Original Article

Comparative analysis of nucleomorphometric parameters in methyl green-pyronin-stained sections of oral epithelial dysplasia, oral submucous fibrosis and oral squamous cell carcinoma

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Abstract

Context: The diagnosis and grading of epithelial dysplasia is based on a combination of architectural and cytological changes. A gradual increase in quantitative DNA aberrations has been found to correlate with increasing degree of dysplasia in oral mucous membranes.

Aims: The aim of this study is to assess nuclear parameters in potentially malignant and malignant lesions of the oral cavity and to assess cytomorphometric changes in the nucleus and nucleolus in oral epithelial dysplasia (OED), oral submucous fibrosis (OSMF), oral squamous cell carcinoma (OSCC) and normal oral mucosa using methyl green-pyronin staining to determine its suitability for detecting potentially malignant lesions and the stage of carcinogenesis.

Methods: Forty-five archival sections of OED, OSMF and OSCC and 5 cases of normal oral mucosa as the control group were stained according to methyl green-pyronin-staining protocol. Cytomorphometric parameters such as nuclear diameter, nucleolar diameter, number of nucleoli and cytoplasmic RNA were assessed.

Statistical Analysis Used: The study was subjected to statistical analysis to evaluate the association between morphometric parameters using analysis of variance test, followed by Bonferroni's *post hoc* analysis.

Results: A progressive increase in the nuclear parameters as well as cytoplasmic RNA content was observed between normal mucosa through dysplasia and OSMF to OSCC.

Conclusion: This study serves as an effective diagnostic aid in assessing nuclear parameters in potentially malignant and malignant epithelial lesions affecting oral cavity.

Keywords: Dysplasia, methyl green-pyronin, nucleolus, nucleus, oral submucous fibrosis, squamous cell carcinoma

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INTRODUCTION

Oral potentially malignant lesions represent a range of mucosal alterations at the tissue and cellular level with reports showing a 6%–36% risk of progression to cancer.[1-3] Quantitative parameters such as nuclear and cytoplasmic morphometric and nuclear protein analysis have shown measureable changes in cells correlating with the malignant potential of a tumor.[4-6]

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High rate of glycolysis and disrupted fidelity of ribonuclease are some of the alterations caused by mitogens.[7,8] DNA is localized in the nucleus whereas RNA in the cytoplasmic ribosomes and nucleolus.[9,10] Leon *et al.* demonstrated that circulating DNA concentration correlates positively with the tumor proliferative potential.[11,12] MicroRNAs are short RNA transcripts that exhibit a 10–100-fold increase during cancer progression. Thus, DNA and RNA assay can act as a potential biomarker for malignant transformation of oral premalignancy.[13,14]

Histological examination of tissues is considered the gold standard for diagnosis of oral lesions.[15] A staining technique that allows simultaneous examination of multiple tissue elements preserving their topographical relations would save time and resources.[16] Hematoxylin and eosin (H and E) and methyl green-pyronin (MGP) can provide a simple and cost-effective histological staining method.[17,18] Methyl green is peculiar among cationic dyes as it has two positive charges.[19] The mechanism of action of the stain depends on the degree of polymerization of nucleic acids, thus highly polymerized nucleic acids (i.e., DNA) stain with methyl green, while low polymers (i.e., RNA) stain with pyronin.[20] MGP allows selective and simultaneous staining of nuclei and nucleolus of tumor cells, enabling a clear differentiation of the nucleic acids that can be reproduced by computerized image analysis.[21,22] Analysis of nucleic acids at the level of individual cell and within the context of tissue microenvironment may aid in the diagnosis of difficult lesions.

Hence, the aim of the study was to assess cytomorphometric changes in the nucleus and nucleolus in oral epithelial dysplasia (OED), oral submucous fibrosis (OSMF), oral squamous cell carcinoma (OSCC) and normal mucosa in the oral cavity, using methyl green-pyronin staining to determine its suitability for detecting potentially malignant lesions and the stage of carcinogenesis.

METHODS

The retrospective study consisted of fifty formalin-fixed paraffin-embedded tissue blocks retrieved from the archives of the Department of Oral and Maxillofacial Pathology. The samples comprised 15 diagnosed cases of dysplastic oral mucosa, 15 diagnosed cases of OSMF and 15 diagnosed cases of OSCC. Five cases of normal oral mucosa were taken as the control group. Four-micrometer sections were prepared from each block, and slides were prepared. The sections were then deparaffinized and hydrated in distilled water. Staining of the sections was performed by a solution of 2% methyl green: 2% pyronin (9:4), pH – 4.8. Sections were placed in the solution for 25 min, followed by rinsing in acetate buffer. Excess stain was blotted dry from the slides. Sections were then dehydrated in alcoholic solution followed by dipping in xylene for 2 min and finally mounted. The slides were observed at ×1000 magnification under an oil-immersion light microscope. Twenty epithelial cells were selected randomly in different fields for each biopsy section, and the following parameters were evaluated: (1) nuclear diameter, (2) nucleolar diameter, (3) number of nucleoli was counted in 10 high-power fields and (4) cytoplasmic RNA [Figures 1-5]. One-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* analysis, was used to compare the parameters assessed in the groups studied. A value for $P < 0.001$ was considered as statistically significant.

RESULTS

The nuclear area, nucleolar area, mean number of nucleoli and cytoplasmic RNA content exhibited a statistical increase from normal oral mucosa through dysplasia to OSCC with OED and OSMF showing insignificant differences among them [Figures 1-8].

The mean nuclear area and related statistical measures in the studied groups are shown in Table 1. For calculation of significant values, comparisons were made among mean ± standard deviation. The differences in mean nuclear area were statistically significant when OSCC was compared with OSMF, OED and normal oral mucosa ($P < 0.001$).

The mean nucleolar area and related statistical measures are summarized in Table 1. ANOVA confirmed a significant difference between the mean nuclear diameters ($P < 0.001$).

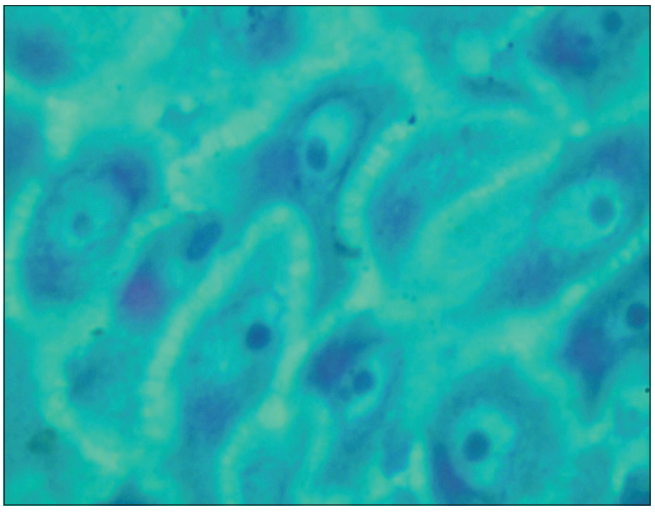

Figure 1: Photomicrograph of methyl green-pyronin-stained section of normal oral mucosa showed nuclei with regular nuclear membrane outlines and round symmetrical nucleoli, with cytoplasm showing even staining of pyronin (Original magnification, ×1000)

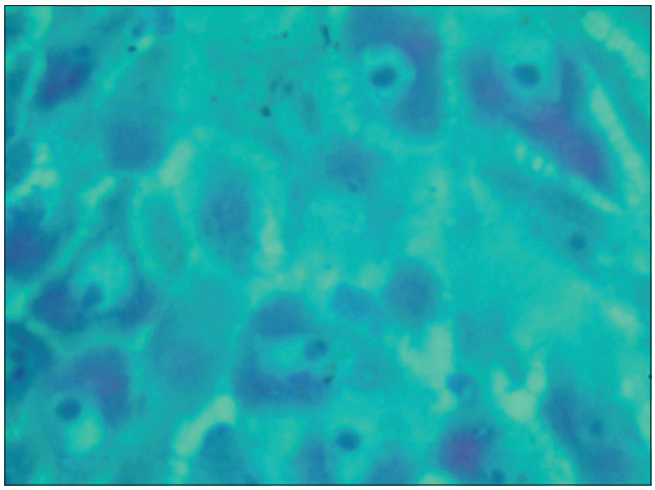

Figure 2: Photomicrograph of methyl green-stained section of oral epithelial dysplasia showed variation in the regular outline of nucleus from round to oval in shape, with cytoplasm showing irregular staining of pyronin (Original magnification, ×1000)

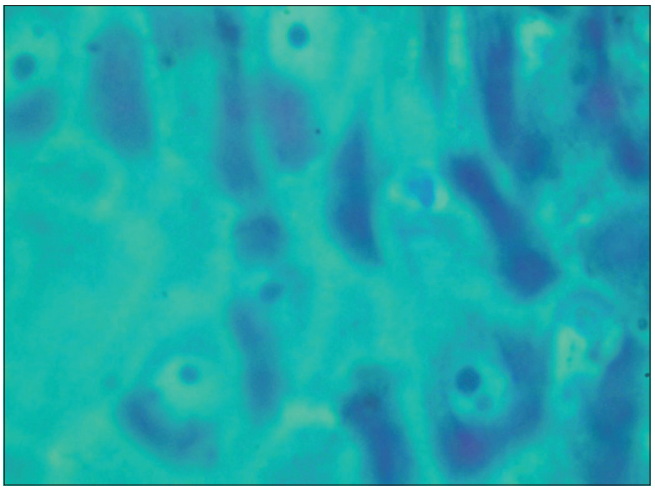

Figure 3: Photomicrograph of methyl green-pyronin-stained section of oral submucous fibrosis showed nuclei with irregular outline and variable number of nucleoli. Cytoplasm shows uneven staining of pyronin (Original magnification, ×1000)

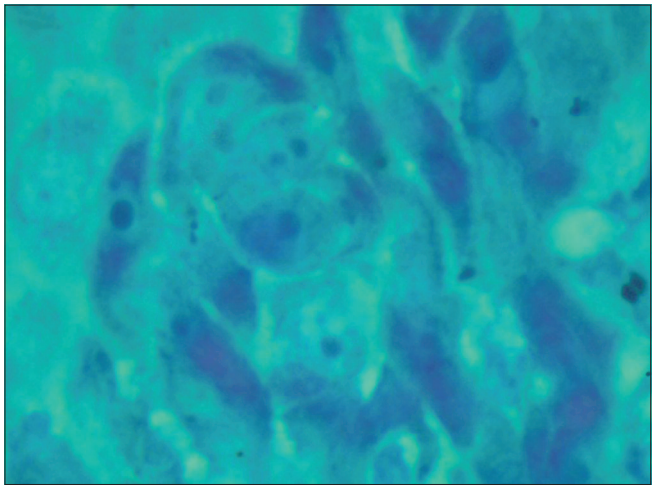

Figure 4: Photomicrograph of methyl green-pyronin-stained section of oral squamous cell carcinoma showed elongated nuclei and nucleoli with inconspicuous borders. Few nuclei show multiple nucleoli. Cytoplasm shows patchy staining of pyronin (Original magnification, ×1000)

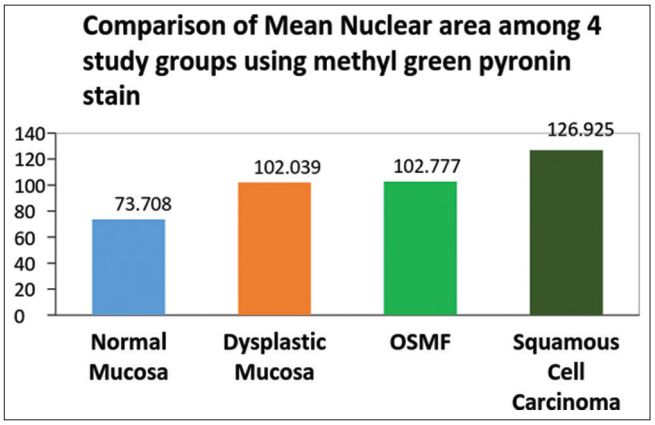

Figure 5: The mean nuclear area increased from normal mucosa through squamous cell carcinoma. Dysplastic mucosa and oral submucous fibrosis showed little variance

The values were found to be increased in OSCC when compared to normal oral mucosa, and these values increased gradually through OED and OSMF.

The mean area of cytoplasmic RNA is shown in Table 1. The data showed significant differences when assessed by one-way ANOVA, followed by Bonferroni's *post hoc* test ($P < 0.001$).

As shown in Table 1, differences in mean number of nucleoli in the studied groups were statistically significant ($P < 0.001$). The values obtained were highest for OSCC and lowest for normal oral mucosa with intermediate values for OED and OSMF.

Thus, a progressive increase in mean nuclear area, mean nucleolar area, number of nucleoli and cytoplasmic RNA content was seen from normal oral mucosa through OED and OSMF to OSCC. *P* values were highly significant on comparison of OSCC with OED, OSMF and normal oral mucosa. On comparison of OED and OSMF, the values did not show a characteristic difference. The values obtained under the four parameters were ranked as OSCC > OSMF > OED > normal oral mucosa.

DISCUSSION

Quantification of nucleic acids and detection of nuclear abnormalities can be used as an important tool to study tumor biology and assess disease progression from potentially malignant disorders to carcinomas.[23] History of methyl green can be traced backed to 1877 when it was introduced into microtechnique by Calberla and its affinity

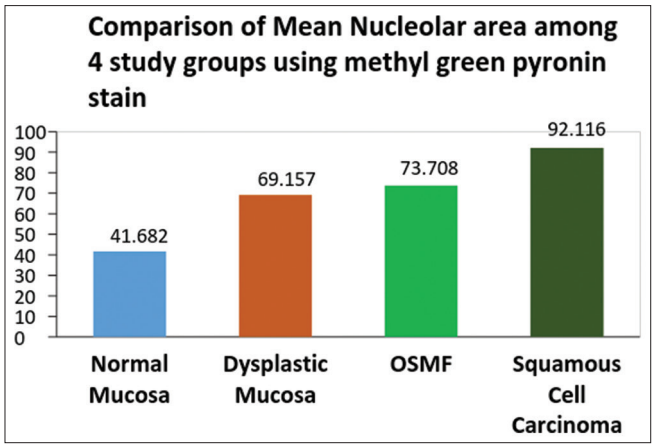

Figure 6: Mean nucleolar area also exhibited similar results as nuclear area

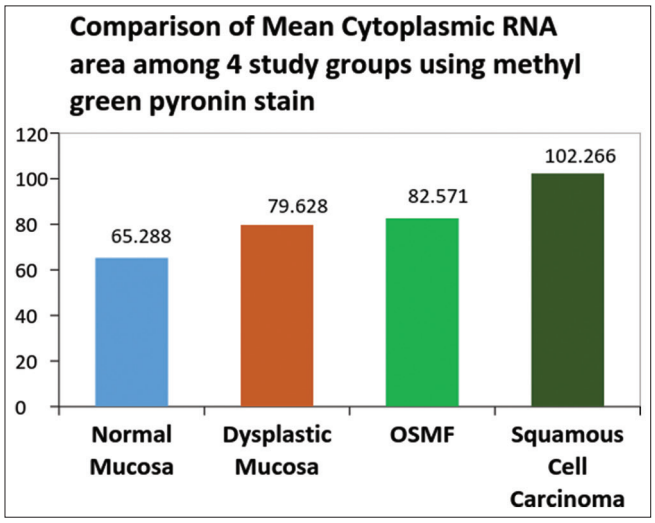

Figure 7: Cytoplasmic RNA content was ranked as minimum in normal mucosa < dysplasia < oral submucous fibrosis < squamous cell carcinoma

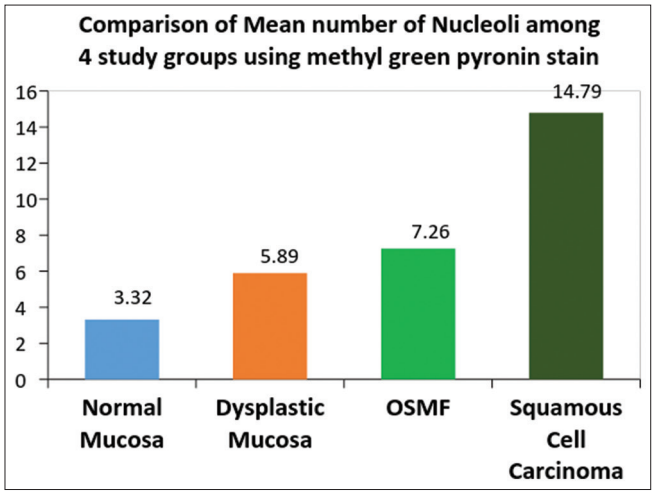

Figure 8: Mean number of nucleoli showed significant variance among the four groups with highest among squamous cell carcinoma and lowest in normal mucosa

for nuclein was introduced by Carnoy in 1884. MGP as a differential stain was introduced by Pappenheim in 1899 which was modified by Unna in 1902. In 1940, Brachet demonstrated that methyl green stains DNA specifically and pyronin staining was a direct indication of the presence of RNA.[19,20,22] Carnoy's fluid is the preferred fixative for the tissues, and butyl alcohol is the suitable differentiating solution following wash in ice-cold distilled water. Routine formalin fixation may lead to artifacts and background staining; hence, proper fixation, acetone dehydration and the use of pure methyl green dye and pyronin dye in standard concentration are recommended.[24-26] As methyl green is often contaminated with methyl violet and requires repeated extractions with chloroform, malachite green can be used instead which produces similar results.[19] Henceforth, numerous studies have been performed to evaluate the efficacy and efficiency of MGP for different tissues.

Elias recommended that rinsing the sections in ice cold water is a crucial step in MGP staining for qualitative nucleic acid differentiation.[27] Potvin described the staining methodology for MGP staining using samples of immunoblastic lymphadenopathy. According to the author using pure dye form of the stain with the correct color index number is crucial to obtain better results.[28] Lavarack stained frozen-dried tissues from albino rat using MGP and obtained reliable results of DNA and RNA demonstration.[10] Perry and Reynolds demonstrated that MGP provides a dynamic picture of white cell physiology in peripheral blood and bone marrow smears when compared with other conventional stains.[29] Iseki and Mori stated from their study on frozen sections of tissues that MGP stain can demonstrate proliferating states of cells and may possibly be applied to cancer tissues.[30] Schulte *et al.* compared MGP-staining technique with the gallocyaninchromalum and Feulgen procedures using image cytometry.[17] They concluded that standardized MGP stain is a reliable and simple method for the simultaneous quantitative assessment of both RNA and DNA. Karpinska correlated morphometric parameters in invasive ductal cancer cells and stated that MGP technique enables a standardized and reproducible examination of the tissue structures with computerized image analysis.[21]

Our findings were consistent with those reported by Mohtasham *et al.*, who demonstrated a progressive increase in mean number of nucleoli and mean nuclear diameter from normal oral mucosa to poorly differentiated SCC. They concluded that nucleic acid detection by MGP can be used as an adjunct to routine H and E staining for the determination of malignancy.[31] In another study, Jahanshah

Table 1: Results obtained from analysis of variance followed by Bonferroni's *post hoc* test

Parameter	Group	*N*	Mean	SD	SE	95% CI		Min	Max	*P*-value	Sig. diff.	P-Value
						Lower	Upper					
Nuclear area	Normal mucosa	52	73.708	6.995	0.970	71.760	75.655	56.510	89.120	<0.001*	S vs D	<0.001*
	Dysplastic mucosa	58	102.039	17.536	2.303	97.428	106.650	78.580	148.970		S vs N	<0.001*
	OSMF	69	102.777	16.340	1.967	98.851	106.702	69.630	134.240		S vs O	<0.001*
	Squamous cell carcinoma	62	126.925	9.481	1.204	124.517	129.333	101.040	143.400			
Nucleolar area	Normal mucosa	60	41.682	6.411	0.828	40.026	43.338	30.090	55.300	<0.001*	S vs D	<0.001*
	Dysplastic mucosa	83	69.157	5.545	0.609	67.947	70.368	53.850	79.290		S vs N	<0.001*
	OSMF	77	73.708	3.090	0.352	73.007	74.410	63.030	79.810		S vs O	<0.001*
	Squamous cell carcinoma	75	92.116	3.805	0.439	91.240	92.991	81.460	99.390			
RNA	Normal mucosa	48	65.288	13.455	1.942	61.380	69.195	47.640	98.570	<0.001*	S vs D	<0.001*
	Dysplastic mucosa	42	79.628	11.838	1.827	75.939	83.317	67.350	110.990		S vs N	<0.001*
	OSMF	49	82.571	12.729	1.818	78.915	86.227	59.380	114.910		S vs O	<0.001*
	Squamous cell carcinoma	40	102.266	8.277	1.309	99.619	104.914	91.240	123.700			
Nucleoli	Normal mucosa	19	3.32	0.946	0.217	2.86	3.77	2	5	<0.001*	S vs D	<0.001*
	Dysplastic mucosa	19	5.89	1.329	0.305	5.25	6.54	3	8		S vs N	<0.001*
	OSMF	19	7.26	0.933	0.214	6.81	7.71	6	9		S vs O	<0.001*
	Squamous cell carcinoma	19	14.79	1.843	0.423	13.9	15.68	12	18			

OSMF: Oral submucous fibrosis. S: Oral squamous cell carcinoma, D: Oral dysplasia, N: Normal oral mucosa, O: Oral submucous fibrosis.
*$P<0.001$=Statistically significant

et al. evaluated the association between morphometric nuclear parameters in MGP-stained cells of clinically normal oral epithelium and cells altered by smoking. They found a progressive increase in the values. According to them, MGP-staining method can be used to establish a link to premalignant and malignant transformation before a lesion is noted.[32] Metgud *et al.* obtained results similar to Mohtasham *et al.* when assessing nuclear parameters using MGP in tissue sections as well as smears of premalignant and malignant lesions. In addition, they compared MGP staining with Feulgen staining and obtained better results with MGP stain, as Feulgen staining did not demonstrate nucleoli or ribonucleoprotein in cytoplasm.[25,33] Sumedha *et al.* used MGP-staining technique for detecting apoptotic cells in malignant and potentially malignant lesions of oral mucosa. They obtained better staining results with MGP when compared with H and E.[18] A similar pilot study of apoptotic cells was carried out by Nayak *et al.* and concurrently by Simila *et al.* They concluded that MGP staining can be used as a cost-effective routine basic laboratory-staining technique.[34,35]

In the present study, the evaluation of nuclear parameters exhibited a consistent increase from normal mucosa through premalignancy to malignancy. The shape of the nucleus in normal mucosa was round and regular, whereas in dysplasia and squamous cell carcinoma, it gradually became oval with irregular borders. The number of nuclei, nucleoli and cytoplasmic RNA content increased consistently from normal mucosa through dysplasia and OSMF to SCC, with dysplasia and OSMF showing little variance among them.

OSCC is recognized as the most common malignant epithelial neoplasm of the oral cavity. Normal oral mucosa adjacent to OSCC is an interesting model for studying the biology of epithelia, as it may have a higher risk for malignant change according to the premalignant hypothesis.[36] Dysplasia represents a spectrum of abnormal epithelial maturation (dysplasia) and cellular aberrations (atypia) with an increased risk of malignant transformation.[37] Yet, the diagnosis and grading of epithelial dysplasia is highly subjective.[4] Several studies done on OSMF have proposed that cytomorphometric changes could be the earliest indicators of cellular alterations in OSMF. There is a progressive increase in cellular and nuclear diameter which serves as a sensitive parameter in the diagnosis of OSMF.[38] Abnormal nuclear morphology is a hallmark of neoplasia with DNA and RNA playing a role as prognostic biomarkers.[39,40] The average number of nucleoli per five high-power fields can be used as a definitive indicator toward disease severity.[4]

The combination of methyl green with pyronin can differentiate between RNA and DNA as the DNA appears green and RNA red. It can also distinguish between single- and double-stranded DNAs, as following denaturation, single-stranded DNA is known to become pyroninophilic.[30] A limitation of routine hematoxylin staining is its incompatibility with immunofluorescence. Methyl green can be used for fluorescent staining of fixed biological tissues.[41,42] Therefore, MGP can be used as an adjunct to routine H- and E-staining procedures and successfully determine the extent of proliferation and differentiation of cells.

CONCLUSION

Nucleo-cytomorphometric analysis is the preliminary step for the evaluation of cells progressing to malignancy.

The aforementioned benefits of MGP make it a suitable staining method which can be used as a supplemental option to evaluate the extent of proliferation and differentiation of cells which is not accurately reproduced by routine H&E. in addition to morphometric parameters we have also made an attempt to evaluate cytoplasmic RNA content which can be used secondarily to examine disease progression.

Technical considerations

Tissue staining is dependent on the handling and processing of the tissues before staining. Purity of methyl green, concentration of the two dyes and pH of dye solution have to be controlled.[43,44]

Future prospects

A standard staining protocol has to be devised so as to improve the shortcomings of the staining results. It can be used as a surrogate marker to many DNA and RNA IHC markers.

Since it is cost-effective, it can be used in screening larger population, especially in Asian subcontinent.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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