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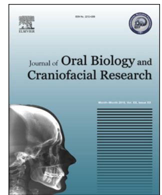

Original Article

Image analysis assisted study of mitotic figures in oral epithelial dysplasia and squamous cell carcinoma using differential stains

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ABSTRACT

Background: Mitosis is a process of cell division resulting in two genetically equivalent daughter cells. Excessive proliferation of cells due to mitosis is the hallmark in pre cancer and cancer.
Aims: This study was conducted to count the number of mitotic figures in normal oral mucosa, oral epithelial dysplasia and squamous cell carcinoma in both Hematoxylin and Eosin (H&E) and Crystal Violet stained sections. Also the overall number of mitotic figures with both stains were compared along with the evaluation of staining efficacy of both the stains.
Methods and material: The present study was conducted on 20 specimens each of the three categories. These were further divided into two groups for staining with H&E and with 1% Crystal Violet respectively. Images were captured and analyzed using image analysis software Dewinter Biowizard 4.1.
Results: Comparison of mitotic figure count in three categories in sections stained with both stains showed statistically significant difference ($p < 0.001$). The mean number of mitotic figures seen in Crystal Violet reagent were significantly higher as seen in H&E stain ($p < 0.001$). The overall diagnostic efficacy of Crystal Violet was 87.6%. Crystal Violet scored over H&E stain and also helped to better appreciate metaphases in Squamous cell carcinoma and telophases in dysplasia.
Conclusion: Number of mitotic figures progressively increase with the advancement of the pathology. Use of 1% Crystal Violet provides better appreciation of mitotic figures and can be employed as a selective stain in routine histopathology.

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1. Introduction

Normal growth and maintenance of oral mucosa requires that birth, differentiation and loss of epithelial cells are regulated and coordinated. It is the disruption of normal growth control mechanisms that leads to neoplastic transformation of epithelium and the development of carcinomas.[1]

Preneoplasia is arguably the most important disease entity of modern man.[2] Head and neck cancer annually, represents 650,000 new cases and 350,000 deaths worldwide.[3] Oral cancer in India constitutes about 10% of all cancer cases and oral squamous cell carcinoma represents 90–95% of them.[4] This ranks as the sixth most common cancer worldwide[5,6] and the third most common form of malignancy in developing countries.[7] This emphasizes the importance of early identification of precursor lesions that will develop into carcinomas.[8]

Cell division is required to maintain tissue integrity.[9] Dysregulation of cell cycle machinery is a fundamental hallmark of cancer progression[10] and hyper-proliferation is thought to be an early, marker of disorderly growth.[11] Mitosis is a process wherein a mother cell divides exactly into two identical daughter cells. The various phases of mitosis are prophase, metaphase, anaphase and telophase, some of which are seen in tissue sections.[9] Mitotic figures are defined as atypical if they demonstrate an abnormal chromosomal distribution or an excessive number of mitotic spindles with a multipolar morphologic appearance.[12]

Errors in identifying a mitotic cell can weaken the reliability of histological grading due to the loose use of morphologic criteria. Combination of stains and modification of the existing histochemical techniques can overcome these problems. Crystal Violet is a basic dye which has a high affinity for the highly acidic chromatin of mitotic cells.[9] The stain was primarily developed for the quantitative analysis of mitotic figures in developing

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brain, but lends itself to any tissue where mitotic counts are required.[13]

Screening for rare mitotic cells in a tumor cell population requires intensive effort at fairly high magnifications to ensure correct identification and classification of mitotic cells.[14] Some authors have sought to remove this problem by using quantitative methods like point counting applying to prognostic evaluation of oral cancer.[15]

The present study was therefore undertaken to evaluate the mitotic figures in normal oral mucosa, oral epithelial dysplasia and squamous cell carcinoma with Crystal Violet staining over routine H&E staining in order to judge its reliability in early detection of oral premalignancy and malignancy.

2. Materials and methods

The study was conducted on tissues/wax blocks of clinically and histologically proved cases of oral epithelial dysplasia and squamous cell carcinoma. A total of 20 specimens each of normal oral mucosa, oral Epithelial Dysplasia and Squamous Cell Carcinoma (SCC) comprised the study population. Each specimen was further divided as to be used for two different staining procedures. After routine staining procedure with Hematoxylin and Eosin (H&E); preparation for Crystal Violet (CV) staining was done.

3. Crystal Violet staining

3.1. Preparation of stain – Hucker and Conn (1928)[16]

Dissolve 2 g of Crystal Violet in 20 ml of 95% alcohol. Add 80 ml of 1% aqueous ammonium oxalate. Dissolve using minimum of heat. Cool, filter and store in amber colored bottle. The solution was used as 1% aqueous Crystal Violet for staining using the Fraser FJ modified13 procedure that has been illustrated in Table 1.

Table 1
Crystal Violet staining procedure (Fraser FJ method modified).[21]

Xylene	3 changes 15 min each
Alcohol	Hydrate through graded alcohols (100%, 90% and 80%)
Water	Wash in water
Hydrolyze	1 N HCl at 56–60 °C for 12 min
Distilled water	Wash in three changes of distilled water
Alkaline water	Wash in two changes of alkaline water
1% aqueous Crystal Violet	Stain for 30 min in Crystal Violet
1% acid alcohol	Dip for 5–10 s to differentiate
Alcohol	Dehydrate quickly through graded alcohols (80%, 90% and 100%)
Xylene	Clearing for 10–15 min
DPX	Mounting

For analysis, images were captured using digital camera and trinocular research microscope with a 40× objective in both Hematoxylin and Eosin and Crystal Violet stained sections. The actual counting was done manually using the Dewinter Biowizard 4.1 after accurate calibration. Images were captured, stored and arranged according to the study groups. Microscopic fields were selected starting from most mitotically rich area and then moving the stage to the next field, and continuing the selection to include a minimum of 3 fields from each section. Each selected field included both basal and parabasal cell compartments. In sections pertaining to squamous cell carcinoma the counting was also performed in islands, cords and sheets of tumor cells infiltrating the connective tissue. The mitosis counting was done using Dewinter-Biowizard 4.1 on the fields stained with both Hematoxylin and Eosin and Crystal Violet stains (Fig. 1).

The criteria given by Van Deist et al.[9] was used to assign a structure as a mitotic figure in this study:

1) The nuclear membrane must be absent indicating that cells have passed the prophase.

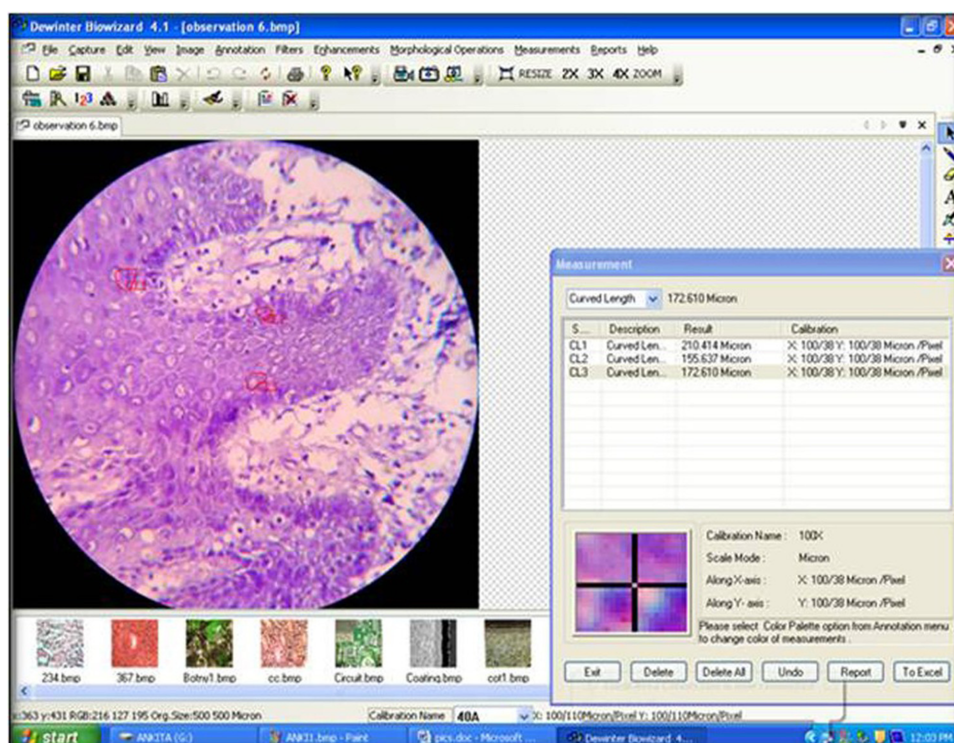

Fig. 1. Method for counting mitosis using Dewinter Biowizard 4.1 in section stained with Crystal Violet stain.

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