonionic forces. The nonionic component could be due to intercalation of the dye between the purine and pyrimidine bases. Therefore, methyl green, azure B, or toluidine blue O allows reliable assessment of DNA contents in the tissue, while the pyronin Y method is specific for detection of RNA (10,11).

Feulgen stain contains fuchsin or magenta I, a decolorized dye that has a strong affinity for DNA, producing a red color. The feulgen reaction is specific for deoxyribonucleoprotein; thus, nuclear chromatin and chromosomes are stained. Nucleoli and cytoplasm, which contain ribonucleoprotein, are feulgen negative (12).

As mentioned above, AgNOR and pyronin Y staining methods are specific for nucleolar proteins and RNA, respectively, while toluidine blue O, azure B, methyl green and feulgen are used to identify DNA. It should be noted that using the methyl green-pyronin method, differentiation between DNA and RNA is possible, as both are stained.

In this dichromatic solution, Pyronin Y binds preferentially with RNA, whereas methyl green reacts strongly with DNA. This results in selective staining of the nuclei and nucleoli, which is the advantage of this technique.

Immunohistochemistry staining also has potential to determine the percentage of tumor cells immunoreactive to proliferative markers. Immunohistochemical expression of cell proliferation proteins, such as PCNA and p53, in lesions having malignant transformation potential, such as epithelial dysplasia, was reported by Sousa et al. (13). It has been shown that the expression of p53 and ki-67 was significantly higher in oral SCC than in premalignant dysplasia, hyperkeratosis and normal mucosa (14).

In our study, evaluation of the mean number of nucleoli, mean nuclear diameter and mean areas demonstrated a progressive increase from normal mucosa to SCC III. Therefore, methyl green-pyronin appears to be a reliable method for acid nucleic detection, in which DNA is green while RNA is red (15). This technique can therefore be used as an adjunct to routine H&E staining for determining malignancy. It is also useful for investigating therapeutic results in cancers, as it has been demonstrated that the proliferation and differentiation of cells can be monitored by methyl green-pyronin staining after therapeutic procedures in esophageal cancer (16).

Because of the size and rigidity of nucleoli, they are particularly attractive cell structures for quantification (17). In our study, as nuclear RNA was stained with pyronin, the number of nucleoli was determined by counting. The morphology of nucleoli is altered during the cell cycle, and they become smaller at the primary phase of mitosis (18). As most cells are probably evaluated at the primary phase of mitosis, there were no statistically significant differences between the mean diameter of nucleoli and type of studied mucosa in the present study.

In conclusion, in this study, methyl green-pyronin staining was applied to cases of SCC (Grade I, II, III), and dysplastic and normal mucosa in the oral cavity. The results showed significant cytomorphometric changes in the studied groups. Therefore, this technique can be used for evaluating changes in the nucleus and nucleolus in premalignant lesions and during carcinogenesis. In future studies, using methyl green-pyronin staining, therapeutic results in oral SCC, as well as correlations between cytomorphometric changes in the nucleus and nucleolus, and stage of oral SCC will be evaluated.

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