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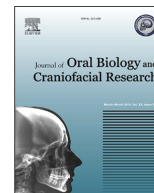

Original Article

Correlation of mitosis obtained by using 1% crystal violet stain with Ki67LI in histological grades of oral squamous cell carcinoma

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ABSTRACT

Aim: The purpose of this study was to use a simple, cost-effective technique for studying mitosis in various grades of oral squamous cell carcinoma (OSCC) using 1% Crystal-violet stain and to correlate mitotic frequency index (MFI) obtained by it with Ki67 labeling index (Ki67LI) so as to validate its usefulness as a selective stain for evaluating proliferation.
Materials and Methods: The invasive front grading score (IFG Score) was recorded in 40 patients of OSCC. Mitotic figures were assessed in hematoxylin and eosin (H and E) stained section as well as in 1% crystal-violet stained section using MFI (MFI = Mitosis/total number of cell counted X100). Comparison between MFI obtained by 1% crystal violet stain and H and E stain was done. Ki67LI was assessed using Ki67 immunohistochemical (IHC) marker. Correlation between Ki67LI and MFI obtained by using 1% crystal-violet stain was performed.
Results: There was statistically significant increase in MFI obtained by using 1% crystal-violet stain compared to routine H and E stain. Statistically significant positive correlation was observed between Ki67LI and mitosis in well and moderately differentiated OSCC. Positive correlation was also observed in poorly differentiated OSCC, but it was not statistically significant. Both mean MFI and mean Ki67LI significantly increased from grade I to grade II to grade III OSCC.
Conclusion: 1% crystal violet stain provides a definite advantage over the H and E stained sections. Thus crystal violet stain is easy and cost effective to evaluate proliferation when compared with expensive proliferative IHC marker.

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1. Introduction

Oral Squamous Cell Carcinoma (OSCC) is the commonest malignant tumor of the oral cavity, accounting to about 96% of all oral malignancies in both males and females. It is one of the leading causes of death in developing countries.[1,2] In order to improve the prognosis to some extent, it is important to govern and regulate the aggressiveness and prognostic factors that will aid in appropriate treatment plan. Although many histopathologic characteristics of OSCC have been known as prognostic factors, accurate and unambiguous factors have not yet been clearly identified.Oral carcinogenesis is a multifactorial process involving numerous genetic events which is the root cause for the progression from a normal cell to a cancer cell, referred to as multi-step carcinogenesis. This event may be concomitant with deregulated control of cell proliferation and apoptosis.[3]Thus proliferation is one of the most significant features exemplifying the malignant phenotype and aggressive biological feature.

The assessment of cell proliferation activity in tumors is an important tool for diagnosis, clinical behavior, and therapy. Numerous techniques have been established to identify and measure proliferating cells. The pioneer method was based on counting of mitotic figures using a light microscope. Other techniques include tritium labeled thymidine, the use of another pyrimidine analogue, the bromodeoxyuridine.[4] Newer prognosticators like flow cytometry, autoradiography, DNA ploidy measurements are now at the leading edge.[5] These techniques are

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challenging, expensive, time consuming and often requiring sophisticated equipment, restraining their routine use by pathologists.[4] Immunohistochemistry is another advanced technique in use. Ki67, is a reliable immunohistochemical (IHC) marker which shows a marked variation within different tumor grades, indicating that it has a crucial role in the aggressive behavior of malignant tumors and thus can be used for assessment of proliferation.[6,7-] Though it is practical compared to other methods of proliferation, it is quite expensive when compared to counting of mitotic figures. Thus cost and time factor makes it less feasible for many laboratories.

Counting of mitotic figures is the oldest, economical way of assessing proliferation. Increased number and abnormal mitosis indicate genetic damage. Thus identification and quantitation of mitotic cells forms an indivisible part of histological grading systems which is further valuable in the assessment of prognosis. However strict and precise morphologic criteria should be applied for recognition of mitotic figures.[5]They can be identified and counted under conventional light microscope. However routine Hematoxylin and Eosin (H and E) stained section may pose a problem in distinguishing mitotic cells from apoptotic cells. This may further weaken the reliability of histological grading because of inappropriate use of morphologic criteria. Therefore, to obtain more reliable and reproducible count, appropriate histochemical techniques needs to be standardized which may include combination or modification of stains. Stains to visualize atypical and typical mitotic figures include H and E, Crystal violet, Giemsa stain and fluorescent microscopy.[8,9]These stains have been used in tissues apart from oral tissue.[10,11]Crystal violet is a basic dye having high affinity for the highly acidic chromatin of mitotic cells which are stained magenta and stand out clearly against a light blue background of resting cells.[9]Literature has revealed very few studies which used these special stains for studying mitosis in oral dysplasia and oral carcinoma.[9,12,13]However, these studies neither compared their results with established proliferative marker like Ki67 nor they compared mitosis in different grades of OSCC.

The aim of this study was to validate the usefulness of Crystal violet as a special stain for evaluating proliferation. This was done by correlating mitotic frequency index (MFI) obtained by it with that obtained by H and E stain as well as with Ki67 labeling index (Ki67LI), an established proliferative IHC marker, in various grades of oral squamous cell carcinoma.

2. Materials and methods

The study was carried out at the Department of Oral and Maxillofacial Pathology and Microbiology, MGV's KBH Dental College, Nashik, Maharashtra, India. The study protocol was approved by the Institutional Ethics Committee. 40 patients with lesions histologically diagnosed as oral squamous cell carcinoma were included in the study. After clinical examination and prior informed consent of the patient, biopsy was performed as a part of the treatment protocol and in order to obtain histopathological diagnosis of the lesion. Grading of the subjects was done according to Bryne (1991)[14] using invasive front grading score (IFG Score). Accordingly patients were grouped as grade I OSCC (IFG score<5), grade II OSCC (IFG score 5-12) and grade III OSCC (IFG score>12). Table 1 summarizes clinical records of the patients studied. Three histological sections of each case were made. First two sections were stained with H and E stain and with 1% Crystal violet stain respectively. Mitotic figures were evaluated in both the sections.

Third section was stained with antibody against Ki67 nuclear antigen, and Ki67LI was assessed. Using binocular compound microscope, both H and E stained andCrystal violet stained sections were studiedat 40 X.To assess inter-observer variation in interpretation, counting of mitotic figures was done by two examiner and no statistically difference was observed between the readings.

Table 1
Clinical And Pathological Data Of The Patients Studied.

			n	%
Age	Mean	48.25 years	40	
	Range	20 to72 years		
Sex	Males	26	40	
	Females	14		
T	Mean	2.02 cm		
	Range	1 to 3		
N	Positive		31	77.5
	Negative		9	22.5
M	Positive		0	0
	Negative		40	100
Stage	I		4	10
	II		6	15
	III		20	50
	IV		10	25

T – tumor size.
N – node.
M – Metastasis.

The areas of the tissue with most invasive and cellular part were selected whereasareas demonstratingtissue folds, necrosis, inflammation, and calcifications were excluded.Following ofprecise protocols are of great help in determining the accuracy of mitotic counting.[15]Therefore they were recognized according to the criteria as given by Vandiest et al (1998).[16]Mitosis was studied using MFI which was expressed as the percentage of mitotic nuclei.

MFI = Number of mitotic figure $\times$ 100/Total number of cells observed.

Counting the 1000 tumor cells was simplified by placing 10 $\times$ 10 squared grid. Only clear nuclei within the grid squares and/or hit by the right or bottom line of the squares were counted in order to avoid double count.

For preparing crystal violet stain, solution A was prepared by mixing 2 grams of crystal violet (HiMedia Laboratories Pvt. Ltd. Crystal violet powder) and 20 ml of 95% ethyl alcohol. Solution B was prepared by mixing 0.8 gram of ammonium oxalate and 80 ml of distilled water. Both the solutions, A and B were mixed and kept for 24 hours. The resulting mixture was filtered and stored in an amber colored bottle. 1N HCl was prepared by adding 8.7 ml of 35%-37% HCl in 90 ml of distilled water. The sections were deparaffinized in hot air oven which was followed by two changes of xylene. Then the sections were hydrated through descending grades of absolute alcohol (90%, 80%, 70%, 60%, and 50%) for 5 minutes each.The sections were further washed well in distilled water for about 10 minutes and were hydrolyzed in 1 N HCl at 60 °C for 10 min (not an essential step). Thereafter the sections were washed well in distilled water for about 10 minutes and stained with in 1% crystal violet solution for 30 min. Finally the sections were again washed in distilled water and were differentiated in 30% ethanol containing 1 to 2 drops of 35%–37% HCL, cleared in xylene and mounted in DPX. Known hyperplastic lymphnode was used as positive control whereas negative controls were not used.

For immunohistochemistry, the sections were deparaffinized with xylene and hydrated in graded alcohol. Antigen retrieval for Ki67 was performed by heating tissue sections in microwave oven in citrate buffer (pH 6.0). In order to inhibit internal peroxidase activity, the sections were incubated with 3% H2O2 in methanol for 30 minutes. Further they were incubated with 10% serum to prevent non-specific reactions and lastly with Ki67 antibody (Prediluted, Clone MIB-1; Product code: N1633, Dako, Denmark) for 90 min. After cleaning the sections in PBS, the HRP Labelled Polymer Antimouse (DakoEnVision + System HRP Labelled

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