

Detection of Apoptosis in Leukoplakia and Oral Squamous Cell Carcinoma using Methyl Green Pyronin and Hematoxylin and Eosin

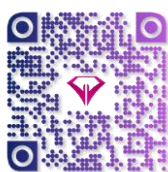
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KEYWORDS

Apoptosis,
Hematoxylin,
Leukoplakia,
Methyl green,
Squamous cell carcinoma

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Main Subjects:
Oral Pathology

Received 04 May 2019;

Accepted 06 March 2020;

Published Online 23 May 2020;

 [10.30699/ijp.2020.107263.2115](https://doi.org/10.30699/ijp.2020.107263.2115)

ABSTRACT

Background & Objective: Cell population and turnover are controlled by a balance between cell proliferation and apoptosis. Detection of apoptosis in oral cancer contributes to its better prognosis and improved management. This study aimed to quantify apoptotic cells in leukoplakia and oral squamous cell carcinoma (OSCC) using methyl green-pyronin (MGP) and hematoxylin and eosin (H & E) staining.

Methods: The sample included a total of 130 subjects (comprising 108 males and 22 females). Formalin fixed and paraffin embedded tissues were used and categorized into three groups of normal oral mucosa (n=10), leukoplakia with dysplasia (n=60), and OSCC (n=60). The number of apoptotic cells and apoptotic index (AI) were calculated after staining with MGP and routine H & E stained slides.

Results: MGP stained the condensed chromatin of apoptotic cells. Statistically significant difference ($P \leq 0.001$) was observed among various study groups in terms of numbers of AI and apoptotic cells. Also, AI increased with increasing grades of dysplasia, and it was the highest in well differentiated OSCC. Results were statistically significant in both H & E and MGP stained sections ($P \leq 0.001$). A good correlation was found between MGP and H & E staining results.

Conclusion: MGP is more specific and can lead to intense staining for chromatin in apoptotic cells. Accordingly, it can provide a good alternative to H&E in identifying apoptotic cells.

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Introduction

The origin of the term “apoptosis” roots back to Greek literature, with intellectual meaning of “falling off”. The term was initially coined by Hippocrates (460-370 BC) with reference to the “falling of bones” and later Gallen extended its meaning to “dropping off scabs” (1). It is a physiological cell death pathway, which is triggered by a tightly regulated cascade in which cells are destined to die. In this process, enzymes are activated that degrade the cell’s nuclear DNA together with nuclear and cytoplasmic proteins, which cause blebbing of the cell membrane, cell shrinkage, chromatin condensation, and DNA fragmentation followed by engulfment of surrounding cells. Apoptosis can be observed and analyzed under microscope by identifying presence of spherical or ovoid acidophilic globules rich in intensively basophilic nuclear fragments. These acidophilic globules are known as as apoptotic bodies (2).

In healthy tissue, the normal cell population is maintained by a balance between cell proliferation and

apoptosis. Disparity between the two processes causes various pathological conditions. Analysis of apoptosis in cases of oral cancer has improved its diagnosis and prognosis, and has enabled exploration of better ways to manage this cancer (3).

Oral squamous cell carcinoma (OSCC) accounts for at least 90% of all oral malignancies; it is the 11th most common cancer worldwide. OSCC is more frequent among men than women (1.5:1) globally (4). It exhibits significant mortality and morbidity rates. Therefore, it is important to investigate factors with prognostic relevance in order to develop the management of OSCC, improve survival rate, and decrease complications.

Virtually, all oral cancers exhibit two stages. A two-step process of cancer progression, i.e., an initial

precancerous stage that subsequently evolves into final stage of oral cancer is well established. The precancerous stages referred to as potentially

malignant disorders (PMDs) are defined as “a family of morphological alterations among which some may have increased potential for malignant transformation” (5). Among different PMDs of oral cavity like leukoplakia, erythroplakia, sideropenic dysphagia, oral lichen planus (OLP), and oral submucous fibrosis (OSMF), oral leukoplakia is the most common and prevalent form. To treat oral cancer effectively, an early diagnosis of these PMDs is essential. To do this, tissue alterations that occur in these disorders must be recognized clinically and histologically, and at the molecular level as early as possible. A significant transformation at cellular level is the appearance of apoptosis. Quantification of apoptosis helps determine the tissue turnover rate, which helps determining the prognosis of the cancer. Therefore, identification of apoptosis could serve as a prognostic marker for the biological behavior of the cancer (3).

Identification of apoptotic cells is feasible, because their characteristic morphological features can be observed by light microscopy with routine stains (2). Other techniques for identifying apoptotic changes include terminal deoxynucleotidyl transferase mediated d-UTP nick-end labeling (TUNEL), DNA diffusion assay, and cytofluorometric analysis. Although technically sophisticated, these techniques have limitations including cost, insensitivity, and production of artifacts. Alternatively, histochemical stains such as Methyl Green-Pyronin Y (MGP) and Feulgen have been used (6).

Our search results showed that there is only one study in which MGP staining in PMD and OSCC has been conducted to identify apoptotic cells. Therefore, the present study aims at quantifying apoptotic cells in leukoplakia and OSCC using MGP and H & E staining. Meanwhile, an attempt is made to quantify apoptotic count in different grades of dysplasia.

Materials and Methods

This study was conducted at Post Graduate Institute of Dental Sciences, Rohtak, Haryana, India from Feb 2017 to Apr 2018. We examined 130 histopathologically diagnosed cases of normal oral mucosa, leukoplakia, and OSCC. Accordingly, 10 cases of normal oral mucosa (group 1), 60 cases of leukoplakia (group 2), and 60 cases of OSCC (group 3) were included. This study was approved by the Institutional Ethics Committee of Post Graduate Institute of Dental Sciences, Rohtak. Well informed oral and written consents were taken from all patients prior to their participation in the study.

The exclusion criteria were: history of chemotherapy or radiotherapy, history or symptoms of systemic illness, recurrence of carcinoma, and unwillingness to undergo biopsy procedure. Normal oral mucosa procured during gingivectomy or periodontal procedures were used as positive controls.

Leukoplakia cases were categorized further into different grades of dysplasia, whereas OSCC cases were graded according to Broder's grading system.

Specimens of normal oral mucosa, leukoplakia, and OSCC were processed using automatic tissue processor (Thermo Microm STP 120 tissue processor, thermo scientific, GmbH, Germany) and formalin fixed and paraffin embedded tissue blocks were prepared. Required archival paraffin embedded blocks were also retrieved and two serial sections were cut at 4 μ m using a semiautomatic microtome (325 HM microtome, thermo scientific, GmbH, Germany). One section was stained with routine H & E and the other one with MGP. This stain (Sigma Aldrich; Bengaluru, India) was prepared according to method recommended by Mohtasham (7). The pH of the staining solution was maintained at 4.2–4.5. Staining by MGP was performed using the procedure recommended by Potvin *et al.* (8). Plasma cells in lymph node sections served as the positive control for MGP staining having intensely red cytoplasm and green to blue-green nuclei.

Apoptotic cells exhibit condensed chromatin, which is composed of DNA and RNA. Since MGP is a special stain used to identify DNA and RNA, we used it to visualize condensed chromatin in apoptotic cells. The criteria proposed by Kerr *et al.* were used for scoring apoptosis as follows: a) condensation of the chromatin to clearly delineated granular masses along the nuclear envelope, b) cell shrinkage, c) convolution of the cellular and nuclear outlines, and d) fragmentation of the nucleus (9).

Apoptotic cells were counted by three observers in 10 random fields using high magnification (400 x) in each slide stained with H & E and MGP. Identification of apoptotic cells was based on a referral chart (control) provided to the observers (Figure 1). In each section, 1000 cells were evaluated for apoptosis. To remove any bias, mean of the observations was considered as the mean number of apoptotic cells for each subject. AI was then calculated as the number of apoptotic cells expressed as a percentage of the total number of cells counted for each specimen.

Statistical Analysis

The data were interpreted using SPSS 21 (SPSS Inc., Chicago, Ill., USA). All the three study groups were compared by non-parametric Kruskal-Wallis test while intergroup comparison was done by Mann-Whitney test. Values for $P \leq 0.001$ were considered significant. Correlation between H & E and MGP staining was derived by spearman's coefficient (>0.75), which showed a good correlation between them.

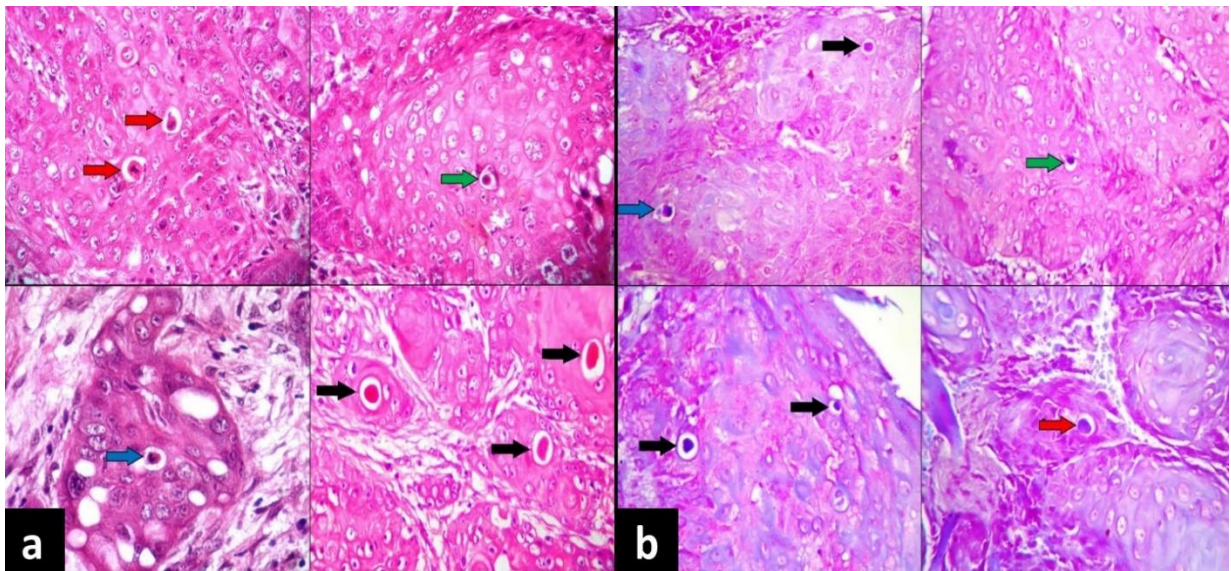


Fig. 1. Control chart for identifying apoptotic cells for (a) H & E; $\times 400$ and (b) MGP; $\times 400$. Red arrow indicates apoptotic cell with nuclear remnants in the center of cell. Green arrow indicates apoptotic cell with inconspicuous nuclear remnant. Blue arrow indicates apoptotic cell with eccentric nuclear remnant placed. Black arrow indicates apoptotic cell with condensed homogenous eosinophilic material.

Results

Out of 130 cases in our study, 83% were males and 17% were females; male predominance was also evident in group 1 (70%), group 2 (95 %) and group 3 (73%). The mean age for group 1 was 40.10 years (26–54 years), group 2 was 45.38 years (21–67 years), and group 3 was 55.60 years (24–80 years).

Various sites affected by lesions were also analyzed. The most common site was the buccal mucosa (55%) followed by retrocommissural area (31.67%) in group 2. In group 3, the retromolar trigone was affected most commonly (28.33%). Other sites included the lower alveolus (18.33%) > buccal mucosa (15%) > tongue (10%) > floor of mouth (8.33%). More than one site was involved in 11.67% of cases.

In group 2, the most common presentation of leukoplakia was non-homogeneous (48.33%) > homogenous (33.33%) > erythroleukoplakia (13.33%) > granular verruciform leukoplakia (5%) type. In group 3, OSCC was seen most commonly in the form of ulceroproliferative lesion (45%) > ulcerative (16.67%) > swelling (6.67%) > sessile growth type (1.67%).

The mean number of apoptotic cells in H & E and MGP stained sections was counted for each group and subgroup (Figure 2). Group 2 included 60 cases that were sub-grouped into 41 cases of mild (2a), 12 cases of moderate (2b) and seven cases of severe dysplasia (2c) (Figure 3), whereas group 3 included only well differentiated OSCCs. The differences among different study groups in terms of apoptotic cell count and AI were highly significant ($P < 0.001$) (Table 1, Figure 4).

Table 1. Apoptotic index and mean apoptotic count among different study groups and subgroups in both H&E and MGP stained sections

Groups	Total number of cases	Apoptotic index \pm SD		Mean apoptotic cell count \pm SD	
		H&E	MGP	H&E	MGP
Normal oral mucosa	10	0.44 \pm 0.33	0.38 \pm 0.20	4.40 \pm 3.26	3.80 \pm 2.04
Leukoplakia	60	0.23 \pm 0.19	0.26 \pm 0.22	2.34 \pm 1.95	2.62 \pm 2.26
Mild dysplasia	41	0.22 \pm 0.21	0.25 \pm 0.24	2.24 \pm 2.14	2.45 \pm 2.46
Moderate dysplasia	12	0.21 \pm 0.13	0.34 \pm 0.18	2.06 \pm 1.25	3.05 \pm 1.76
Severe dysplasia	7	0.34 \pm 0.16	0.21 \pm 0.19	3.43 \pm 1.60	2.80 \pm 1.89
OSCC	60	1.41 \pm 0.58	1.23 \pm 0.45	14.06 \pm 5.76	12.33 \pm 4.48
P-value		< 0.001*	< 0.001*	< 0.001*	< 0.001*

* $P < 0.001$; highly significant

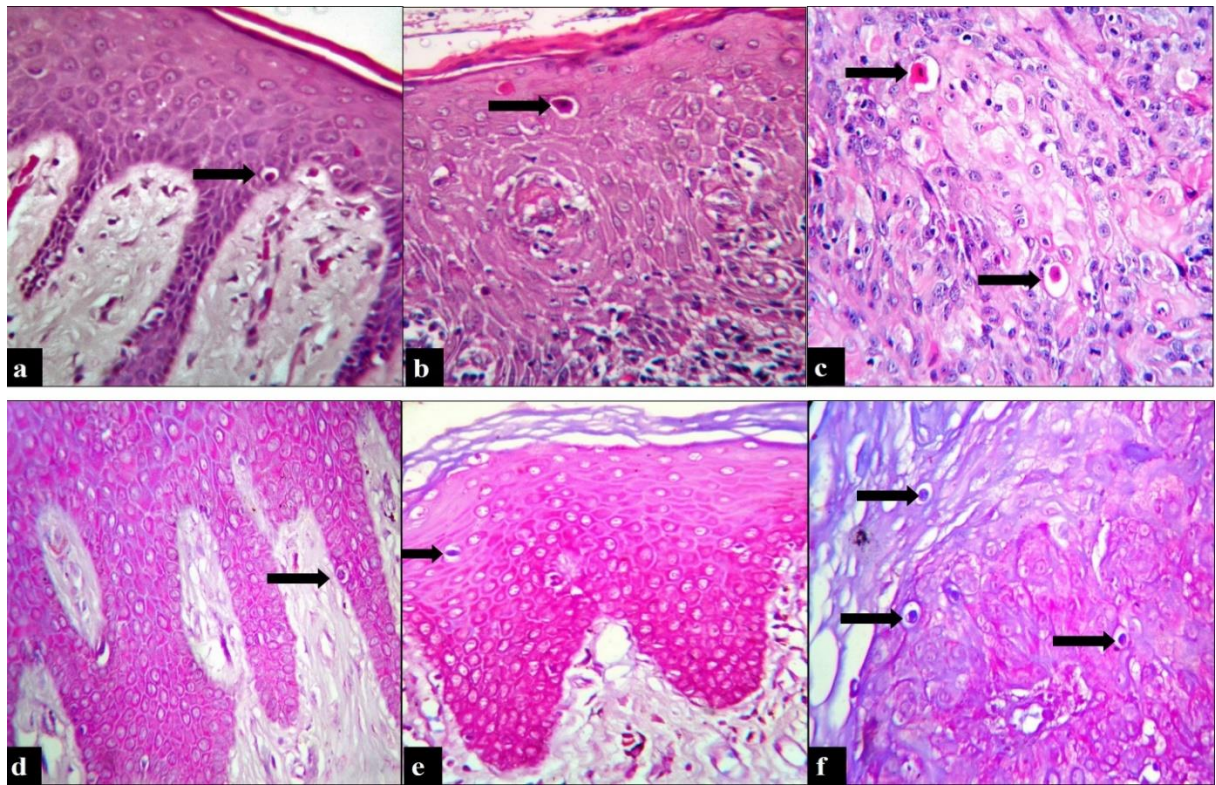


Fig. 2. Apoptotic cells in (a) normal oral mucosa, (b) leukoplakia, (c) OSCC. H & E; x400, and (d) normal oral mucosa, (e) leukoplakia, (f) OSCC. MGP; x400.

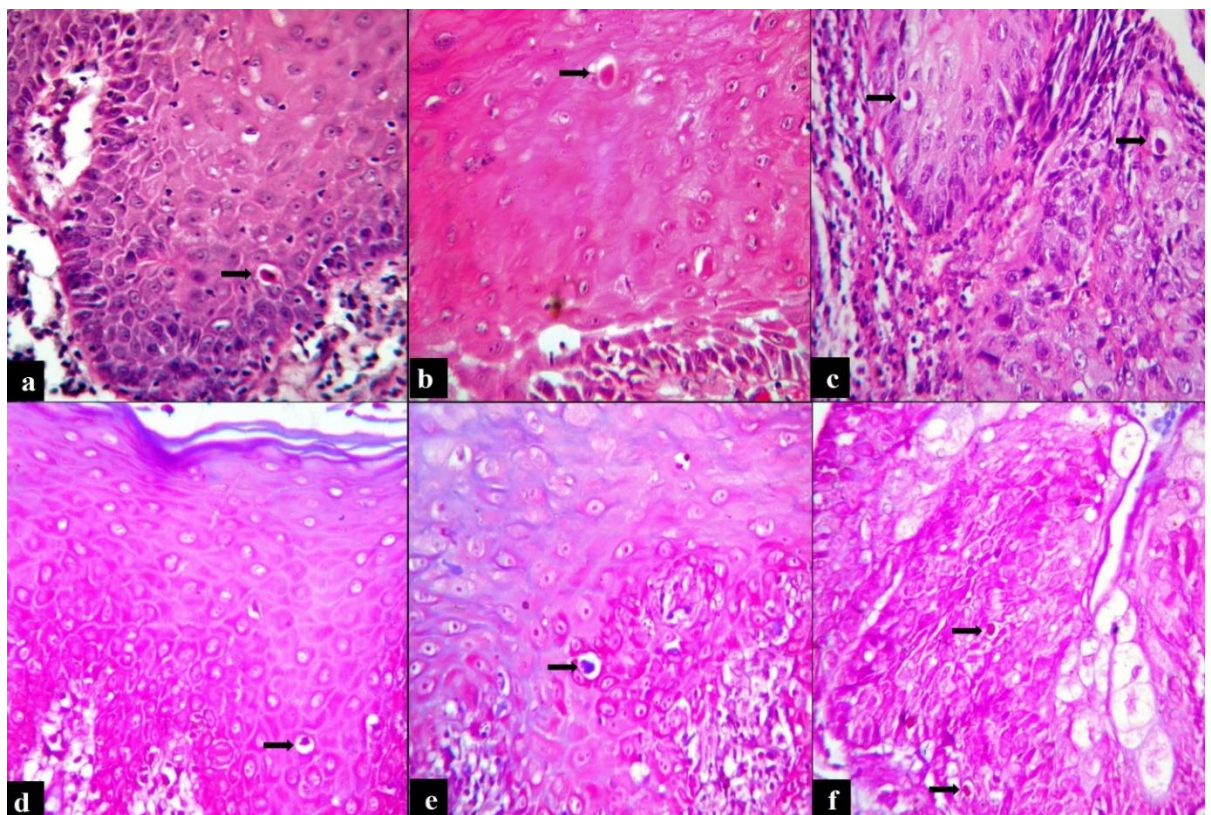


Fig. 3. Apoptotic cells in different histological grades of leukoplakia. a) Mild dysplasia. b) Moderate dysplasia. c) Severe dysplasia. H & E; x 400. d) Mild dysplasia. e) Moderate dysplasia. f) Severe dysplasia. MGP; x 400.

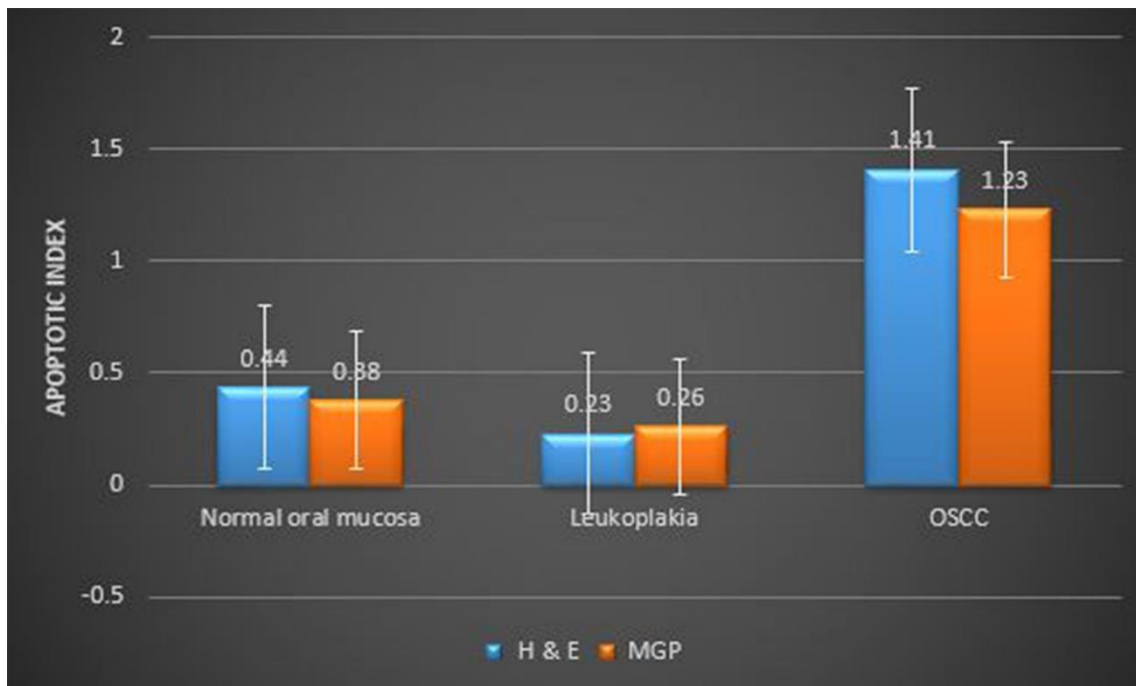


Fig. 4. Apoptotic index among study groups for both H & E and MGP stained sections.

Also, intergroup comparison of mean apoptotic cell count and AI was done. Results of various study groups in terms of mean apoptotic cell count and AI were found to be statistically significant in groups 1 & 2 in

H & E (<0.05) but non-significant in MGP (>0.05). In addition, a highly significant (<.001) difference was observed in relation to AI in Groups 2 & 3 and 1 & 3 with both H & E and MGP stained sections (Table 2).

Table 2. Intergroup comparison among study groups

Groups	Mean apoptotic cell count ± SD		Mean apoptotic cell count ± SD	
	H&E	P-value	MGP	P-value
Normal oral mucosa vs Leukoplakia	4.40 ± 3.26	.029 (<0.05)*	3.80 ± 2.04	.064 (>0.05)
Leukoplakia vs OSCC	2.34 ± 1.95		2.62 ± 2.26	
Leukoplakia vs OSCC	2.34 ± 1.95	< 0.001*	2.62 ± 2.26	< 0.001*
Normal oral mucosa vs OSCC	14.06 ± 5.76		12.33 ± 4.48	
Normal oral mucosa vs OSCC	4.40 ± 3.26	< 0.001*	3.80 ± 2.04	< 0.001*
	14.06 ± 5.76		12.33 ± 4.48	

* P<0.001; highly significant, P<0.05; being significant.

Discussion

Apoptosis is readily identified as eosinophilic cells which are observed routinely in the supra-basal and basal regions of normal oral mucosa. Apoptotic cells increase in number when mucosa undergoes premalignant or malignant conversion (10).

We found that the mean age for leukoplakia and OSCC groups was 45.38 and 55.60 years, respectively. Napier *et al.* reported that leukoplakia was more common in middle aged subjects (11). Others have reported that OSCC was most common during the fifth and sixth decades of life (12, 13). All these findings

suggest earlier occurrence of leukoplakia than OSCC and that about one decade is required for conversion of leukoplakia into OSCC.

We also found that precancer and cancer were more common in males (83%). In the Indian population, Mehta *et al.* (1969) reported 95–98% cases of leukoplakia in males (14). Other investigators reported similar results in Hungarian and American populations (15). We found male predominance of OSCC with a male to female ratio of 2.7:1, which is consistent with some earlier reports (12,16). The predominance of males

in all reports is probably related to the higher predominance of tobacco consumption by males worldwide.

The most commonly affected site in group 2 of our study was buccal mucosa (55%), which is consistent with earlier reports (14,17). By contrast, in Andhra Pradesh, most (71.3%) leukoplakias occurred on the palate owing to prevalence of reverse smoking (14). Similar to other studies, predominance of leukoplakia on buccal mucosa was also seen in our study. Buccal mucosa covers maximum surface area of oral cavity, which makes it more vulnerable to chemical and mechanical insults (18). We found that the most common site affected in OSCC cases in our study was the retromolar trigone region (28.33%), which differs from reports by Leite *et al.* and Feller *et al.* concerning Western countries (12,19).

We found that the most common clinical type in group 2 was non-homogeneous leukoplakia (48.33%). Pindborg *et al.* and Silverman *et al.* reported that most leukoplakia cases were speckled leukoplakia (19, 20). In contrast, others classified the maximum number of leukoplakia patients as homogeneous (21). In group 3, the most common clinical presentation of OSCC was ulceroproliferative (75%); these results were consistent with other earlier reports (12,13).

Group 1 consisted of normal gingival tissues with keratinized epithelium. Apoptotic cells were confined mostly to the supra-basal layer in this group. Others have reported a similar distribution pattern (22). We calculated AI as 0.44 in H & E and 0.38 in MGP stained sections, whereas Sumedha *et al.* observed AI as 0.16 and 0.28 in H & E and MGP stained sections, respectively. Similar findings have been reported by Birchall *et al.* of AI being 0.12 in H & E stained sections (23). Viswanathan *et al.* revealed AI as 0.09 in normal oral mucosa (22). The variation in the AI of our study from AI of Sumedha *et al.* and others could be due to our use of only gingival tissues.

We calculated AI in leukoplakia as 0.23 and 0.26 in H & E and MGP stained sections, respectively; this is consistent with a report by Sumedha *et al.* These researchers reported AI in leukoplakia as 0.2 and 0.22 in H & E and MGP stained sections, respectively. Birchall MA *et al.* recorded AI of 0.28 in dysplasia (23). Another study by Jain *et al.* observed an AI of 0.52 in dysplasia in H & E stained sections (24). Viswanathan *et al.* reported AI in leukoplakia as 0.11, whereas Kesarwani *et al.* calculated AI in oral epithelial dysplasia as 0.41 in H & E stained sections (22,25). Nambiar *et al.* reported AI as 0.74 in oral epithelial dysplasia (26). We found a slight, but not significant, decrease in AI from normal to leukoplakia cases, whereas above mentioned studies by Birchall, Jain, Kesarwani, Viswanathan and Nambiar *et al.* reported a slight increase from normal to leukoplakia. Exclusion of lymphocytes mimicking apoptotic cells in basal and suprabasal layer of inflamed tissues might have reduced false positive count and would have led to this drop in apoptotic cell count.

In H & E sections, AI in mild, moderate, and severe dysplasia was 0.22, 0.21, and 0.3, respectively; and in MGP stained sections, AI in mild, moderate, and severe dysplasia was 0.25, 0.34, and 0.21, respectively. Kesarwani *et al.* used H & E staining and reported AI as 0.23, 0.46, and 0.60 in mild, moderate, and severe dysplasia, respectively (25). Viswanathan *et al.* reported AI as 0.14, 0.17, and 0.14 in mild, moderate, and severe grades of dysplasia, respectively (22). In present study AI increased with increasing grades of dysplasia. However, few research papers also stated that there is no definite pattern being established for AI in different grades of dysplasia. This difference could be explained on basis that there were less number of moderate and severe dysplasia in our study in comparison to mild dysplasia. Ohbu *et al.* observed AI in keratinizing (15.68) and nonkeratinizing epithelium (6.79) and noticed a statistically significant difference between them (27). This difference in AI between keratinizing and non-keratinizing mucosa was also found in our study, which is similar to the findings of Ohbu *et al.*

Group 3 comprised of only well differentiated OSCC. Apoptotic cells were counted in the tumor mass and the apoptotic cells near the areas of inflammation and necrosis were excluded. AI was 1.41 and 1.23 for H & E and MGP stained sections, respectively. Sumedha *et al.* reported AI as 0.53 and 0.83 in H & E and MGP stained sections, respectively.

Most investigators have used MGP to distinguish DNA and RNA. Only Sumedha *et al.* used this staining to detect apoptotic cells in tissue sections. We found that MGP staining of apoptotic cells made their identification easy even at low magnification. MGP stained apoptotic cells were lesser in number as compared to cells with H & E of similar tissues, which was in contrast to the results of Sumedha *et al.* They observed more apoptotic cells and higher AI in MGP stained sections than our study. The difference could be due to strict adherence by all three observers to the referral chart eradicating subject bias employed in our study. We also found that MGP staining was more specific than H & E. The present study appears to be the second research after the study by Sumedha *et al.* that used MGP for identification of apoptotic cells. We encountered some difficulties with standardization of MGP staining despite its reputed simplicity.

We also found that apoptotic cells were identifiable more easily in MGP stained sections than in H & E stained sections. MGP staining of apoptotic cells made their identification possible even at low magnification. A good correlation between MGP and H & E was observed statistically, which suggests that MGP is a useful substitute for H & E in recording apoptosis of tissue sections.

Conclusion

According to our results, it is recommended that quantifying apoptotic cells can be a good prognostic factor for various oral PMDs and OSCC. We observed

significant increase in apoptosis through increasing grades of dysplasia and oral cancer. This information might better inform surgical intervention and development of anti-cancer therapies. MGP is time-consuming and tedious to perform routinely. Also, the cost of the stain is not an advantage compared to H & E. Since MGP stains apoptotic cells precisely, it is recommended as a good substitute for H & E.

Acknowledgements

Our special thanks to Dr. Adarsh for statistical analyzing.

Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this article.

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Sharma, P., narwal, A., kamboj, M. Detection of Apoptosis in Leukoplakia and Oral Squamous Cell Carcinoma using Methyl Green Pyronin and Hematoxylin and Eosin. *Iranian Journal of Pathology*, 2020; 15(3): 189-196. doi: 10.30699/ijp.2020.107263.2115