ORIGINAL RESEARCH

Mitotic Index in Oral Epithelial Dysplasia: A Histopathological Comparison of Feulgen, Giemsa and H & E stains

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Abstract

Background: Oral cancer is ranked as the sixth most frequent malignancy worldwide. Oral malignancies are usually preceded by Oral Potentially Malignant Disorders (OPMDs), which manifest as Oral Epithelial Dysplasia (OED) in the early stages. Carcinogenesis occurs due to genetic alteration of DNA occurring as dysregulated mitosis. Mitotic figures is a significant hint in determining the severity of dysplastic lesions and hence the purpose of this study was to quantify the Mitotic Index in such cases using specific stains such as Feulgen and Giemsa, and to compare the results with standard H&E stain.

Aim: To compare and corelate the efficacy of different histopathological stains, H&E, Feulgen and Giemsa in assessing the Mitotic Index in different grades of OED.

Materials and Methods: A retrospective study was carried out to assess the mitotic index in Oral Epithelial Dysplasia. A total of 30 histopathologically confirmed cases of OED were included in the study and staining was done using Feulgen and Giemsa. The mitotic index was calculated and the mitotic index was compared between the routine H&E, Feulgen and Giemsa stains.

Results: An increase in the mean Mitotic Index was noted with those cases of OED that were stained using Giemsa staining when compared to those cases of OED that were stained using Feulgen staining and routine H & E staining.

Conclusion: The proliferative activity of a cell can be best identified using mitotic index. In our study, Giemsa staining showed the highest mitotic index, significantly better than Feulgen and routine H&E, suggesting its superior utility in identifying proliferative activity.

Keywords: Feulgen, Giemsa, Mitotic Index, Oral Epithelial Dysplasia.

Introduction

The term dysplasia was derived from two Greek words which refers to abnormal or atypical proliferation. It was first introduced in the context of histology by J. W. Reagon in 1958 to describe the abnormal cells exfoliated from uterine cervix lesion. Later in 1977, Pindborg defined epithelial dysplasia as "a lesion in which part of the thickness of the epithelium is replaced by cells showing varying degrees of cellular atypia".[1] It is a reversible condition, and is hence cannot be considered cancerous. In most cases, epithelial dysplastic changes are the earliest microscopic evidence that represent the likely progression towards malignancy.[2]

Mitotic figures is an important parameter in the assessment of cellular proliferation and also a prognostic sign in OED and Oral squamous cell carcinoma (OSCC).[3] Mitosis involves the division of a mother cell into two identical daughter cells, further subdivided into prophase, metaphase, anaphase, and telophase, which are seen in the histological sections. Atypical Mitotic Figures (AMF) refer to abnormal, irregular assembly of nuclear materials within dividing cells which reflects the genomic abnormalities underlying it such as chromosomal instability, telomere dysfunction and aneuploidy.[4] AMF along with an increase in number of mitotic figures are included in the grading of OED. Special stains such as Feulgen and Giemsa enhance the visibility of mitotic figures, making their identification and localization more accurate and reliable.

Feulgen stain aids in the visualization of DNA on tissue sections and in cells. The principle mechanism behind is the

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How to cite the article: D. Angelin, George B., L. Sambath, Mary J S., R. Franklin, M P Rajalekshmi. Mitotic Index in Oral Epithelial Dysplasia: A Histopathological Comparison of Feulgen, Giemsa and H & E stains.Oral Maxillofac Pathol J 2026; 17(1); 41-44.

Source of Support: Nil

Conflict of Interest: None

Acknowledgement: We sincerely thank our principal Dr. Elizabeth Koshi for granting permission for the study. We are also thankful to Dr. Malarkodi Chitra for the support and guidance with reviewing the slides and writeup corrections.

dissociation of the two strands of DNA through hydrolysis, which makes the deoxyriboses accessible so that fuchsin (Schiff's reagent) can react with the aldehyde groups resulting in the formation of a red precipitate. This staining is more or less intense depending on the degree of chromatin spiralization and is therefore particularly suitable for highlighting the chromosomes in the nucleus during mitosis.[5] Wright-Giemsa stain, a mixture of basic dye, methylene blue and acidic dyes, eosin that stains the acid components of the cell (nucleus, cytoplasmic RNA, basophilic granules) as blue or purple, and basic components of the cell (haemoglobin, eosinophilic granules) as red or orange.[6]

Counting mitotic figures and presence of AMF is an important way to assess how actively cells divide, making it a valuable tool in tumor diagnosis. Evaluating mitosis and overall cellular proliferation not only supports initial diagnosis but also helps in predicting the tumor's behaviour and prognosis. This remains one of the simplest and fastest methods to gauge proliferative activity.

Materials and Methods

The current study is a retrospective quantitative analysis of the proliferative activity of a cell based on the mitotic index and special staining techniques such as Feulgen and Wright's Giemsa stains. The study was conducted in the Department of Oral and Maxillofacial pathology, and paraffin embedded blocks of histopathologically proven cases of OED of different grades are retrieved. 4 - 5 µm thick sections were made and stained with H & E. Cases with areas showing necrosis, inflammation, tissue folds and calcifications were excluded. 10 cases of OED fulfilling the inclusion criteria were selected and 3 sections were made from each case and stained with H & E, Feulgen and Wright's Giemsa stains. A total of 30 samples were divided into 3 groups according to the stains used.

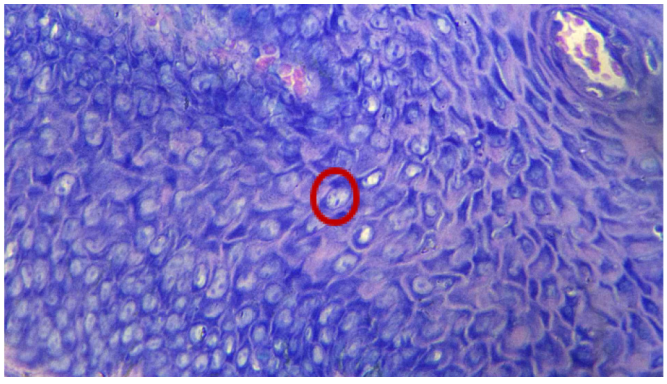

Fig. 1: Microscopic evaluation of mitotic figure in Feulgen staining (Red circle indicates mitotic figure, 40x).

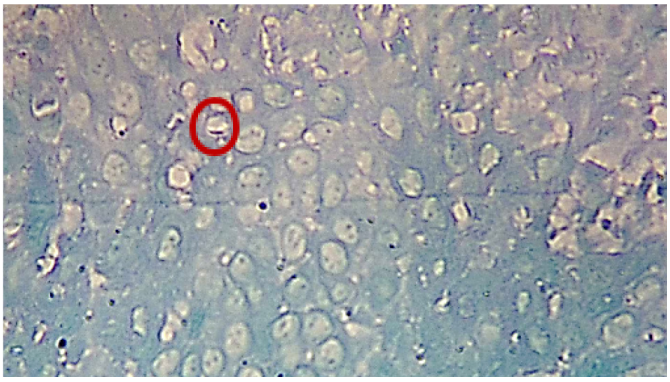

Fig. 2: Microscopic evaluation of mitotic figure in Giemsa staining (Red circle indicates mitotic figure, 40x).

Feulgen staining procedure

The sections were deparaffinized, rehydrated through grades of alcohol, and brought to water. The sections were then rinsed in 1M hydrochloric acid (HCl) at room temperature and placed in 1M HCl at 60° and again rinsed in 1M HCl at room temperature for 1 min. followed by the transfer to Schiff's reagent for 45 min and it was placed in bisulfite solution for 2 min, and this step was repeated twice, after which the sections were rinsed in distilled water. Counterstaining was done with 1% light green for 2 min and dehydrated through alcohols to xylene and mounted with DPX.

Giemsa staining procedure

The paraffin sections were placed in xylene for 2–3 minutes and repeated in fresh xylene for another 2–3 minutes to remove paraffin completely. Then the sections were transferred to 100% alcohol for 2 minutes. Passed through a series of graded alcohols (95%, 70%, 50%) for 1-2 minutes each. The sections were rinsed in distilled water for 2 minutes and stained using Giemsa solution for 15–30 minutes. The slides were then rinsed in phosphate buffer (pH 6.8) to differentiate the staining and achieve clearer nuclear and cytoplasmic contrast. Dehydration of the sections were done by passing it through ascending grades of alcohol: 50%, 70%, 95%, and 100%. Clearing of the sections were done by placing them in xylene for 2–3 minutes followed by mounting using DPX.

Counting of mitotic figures

Ocular graticule was calibrated using a light microscope (Leica, DM 1000) by the method described by Culling. The counting of mitotic figures was performed by superimposing

Table 1: showing the Demographic Details

Variables	Categories	Total (%)
Age in years	31-40 years	2 (20%)
	41-50 years	3 (30%)
	>50 years	5 (50%)
Total		10 (100%)
Gender	Males	7 (70%)
	Females	3 (30%)
Total		10 (100%)
Site	Alveolar Mucosa	2 (20%)
	Buccal Mucosa	4 (40%)
	Labial Mucosa	1 (10%)
	Gingiva	2 (20%)
	Tongue	1 (10%)
Total		10 (100%)

the ocular graticule onto the tissue preparation. Each slide was then viewed under a high-power field (400X) for counting of mitotic figures using ocular grid eyepiece. Counting of mitotic figures was done in stepladder fashion in 10 different high-power fields.[1] The mitotic figures were counted as per the criteria given by Van Diest et al (nuclear membrane must be absent, clear, hairy nuclear extensions must be present either clotted in a plane or two separate planes, and two clots in single plane should be considered as one mitotic figure).[8]

Each slide was observed by two separate observers and the Mitotic Index (MI) was calculated based on the given formula,

Mitotic Index = Number of Mitotic Figures / Total number of cells in that field

Statistical analysis: The Statistical tests used for data analysis were Student t-test and the interobserver variability was assessed using kappa statistics. Software(s) used for statistical analysis was SPSS version 27.0.

Ethics Statement: This study was approved by the Institutional Research committee (IRC: Ref No 34/2024) and Institutional Human Ethics Committee (SMIDS/IHEC No: 05/ Protocol No: 31/2024) of Sree Mookarnbika Institute of Dental Sciences. All the procedures were followed according to the Helsinki Declaration of 1975, as revised in 2008. This article does not contain any studies with human or animal subjects performed by any of the authors.

Results

Demographic details

The samples in study groups ranged in age group from 30 to 80 years old, with a mean age of 54. The samples were predominated (50%) by the age group of >50 years, with the remaining cases (50%) in the age group of 30 - 40 years and 40 – 50 years. Among the 10 samples in each of the groups, 3 were from female and 7 were from male patients. In the present study, the cases were distributed among the anatomical sites as follows: buccal mucosa (40%), alveolar mucosa (20%), gingiva (20%), tongue (10%) and labial mucosa (10%). (Table 1)

Expression of Feulgen and Giemsa stains

The mean MI±SD values per 10 grid fields in H&E, Feulgen and Giemsa staining are found to be 2.35±0.76, 2.31±0.94 and 3.12±0.32 respectively. It was found to be statistically significant with a p-value <0.05. A significant increase in the mean values was noticed in Group III (i.e. Giemsa staining) when compared to Group I (i.e. H&E staining) and Group II (i.e. Feulgen staining). (Table 2) The interobserver variability was verified by using kappa statistics to eliminate the interobserver bias and it was found to be statistically significant with a p-value of 1.000. (Table 3)

Discussion

Oral Potentially Malignant Disorders (OPMDs) diagnosed as OED are significantly associated with increased risk of progression of a precancerous lesion to cancer. There are many nuclear abnormalities like pyknotic nuclei, micronuclei, binucleation, an increase in the quantity of mitotic figures, and abnormalities in the number of mitotic figures as a consequence of defects of mitosis.[8] AMF can be either i) lag atypical mitosis (i.e. 2 unattached chromosomes at each side of metaphase plate), ii) spindle multipolarity either tripolar, tetrapolar or multipolar, iii) dispersed mitosis (i.e. dispersed chromosomes with no specific shapes), iv) polar symmetry (i.e. 2 pulled away chromatids that is unequal in size and shape), v) anaphase bridge mitosis (i.e. string of chromatin from one pole to other) and vi) ring mitosis (i.e. chromosomes displaced at the periphery in form of a ring).[4]

The assessment of mitotic figures is commonly employed in the diagnosis and prognosis of OED and OSCC. Despite being more precise, advanced techniques like flow cytometry, IHC, auto radiography is being used to detect mitotic figures but they are expensive and time consuming and so selective stains have been used to stain mitotic figures in tissues.[3,9] These issues can be resolved with a fully standardised histochemical stain and the accurate use of morphological criteria for mitotic cell identification.[10]

Table 2: Showing the mean MI between various groups.

Groups	n	MEAN	S. D	MEAN±SD	p value
I - H&E staining	10	2.35	0.76	2.35±0.76	0.0001
II - Feulgen staining	10	2.31	0.94	2.31±0.94	
III - Giemsa staining	10	3.12	0.32	3.12±0.32	

Table 3: Showing the interobserver variability among the groups

Groups	Observer-1 Score (MEAN±SD)	Observer-2 Score (MEAN±SD)	p value
Group-I	2.35±0.76	2.36±0.50	1.000
Group-II	2.31±0.94	2.30±0.04	1.000
Group-III	3.12±0.32	3.15±0.12	1.000

(*p<0.05 significant compared group-I with other groups, #p<0.05 significant compared group-II with other groups)

H&E stain is most readily available but the distinction between an apoptotic cell, a pyknotic nucleus and a mitotic cell sometimes may be difficult.[11] To study the frequency of mitotic activity, physiological markers for mitotic cells like protein kinases, as well as the accumulation of different molecules such as dyenins and cyclins in the different stages of mitosis can also be used.[12] Special stains like Nissl stain, gallocyanin, toluidine blue and Feulgen have also been used to study mitotic figures.[13] In the present study, we had used the routine H & E, Feulgen and Giemsa Stains for identifying the MI in OED.

In the present study, we found that good contrast in staining and mitotic figures could be appreciated as dark blue in a light pink background even at low magnification. A statistically increased MI value was noted in OED sections that were stained using Giemsa staining (3.12±0.32) when compared to those of the routine H & E staining (2.35±0.76) and Feulgen staining (2.31±0.94). These findings were in concordance with the study done by Varma et al[14] who evaluated the mitotic figures in OED and OSCC cases and concluded that Giemsa was superior for labelling of mitotic index.

In the study by Rao et al.,[3] he had compared the MI using Feulgen, crystal violet and H & E, it was found that Feulgen was better than the other 2 stains used. Radhakrishnan et al.,[15] in his study had compared the efficiency of Crystal Violet and Feulgen found that Feulgen was considered to be reliable and effective in the evaluation of MI.

Based on our findings, we found that Giemsa stain is a highly reliable and useful method for recognising mitotic figures. Its ability to vividly identify mitotic activity makes it particularly important for accurately assessing the mitotic index. The most important drawback of our study was the use of limited number of samples. The direction of the future perspective could be the use of a larger group of study samples which could help us in detection of these mitotic figures.

Conclusion

In our study, mitotic figures were more enhanced with Giemsa staining in comparison to other stains – Feulgen and H & E. This study may be used as an key note or a basis for future researchers and the efficiency of Giemsa staining should be studied in a broader way in order to arrive at a conclusive result.

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