ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 May 31; 7(10):1576-1581.
https://doi.org/10.3889/oamjms.2019.330
eISSN: 1857-9655
Basic Science

Efficiency of Crystal Violet Stain to Study Mitotic Figures in Oral Epithelial Dysplasia

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Citation: Sajjad A, Sajjad SZ, Minhas S, Kashif M, Samad A, Sajid KA, Hasan M, Qureshi I. Efficiency of Crystal Violet Stain to Study Mitotic Figures in Oral Epithelial Dysplasia. Open Access Maced J Med Sci. 2019 May 31; 7(10):1576-1581. https://doi.org/10.3889/oamjms.2019.330

Keywords: Crystal violet stain; Mitotic figures; Oral epithelial dysplasia; Oral cancer

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Received: 28-Feb-2019; **Revised:** 29-Apr-2019; **Accepted:** 30-Apr-2019; **Online first:** 22-May-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: To evaluate mitotic activity in the different grades of oral epithelial dysplasia using 1% crystal violet stain.

MATERIAL AND METHODS: A descriptive study was conducted in the Department of Histopathology of the Post Graduate Medical Institute, Lahore on a total of thirty-three cases of the Oral Epithelial Dysplasia (OED). Fresh, frozen paraffin-embedded archival tissue blocks were collected from Lahore General Hospital, Lahore & Oral & Maxillofacial Surgery Department of Nawaz Sharif Hospital, Yakki Gate, Lahore. The representative sections were taken and, after processing, mounted on glass slides and stained with H&E and crystal violet stains. The stained slides were then examined under an optical microscope. The efficacy of 1% crystal violet stain to identify mitotic figures in the different grades of oral epithelial dysplasia was assessed with the sample t-test. A difference of $p < 0.05$ was considered to be significant.

RESULTS: A comparison of the mitotic figure count in two categories in sections stained with both stains showed a statistically significant difference. An increase in the mean mitotic count was noted in the sections of OED stained with crystal violet in comparison to the sections of OED stained with H&E which was statistically significant ($p = 0.00$).

CONCLUSION: Counting of mitotic cell is the rapid and simplest way of evaluating the proliferative activity of cells. Crystal violet stain can be a rationalised step in the staining of mitotic figures compared to the usual H&E staining and can be employed as a selective stain during routine histopathological procedures.

Introduction

There are many lesions in the oral cavity which are characterised by various morphological and cellular changes. These lesions may proceed to cancer. Dysplastic changes are considered to be the most important of these identifiable changes in the oral cavity [1].

One of the most complex topics of head and neck pathology is the precancerous lesion. The idea of step-by-step development of malignancy in the oral cavity mucosa is well recognised. The presence of dysplastic epithelium may play an even more important role in foretelling the development of cancer compared to the clinical presentation [2].

Dysplasia is a Greek word which means abnormal, atypical proliferation. Reagon used the term “dysplasia” in 1958 to describe cells exfoliated from a lesion of the uterine cervix. It is encountered predominantly in epithelia. 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." In 1981, Burkhart and Maerker specified that the degree of dysplasia is defined "as a measure of tissue and cellular deviation from the normal," whereas Kumar et al., (1992) stated that dysplasia is a "disturbance in the maturational sequence of the stratified squamous epithelium and disturbance in cell kinetics of the proliferative compartment with cytological changes [3].

Freedmen and Kerpel (1995) explained it as a diagnostic term which is used to describe the histopathological changes seen in premalignant disorders of the oral mucosa. Oral Epithelial Dysplasia (OED) is the diagnostic term which describes the histological and cellular changes that are seen in a chronic, progressive and premalignant disorder of the oral mucosa. OED is not linked with any definite clinical picture. However, lesions like leukoplakia and erythroplakia are typically connected with dysplastic changes. Therefore, white, red, or mixed white and red lesions are those which have most commonly revealed OED. It has also been constantly seen in the mucosa next to the malignancy in patients with invasive SCC [4].

There is more of a risk of the development of oral cancer in oral potentially malignant disorders which are diagnosed as OED. The studies have reported a transformation rate ranging from approximately 6.6-36.4% after the mean follow-up periods of 1.5-8.5 years. The definite mechanism of neoplastic transformation is not clearly understood and it is not unavoidable that a dysplastic lesion will lead to malignancy [5].

Epithelial dysplasia is found in 5-25% cases of leukoplakia. It is suggested that a histological report should always describe the absence or presence of epithelial dysplasia and also account for the determination of the severity of epithelial dysplasia in the case of its presence. On the other hand, erythroplakia, which is a less common lesion than leukoplakia, almost consistently reveals epithelial dysplasia [4].

The diagnosis of dysplasia is described in terms of the existence of particular histological and cytological characteristics [6]. A chain of subtle changes occurs in dysplasia, which suggests the development of anaplasia. Dysplasia is a reversible condition, and is hence not yet cancerous [7].

Dysplasia is characterised as unusual, abnormal, proliferation occurring in the epithelium. In most cases, dysplastic changes are the earliest microscopic evidence that represent the progression of malignancy [3]. Mitotic figures play an important role in the assessment of cellular proliferation and act as a prognostic sign in OED and OSCC [8].

Mitosis involves the division of mother cells into two daughter cells that look alike. These are further subdivided into prophase, metaphase, anaphase, and telophase, which are seen in the histological sections. 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. One of the most common findings in OED and OSCC is presence of abnormal mitotic figures or an increase in the number of mitotic figures. Mitotic figures are one of the important criteria that carry importance in the grading of OED [9].

Atypical mitotic figures or an increase in the number of mitotic figures is one of the standards for the grading of OED according to the WHO [8].

Abnormal and increased mitosis reflects genetic injury. Hence, identifying and quantifying mitotic cells is an important aspect of the histological grading system which is used for determining the prognosis of premalignant and malignant lesions [5].

Mitosis serves as the basis for cell proliferation and the study of mitosis plays an important role in determining the aggressiveness and prognosis of lesions. Numerous authors have reported various stains and techniques which can detect or study mitotic activity [10].

Previous studies have shown that there are various selective stains such as toluidine blue, giemsa and crystal violet which can detect chromatin patterns [9]. Among these, crystal violet is the one which is used to study chromosomal pattern in cells based on the hydrolysis of DNA [8]. The objective of the current study was to evaluate mitotic figures in the oral epithelial dysplasia using crystal violet staining over routine H&E staining in order to judge its reliability in the early diagnosis of oral precancerous and cancerous conditions.

Table 1: Histological Grading of Oral Epithelial Dysplasia (Smith and Pindborg, 1969)

	Type of change	Severity of dysplasia		
1	Drop-shaped retepegs	None (0)	Slight (2)	Marked (4)
2	Irregular epithelial stratification	None (0)	Slight (2)	Marked (5)
3	Keratinization of cells below keratinised layer	None (0)	Slight (1)	Marked (3)
4	Basal cell hyperplasia	None (0)	Slight (1)	Marked (4)
5	Loss of intercellular adherence	None (0)	Slight (1)	Marked (5)
6	Loss of polarity	None (0)	Slight (2)	Marked (6)
7	Hyperchromatic nuclei	None (0)	Slight (2)	Marked (5)
8	Increased nucleo-cytoplasmic ratio in basal and prickle cell layers	None (0)	Slight (2)	Marked (6)
9	Anisocytosis and anisonucleosis	None (0)	Slight (2)	Marked (6)
10	Pleomorphic cells and nuclei	None (0)	Slight (2)	Marked (6)
11	Mitotic activity	None (0)	Slight (1)	Marked (5)
12	Level of mitotic activity	None (0)	Slight (3)	Marked (10)
13	Presence of bizarre mitoses	None (0)	Slight (6)	Marked (10)

Material and Methods

The present study was conducted on a total of 33 specimens of OED which were further divided into two groups for staining with H&E and crystal violet, respectively. Here, the 1% crystal violet staining was performed according to the modified Fraser FJ

method (11). The prepared slides were examined under a CX31 Olympus microscope. Histological grading of oral epithelial dysplasia was performed according to the Smith and Pindborg classification system [12] (Table 1 and 2).

TABLE 2: Scoring of dysplasia (Smith and Pindborg, 1969)

Total score (EDI)	Grade
0-10	No dysplasia
11-25	Mild Dysplasia
26-45	Moderate dysplasia
46-75	Severe dysplasia

Criteria for identification of Mitotic Cells

To label a structure as a mitotic figure, criteria given by Van Deist et al. were used in this study [13]:

1. Cells have conceded prophase, indicating the absence of nuclear membrane.

2. The presence of condensed chromosomes (clear, hairy extensions of nuclear material) must exist either in the clotted form (beginning metaphase), in a plane (metaphase or anaphase) or in separate clots (telophase).

3. Two parallel, clearly separate chromosome clots to be counted as if they are separate.

These criteria helped to differentiate between different phases of mitosis from other, commonly seen nuclear changes like apoptosis, pyknotic nuclei, and karyorrhexis.

Counting of Mitotic Figures

Ocular graticule was calibrated using a light microscope (Leica, DM 1000) by the method described by Culling [14].

- A 20 × 20 square (area = 1mm^2) grid eyepiece graticule, engraved on a disc, was placed inside the eyepiece of the microscope.
- Stage micrometre, a 3 × 1 inch slide onto which a 1 mm scale was divided into 100 equal divisions is engraved, was placed under the objective.
- A 10X objective was selected and focused on the stage micrometre scale.
- The number of transverse and vertical segments of ocular graticule squares equal to an exact number of divisions of the stage micrometre scale was determined.
- Every 4 transverse and vertical segments of ocular graticule squares were equal to 10 stage divisions, therefore:

100 stage divisions = 1 mm = 1000 µm.

1 stage division = 1000/100 = 10 µm.

1 stage division = 10 µm.

10 stage division = 10 x 10 = 100 µm.

4 segments of graticule = 10 stage divisions = 100 µm.

1 segment of graticule = 100/4 = 25 µm

- The area of the graticule was calculated by multiplying the calibrated factor 25µm with transverse and vertical segments of 20 small squares of the graticule as follows:

(20 × 25 = 500 µm. or 0.5 mm.) × (20 × 25 = 500 µm. or 0.5 mm.)

or 0.5 × 0.5 = 0.25 mm^2.

- The counting of mitotic figures was performed by superimposing 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. The area selected for the counting of mitotic figures included the most invasive part and the most cellular part of the tissue. The areas showing necrosis, inflammation, tissue folds and calcifications were not considered for counting.

Mitotic count was described as the mitotic count per grid field and the mitotic count per square millimetre.

The mitotic count per grid field was calculated as:

Mitotic count/grid field = Total number of mitotic figures observed/Number of grid fields counted

The mitotic count per square millimetre was calculated as follows:

i. *Area of 1 grid field = 0.25 mm^2* [14]

ii. Mitotic count per square millimetre = Average number of mitotic figures per grid field/0.25 mm^2

Each slide was observed by two separate obs ervers without any exchange of information regarding study sample details.
Observations made by each observer regarding numb er of MFs were recorded separately and average valu e was calculated for both observations. The data was entered and analyzed using the Statistical Package for Social Sciences (SPSS) [Version 20]. Quantitative data was presented as Mean and Standard deviation. Qualitative data was presented as frequency and percentage. Comparison of staining of mitotic figures between crystal violet and H & E staining in oral epithelial dysplasia was done using T-test. p-value ≥ 0.05 was significant.

Results

A total of 33 patients presenting with different grades of OED was collected from Lahore General Hospital, Lahore & Oral & Maxillofacial Surgery Department of Nawaz Sharif Hospital, Yakki gate, Lahore. Out of the 33 patients of OED, n = 23 (69.7%) were males and n = 10 (30.3%) were females, with a male to female ratio of 2.3:1 (Figure 1).

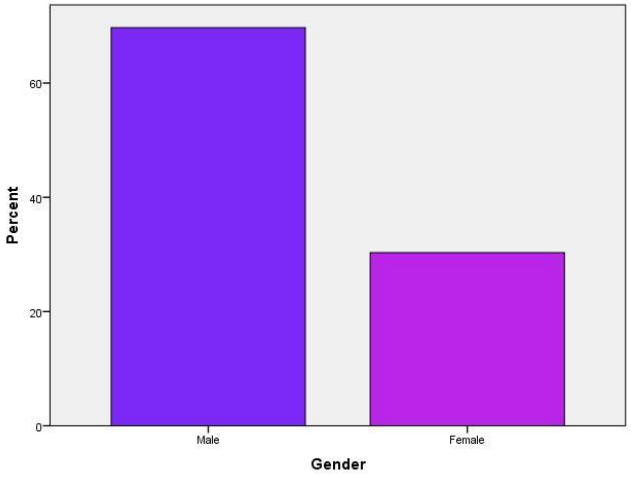

Figure 1: Gender distribution

The overall mean age of the patients reported with OED was 48.81 ± 13.05 years, with most patients presenting between the ages of 40 and 60 years. The age range in females was 35-85 years, with the youngest female patient presenting at the age of 35 years and oldest at the age of 85. A quite similar age range was seen in males, from 33-80 years. The minimum and maximum ages in male patients was 33 years and 80 years, respectively. The data indicate that the age incidences in both genders are quite similar (Figure 2).

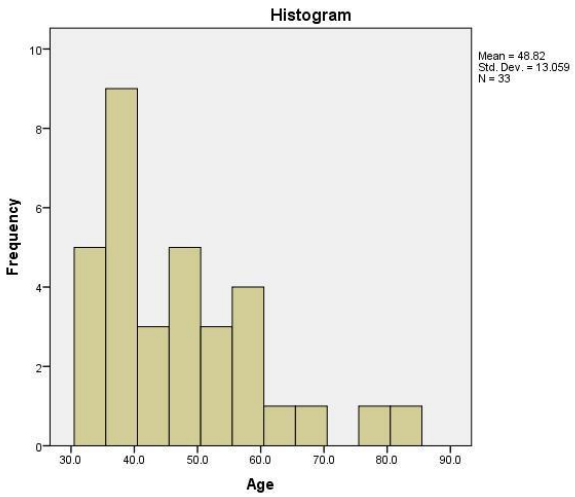

Figure 2: Age incidences in both genders

The predominant site of involvement in OED was buccal mucosa (66.7%), followed by tongue (18.2%), retromolar area (9.1%), floor of mouth (3.0%) and lip each (3.0%) (Figure 3).

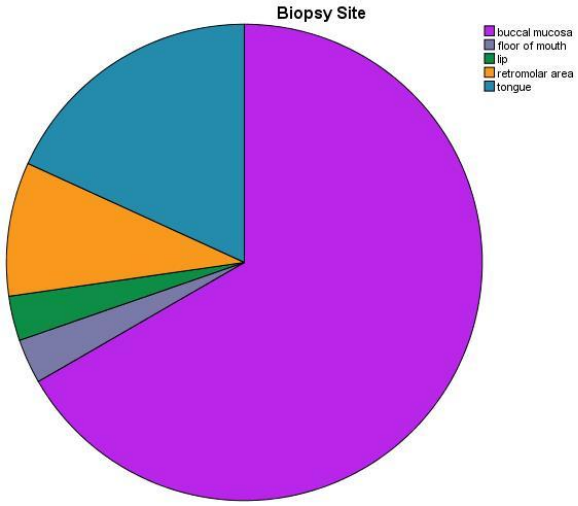

Figure 3: Site of involvement in OED

When the OED were sub-classified on the basis of their histological subtypes, we observed that the most common histological grade among the 33 cases was mild epithelial dysplasia, which was seen in n = 15 (45.5%) cases, followed by moderate epithelial dysplasia in n = 9 (27.3%) and severe epithelial dysplasia in n = 9 (27.3%) cases, respectively (Figure 4).

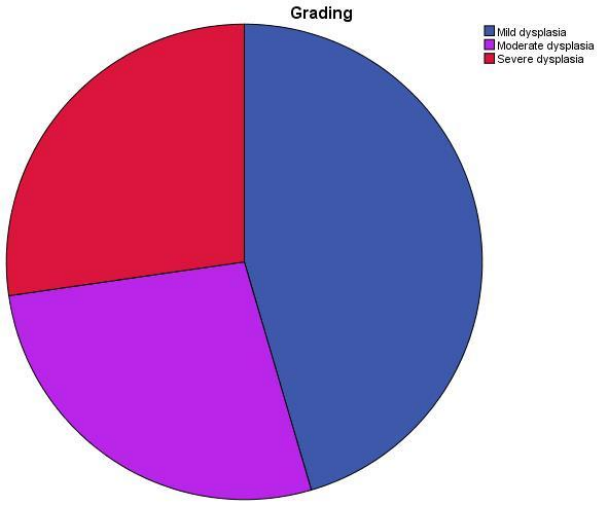

Figure 4: OED sub-classification on the basis of their histological subtypes

The mean (average) mitotic count in 33 cases of epithelial dysplasia was 4.4606/10 grid fields in H&E stained sections compared to 5.3212/^{10} grid fields in the sections stained with crystal violet (p = 0.00) (Tables 3 and 4).

Table 3: Mean mitotic count per square millimetre in OED cases

	N	MEAN
H&E	33	4.4606
CV	33	5.3212

A significantly increased mitotic count was observed in crystal violet-stained sections of the oral epithelial dysplasia when compared with H&E stained counterparts.

Table 3: Mean mitotic count per square millimetre in OED cases

	N	MEAN
H&E	33	4.4606
CV	33	5.3212

Discussion

Divisions of cells are needed to maintain the integrity of tissue [9]. Mitosis indicates genetic damage that is increased and abnormal. This is one of the essential features seen in the precancerous and cancerous condition [15]. The present study was carried out for the localisation of mitotic figures and highlights the importance of mitosis in assessing cell proliferation.

The oldest and simplest way of assessing cell proliferation is the counting of mitotic figures. It is a way to assess cell proliferation and is used as a diagnostic tool in tumour pathology. Cellular proliferation is widely used for primary diagnostic purposes, as well as a guide to prognosis in the assessment of tumours [16].

Thus, the identification and quantitation of mitotic cells plays an important role in histological grading systems which are used for determining the prognosis of premalignant and malignant lesions [9]. Clinical measures of proliferation are often included in the histological grading system as those tumours which exhibit increased proliferation have a tendency to be more aggressive [16].

Over the decades, the quantification of mitotic figures has been on the backseat. Newer prognostic indicators are now on the forefront, for example immunohistochemistry, autoradiography, flow cytometry and DNA ploidy measurements [9]. 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 [17]. Special stains like Nissl stain, gallocyanin, Giemsa, toluidine blue and Feulgen have also been used to study mitotic figures [18].

Although time and cost elements make them less feasible, a well standardised histological stain along with accurate use of the morphologic criteria for the identification of mitotic figures can overcome these difficulties [9].

The present study was carried out to overcome such limitations. The aim of this study was to use easy and economical methodology to study mitotic figures. In this study, 1% crystal violet is used as a selective stain and then compared with H&E staining in thirty three cases of OED.

When determining mitotic activity, methodological errors are of great interest [19]. Therefore, well-standardised procedures were used in the counting of mitotic figures, involving the analysis of multiple microscopic fields in representative areas of the tumour.

The counting of mitotic figures was done and the result in each sample was expressed in terms of the number of mitoses per high power field (hpf) and also in 1 square millimetre of the tumour tissue (mitoses per mm^2). As the microscopic objectives used (numeric aperture, field diameter at specimen level) are difficult to control, mitoses per high power field may be quite variable; because of this, results cannot be compared between various laboratories. Therefore, counting per mm^2 reduced the need for additional standardisation and gave significant findings [11] (Table 3).

In this study, the mean (average) mitotic count in 33 cases of epithelial dysplasia was 4.4606/10 grid fields in H&E stained sections compared to 5.3212/10 grid fields in the sections stained with crystal violet. In the present study, we meant to define a simple, economical and prompt technique to identify the mitotic figures and also to evaluate its role in histological grading of OED and OSCC. It was observed that crystal violet delivered superior staining of mitotic figures compared to H&E stains.

A similar study was conducted by Ankle MR on mitotic figures, which was used to determine the 1% crystal violet stain selectivity by comparing it with the H&E staining method. The findings of the study showed a statistically increased mean mitotic count in the sections of OED stained with 1% crystal violet and OSCC in comparison to sections stained with H&E [9]. From the present study, it can be easily observed that crystal violet provides efficient staining of mitotic figures and assists in its identification.

In another study which was carried out by Jadhav, it was seen that crystal violet provided a clear-cut advantage over H&E-stained sections in the selective staining of mitotic figures. A significant increase in the number of mitotic figures was noticed in both OED and OSCC [15]. The present study is in accordance with the previously conducted studies.

The higher sensitivity of crystal violet could be described due to its basic nature and its high affinity for highly acidic chromatin [9]. The overall higher

diagnostic efficiency of crystal violet could also be explained due to the modification of a staining method by using 1N HCL at 60°C, therefore causing an increase in contrast due to the light staining of the cytoplasm, which is likely to be a result of the decreased RNA content following hydrolysis [11].

From the results of the present study, it is concluded that mitotic cell counting is the quickest and simplest way of assessing the cellular proliferation. Crystal violet provides the efficient staining of mitotic figures and assists in its identification. It can be used for the localisation of mitotic figures and to assess proliferation, even in small scale laboratories, as crystal violet staining is easier, cheaper and more feasible. Crystal violet staining can be a rationalised step in the staining of mitotic figures compared to the usual H&E staining and can be employed as a selective stain during routine histopathological procedures.

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