Original Article

Mitotic Figures Evaluation in Oral Squamous Cell Carcinoma using Crystal Violet and Feulgen Stains - A Comparative Study

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Abstract

Context: Mitosis is the process of nuclear cell division. Increased mitosis causes excessive proliferation of cells which is observed in oral squamous cell carcinoma (OSCC). However, there is always problem in differentiating a mitotic cell from an apoptotic cell, during routine staining procedures, which affect the reliability of histological grading of OSCC. Recently, several methods have been implemented in identifying these mitotic figures (MFs), but they seem to be time-consuming and expensive which makes them less feasible. Thus, an effort was done to evaluate the efficacy of crystal violet and Feulgen stains in identifying MFs along with routine hematoxylin and eosin (H and E) in OSCC. **Aim:** In the identification of MFs in diagnosed cases of OSCC using crystal violet and Feulgen stains and H and E stain. **Settings and Design:** This was a retrospective study. **Materials and Methods:** The study sample includes five tissue sections of moderately-differentiated and well-differentiated OSCC each and four sections of poorly differentiated which were stained with H and E, Feulgen, and 1% crystal violet stains, and the number of MFs was enumerated. **Statistical Analysis Used:** One-way ANOVA was used for statistical analysis. **Results:** The results from our study showed that 1% crystal violet-stained MFs are better than H and E and Feulgen stains. **Conclusion:** Crystal violet stain can be considered as a simple, reliable, cost-effective, and reproducible method of staining MFs.

Keywords: *Crystal violet stain, Feulgen stain, mitotic figures, oral squamous cell carcinoma*

Received: 31-12-2021.
Decision: 05-01-2022.
Accepted: 10-01-2022.
Published: 25-02-2022.

Introduction

In mitosis, a mother cell divides exactly into two identical daughter cells. Tissue sections show different phases of mitosis which are prophase, metaphase, anaphase, and telophase. Nuclear abnormalities such as micronuclei, binucleation, broken egg appearance, pyknotic nuclei, and increased number of and/or abnormal mitotic figures (MFs) occur due to defect in mitosis.[1] These abnormal MFs are commonly seen in oral squamous cell carcinoma (OSCC). Increased numbers of and/or abnormal MFs are important criteria that carry increased importance in the grading of OSCC.[2] Most of the times, in routine hematoxylin and eosin (H and E) staining, there is always an issue in differentiation between a pyknotic nucleus, an apoptotic cell, and a mitotic cell. These MFs have a vital role in assessing cellular proliferation and act as a prognostic indicator in treating OSCC.[3] Advanced methods, such as immunohistochemistry (IHC), flow cytometry, autoradiography, and DNA ploidy, are available for the identification of MFs, but still increased cost and time make them difficult to use.

Hence, special stains can be employed for rapid and easy identification. Toluidine blue, Giemsa, and crystal violet

Access this article online

Quick Response Code:

Website: www.ijofb.org

DOI: 10.4103/ijofb.ijofb_9_21

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How to cite this article: Subashini V. Mitotic figures evaluation in oral squamous cell carcinoma using crystal violet and feulgen stains - A comparative study. Int J Orofac Biol 2019;3:42-4.

stains have been used to stain nuclear DNA.[4,5] Feulgen and crystal violet stains are special stains in histology. This is due to their high affinity toward acidic nuclear components being basic.[6] In this study, Feulgen and 1% crystal violet stains are used as an attempt to aid in the distinct and differential staining of MFs, which will facilitate easy identification also to assess and evaluate the number of MFs in various grades of OSCC.

Materials and Methods

The study sample included retrieval of 14 formalin-fixed paraffin-embedded tissue sections diagnosed for various grades of OSCC from the archives of the Department of Oral Pathology, Saveetha Dental College and Hospital, Chennai. Study samples of 15 diagnosed cases of OSCC were grouped into three categories: Group 1 – well differentiated, Group 2 – moderately differentiated, each group comprising 5 cases, and Group 3 – poorly differentiated comprising 4 cases.

Methodology

Three serial sections of 5 µ each were sectioned and stained with H and E, 1% crystal violet, and Feulgen using the Methodology from Bancroft.[7] Sections were interpreted for MFs under ×40, under a light microscope by two well-experienced oral pathologists. The criteria given by Van Diest *et al*. were strictly adopted to identify MFs to rule out similar looking cells such as pyknotic nuclei and apoptotic cell.

Van Diest *et al*.'s criteria to assess the MFs are as follows:

1. The nuclear membrane must be absent, indicating that the cells have passed the prophase
2. Clear, hairy extension of nuclear material (condensed chromosome) must be present, either clotted (beginning metaphase) in a plane (metaphase/anaphase) or in separate clots (telophase)
3. Two parallel, clearly separate chromosomes clot to be counted individually as if they are separate mitosis.

The entire stretch of the epithelium was observed, and the number of MFs in each field was counted to give the total number of MFs in that particular tissue section.

Areas excluded for counting MFs are as follows:

1. Areas showing necrosis
2. Inflammation
3. Tissue folds and calcifications.

Statistical methods

The entire procedure was blinded using two observers. Data were statistically analyzed using one-way ANOVA test. Interobserver variability was calculated using Kappa statistics is 0.68, which showed substantial agreement between the observers.

Results

- The number of MFs was compared among the three groups.
- A significant increase in the number of MFs was observed in sections stained with 1% crystal violet as compared to H and E and Feulgen stains [Figure 1]
- A significant ($P < 0.01$) increase in the identification of MFs was observed in 1% crystal violet-stained sections as compared to the gold standard H and E. No significant increase in the number of MFs between well, moderate, and poorly differentiated OSCC
- Interobserver variability was calculated to be 0.62 which showed substantial agreement between the observers.

Discussion

Mitosis has a vital role in the growth and maintenance of an organism. It has five stages: prophase, prometaphase, metaphase, anaphase, and telophase.[8]

Carcinogenesis occurs when nuclear DNA undergoes genetic alterations, which results in dysregulation of mitosis. Increased and abnormal mitosis seen in OSCC reveals genetic damage and has a significant role in carcinogenesis. Hence, mitotic activity evaluation under microscope seems to be a most important prognostic indicator. The significance of MFs is as follows:

1. Assess prognosis of various grades of cancer
2. Chromosomal aberration reasons
3. Aids in histological grading by evaluating cellular proliferation.

Thereby, MF is an imperative aid in pathology.

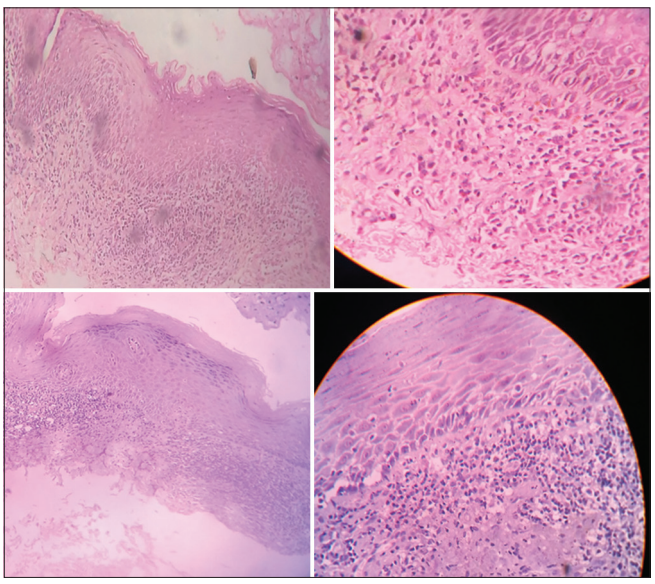

Figure 1: Hematoxylin and eosin stain (×10 and ×40), PAS stain (×10 and ×40)

New inventory techniques, such as IHC using Ki-67, PCNA, and phosphohistone H3, autoradiographic determination of thymidine labeling to see for proliferation, and flow cytometry are also available, but their increased cost and time make them less feasible. A standardized, simple, and quick stain with a set of well-defined criteria for the quantification of MFs will be effective and helpful.[9,10]

In this study, we were involved to find out a stain which could be a simple, cost-effective, and brisk technique for identification of MFs and also to evaluate its role in histological grading of OSCC. We observed that 1% crystal violet stain showed more number of MFs than H and E and Feulgen stain.

In a study conducted by Ankle *et al*. to evaluate the selectivity of 1% crystal violet stain for MFs on normal oral mucosa, oral epithelial dysplasia, and OSCC, the results showed statistically increased mean mitotic counts with 1% crystal violet-stained sections of oral epithelial dysplasia and OSCC as compared to H and E-stained sections.[4]

In another study conducted by Jadhav *et al*., it was seen that 1% crystal violet provided a definite advantage over H and E-stained sections in selectively staining MFs. A significant increase in the number of MFs in crystal violet-stained tissues was observed in oral epithelial dysplasia and OSCC.[5]

A study done by Rao *et al*. observed that Feulgen stain provided superior staining of MFs facilitating its identification compared to 1% crystal violet and H and E stains. Distinguishing an MF from pyknosis, apoptosis, and karyorrhexis is important to prevent false-positive results, and Feulgen stain provided excellent detail and morphology of a mitotic cell.[6]

A study was conducted by the Division of Human Nutrition, Adelaide, South Australia, to evaluate the selective stain for MFs in tissue sections of developing brain, large and small intestine, skin, and liver from rat, sheep, and guinea pigs. The staining was done with 1% crystal violet with nuclear fast red as counterstain. Results showed that tissue MFs were visible in low power and stood out clearly against red background.[11]

However, in the present study, with 1% crystal violet, we could identify MFs better than H and E and Feulgen stains.

Conclusion

Our study suggests the use of 1% crystal violet for distinct and selective staining of MFs.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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