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CLINICAL & BASIC RESEARCH

Evaluation of Mitotic Figures and Cellular and Nuclear Morphometry of Various Histopathological Grades of Oral Squamous Cell Carcinoma

Comparative study using crystal violet and Feulgen stains

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تقييم الإنقسام التفتلي والبنية الخلوية والنووية لعدة درجات مرضية نسيجية من سرطان الخلايا الحرشفية في الفم

دراسة مقارنة ما بين صبغتي البنفسج البلوري و صبغة الفلوجين

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ABSTRACT: ***Objectives:*** The objectives of this study were to quantitatively estimate the number of mitotic figures (MFs) and evaluate the cellular and nuclear features of various histological grades of oral squamous cell carcinoma (OSCC) using Feulgen and 1% crystal violet stains. ***Methods:*** This case-control study took place at the Dr D. Y. Patil Dental College & Hospital in Mumbai, Maharashtra, India, between June and December 2016. A total of 51 samples were retrieved from the hospital archives. Of these, 15 well-differentiated, 15 moderately-differentiated and six poorly-differentiated OSCC samples formed the case group while 15 samples of normal gingival *mucosa* constituted the control group. Each sample was dyed using Feulgen and 1% crystal violet stains and the mitotic count, nuclear area (NA), cellular area (CA), nuclear perimeter (NP), cellular perimeter (CP) and nuclear-to-cytoplasmic (N/C) ratio was calculated using computer-aided morphometry techniques. ***Results:*** The number of MFs visible per field was significantly higher in Feulgen-stained sections as compared to those stained with crystal violet (P = 0.050). In addition, the NA, NP, CA and CP values and N/C ratios of samples in the experimental group increased significantly in accordance with an increase in OSCC grade (P <0.001). ***Conclusion:*** The Feulgen stain is more reliable than 1% crystal violet in terms of the selective staining of MFs. Moreover, the findings of this study indicate that computer-based morphometric analysis is an effective tool for differentiating between various grades of OSCC.

Keywords: Crystal Violet; Feulgen Stain; Mitotic Index; Image Cytometry; Squamous Cell Carcinoma; Oral Cancers.

الملخص: الهدف: الهدف من هذه الدراسة هو التقييم الكمي لعدد التشكلات الإنقسامية بالإضافة إلى تقييم الخصائص الخلوية والنووية لعدة درجات نسيجية من سرطان الخلايا الحرشفية الفموي باستخدام صبغتي البنفسج البلوري وصبغة الفلوجين. **الطريقة:** اقيمت هذه الدراسة في كلية و مستشفى الدكتور د. باتل في موميباي في مهارشترا بالهند، بين شهري يونيو وديسمبر من عام 2016، تم حصر مجموع 51 عينة من سجلات المستشفى، من هذه العينات وُجدت 15 عينة واضحة التمايز، و 15 عينة متوسطة التمايز، و 6 عينات ضعيفة التمايز من سرطان الخلايا الحرشفية الفموي ممثلة مجموعة الحالات، كما اختيرت 15 عينة لأنسجة طبيعية من الغشاء المخاطي المبطن للفم لتمثل مجموعة الشواهد، كل عينة تم صبغها باستخدام إما صبغة الفلوجين أو تركيز 1% من صبغة البنفسج البلوري، تم حساب معدل الإنقسام الخلوي، والمناطق الخلوية، والمحيط النووي، والمحيط الخلوي، بالإضافة إلى النسبة النووية لحجم الهلام مقابل النواة بإستخدام تقنيات مقياس الأشكال بمساعدة الحاسوب. **النتائج:** الدراسة أظهرت أن عدد التشكلات الإنقسامية التي ترى في الحقل الواحد المحدد كانت أعلى بشكل كبير في القطع الملونة بصبغة الفلوجين مقارنة بالقطع الملونة بصبغة البنفسج البلوري ($P = 0.050$)، كما أظهرت أيضاً أن معدلات كل من المناطق الخلوية، المحيط النووي، المحيط الخلوي، والنسبة النووية لحجم الهلام مقابل النواة في مجموعة الحالات زادت بشكل واضح مع زيادة درجة ضعف التمايز الخلوي لسرطان الخلايا الحرشفية الفموي ($P <0.001$). **الخلاصة:** صبغة الفلوجين أكثر موثوقية من صبغة البنفسج البلوري (تركيز 1%) عند استخدامه قي الصبغ الإنتقائي للتشكلات الإنقسامية، بالاضافة الى ذلك تشير نتائج هذه الدراسة إلى أن استخدام تحليل قياس الأشكال بمساعدة الحاسوب يُعد أداة فعالة للتفريق بين الدرجات التمايزية المختلفة لسرطان الخلايا الحرشفية الفموي.

الكلمات المفتاحية: صبغة البنفسج البلوري؛ صبغة الفلوجين؛ مؤشر الإنقسام؛ الصورة الخلوية؛ سرطان الخلايا الحرشفية؛ سرطان الفم.

Advances in Knowledge

- *Feulgen staining was found to provide a more reliable estimation of tumour aggressiveness and progression in comparison to crystal violet stains.*
- *Moreover, there was a consistent increase in the mitotic count and cellular and nuclear measurements of oral squamous cell carcinoma cells according to histological grade.*

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Application to Patient Care

- *Feulgen stains offer an objective basis for grading oral squamous cell carcinomas which may be otherwise missed in routine histopathological examinations, thereby aiding healthcare providers in the provision of appropriate treatment plans.*

Cancer develops from a series of uncontrolled cellular events known as *atypia* in which cellular and nuclear morphometric changes occur as a result of excessive alterations in DNA synthesis accompanied by proliferation and *apoptosis*.[1] *Mitosis* is defined as a process of cell division wherein a single mother cell divides to produce two identical daughter cells; this process occurs in four distinct phases known as prophase, metaphase, anaphase and telophase.[2] Excessive cell proliferation due to increased abnormal *mitosis* is the hallmark of cancerous and precancerous lesions. As such, quantitatively evaluating the number of cells undergoing *mitosis*—also known as mitotic figures (MFs)—can serve as a prognostic indicator for such lesions.[2,3] Unfortunately, routine histochemical procedures cannot differentiate between pyknotic *nuclei*, apoptotic cells and MFs, posing a diagnostic problem.[4] To date, light microscopy remains the gold standard for MF identification, among various other methods such as morphometry, immunohistochemistry, flow cytometry, DNA ploidy and nucleotide radiolabelling.[5]

Malignant cells also exhibit considerable variations in terms of their morphology and dimensions, including pleomorphism, nuclear hyperchromatism and increased nuclear-to-cytoplasmic (N/C) ratios.[6] Various morphometric parameters including the shape and size of cells or cellular structures (i.e. the cellular area [CA], cellular perimeter [CP], nuclear area [NA] and nuclear perimeter [NP]) as well as the N/C ratio have proven useful in differentiating between normal tissues and potentially malignant disorders such as *leukoplakia*, lichen *planus* and oral squamous cell carcinoma (OSCC).[7] Previous studies have deduced that computer-assisted image analysis allows for the assessment of large numbers of specimens with high reproducibility as well as sensitivity and accuracy rates exceeding 99%.[8,9] This method also allows for precise measurement of the size, shape, organisation and quantity of cells and their nuclei, while reducing interobserver discrepancies, thus increasing the reliability of accurate histopathological grading of the lesion.[9]

Special stains such as Feulgen and 1% crystal violet stains which depend on the acid hydrolysis of DNA can be used to identify chromosomal material in cells and tissues.[10] Previous studies have utilised haematoxylin and eosin (H&E), Giemsa, crystal violet, toluidine blue and Feulgen stains to investigate chromatin patterns and identify MFs in brain, uterus and breast tissue.[2,5,11] However, few studies have employed Feulgen stains to stain paraffin-embedded sections of oral dysplasia and OSCC cells.[8,10] This study aimed to compare the number of MFs assessed quantitatively in normal *mucosa* and OSCC samples using Feulgen and 1% crystal violet stains. A secondary objective was to evaluate the mitotic count among various grades of OSCC. In addition, the study aimed to evaluate other nuclear and cellular morphometric variables among various grades of OSCC, including CA, CP, NA, NP and N/C ratio.

Methods

This case-control study was conducted between June and December 2016 in the Department of Oral & Maxillofacial Pathology & Microbiology at the Dr D. Y. Patil Dental College & Hospital, Mumbai, Maharashtra, India. A total of 51 samples were retrieved from the hospital archives. The experimental group comprised 15 samples of well-differentiated, 15 samples of moderately-differentiated and six samples of poorly-differentiated OSCC collected from surgical biopsies sent for diagnosis, while the control group consisted of 15 samples of normal gingival *mucosa* willingly donated by patients undergoing minor oral and periodontal surgeries. The gingival *mucosa* was chosen as the control tissue because the gingival epithelium is active and would thus be an adequate comparison for the highly active and proliferative epithelia of oral cancers and precancers.[5] Two sections from each sample were prepared and stained with Feulgen and 1% crystal violet stains as per established methods.[12,13] Slides were observed under a Leica Microsystems LED Laboratory Microscope (Model DM1000, Meyer Instruments Inc., Houston, Texas, USA) and assessed using image analysis software (Version 3.8.0, Leica Microsystems Image Organizer software, Meyer Instruments Inc.). The sections were subsequently evaluated for MFs along with other cellular and nuclear features.

The four phases of *mitosis* were identified as follows. Cells lacking a nuclear membrane indicated that the prophase stage had begun; subsequently, the metaphase stage was characterised by condensed chromosomes forming clear hair-like extensions of nuclear material in either a clot or along a single equatorial plane. Anaphase was denoted by the clots beginning to separate into two separate parallel chromo-

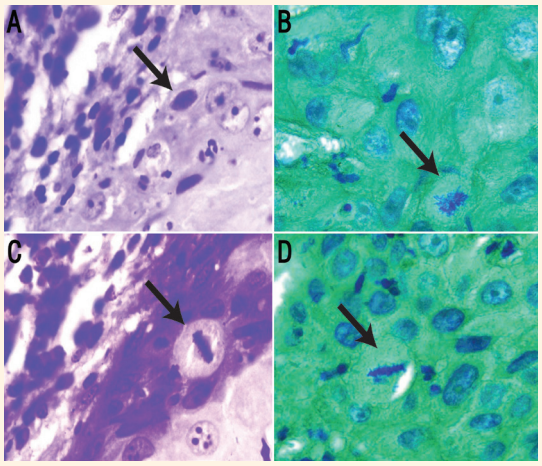

Figure 1: Paraffin-embedded oil immersion sections at x100 magnification showing the (**A and B**) prophase and (**C and D**) metaphase stages of *mitosis* in well-differentiated oral squamous cell carcinoma cells using 1% crystal violet and Feulgen stains, respectively.

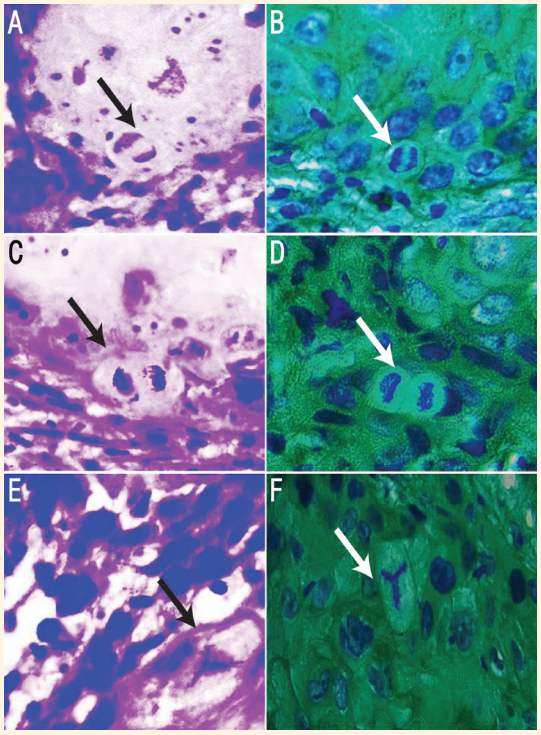

Figure 2: Paraffin-embedded oil immersion sections at x100 magnification showing the (**A and B**) anaphase and (**C and D**) telophase stages of *mitosis* and (**E and F**) tripolar *mitosis* in well-differentiated oral squamous cell carcinoma cells using 1% crystal violet and Feulgen stains, respectively.

somes, while the movement of the chromosomes to separate poles of the cell signified the telophase stage. Each MF was identified as per criteria reported by Van Diest *et al.*[1] The MFs were analysed at various magnifications and counted in 'step-ladder' fashion using an oculometer grid of 15 fields at x40 magnification. The entire stretch of the epithelium was

Table 1: Mean mitotic figure count using Feulgen and 1% crystal violet stains in samples of normal gingival *mucosa* and various grades of oral squamous cell carcinoma (N = 51)

Stain	Mean MF count per field ± SD	T value	*P* value*
Feulgen	3.51 ± 2.93	1.938	0.050
Crystal violet	2.47 ± 2.47		

MF = mitotic figure; SD = standard deviation.
**Using an unpaired two-tailed Student's t-test.*

searched for MFs, excluding areas of inflammation, necrosis, tissue folds and calcification.

After reviewing the number of MFs in the tissue sections, the samples were evaluated for nuclear and cellular morphometric features. The final image of the representative cells in the basal cell layer was captured at x10 magnification using the Leica Microsystems Image Organizer software (Meyer Instruments Inc.), avoiding areas of basal cell hyperplasia and overlapping cells. The outlines of the cells and nuclei were traced on a black and white monitor and the size of the cell and nucleus was calculated by measuring the area and perimeter of the outline.[8] Afterwards, the cellular and nuclear outlines of 5–7 cells with the largest dimensions and the clearest outlines were traced. The CA, CP, NA and NP were then automatically calculated by the Leica Microsystems Image Organizer software (Meyer Instruments Inc.), based on the number of boundary pixels detected. The N/C ratio of the cell was calculated using the following formula:[6]

$$\text{N/C ratio} = \frac{\text{NA}}{\text{cytoplasmic area - NA}}$$

Differential and inferential statistical analyses were undertaken using the Statistical Package for the Social Sciences (SPSS), Version 20.0 (IBM Corp., Armonk, New York, USA) and Microsoft Excel, Version 2007 (Microsoft Inc., Redmond, Washington, USA). Continuous measurements were presented as means and standard deviations. The level of significance was set at $P \leq 0.050$. Continuous parameters were compared between two groups using an unpaired two-tailed Student's t-test, while comparisons between three or more groups were performed using an analysis of variance test which, if significant, was followed by a *post hoc* analysis.

Ethical approval for this study was obtained from the Institutional Review Board of the Dr D. Y. Patil Dental College & Hospital (#DYPUSOD/GS-OP-SAT/28/2017). Written consent to collect oral samples was obtained from all donors in both the control and case groups.

Table 2: Mean cellular area, cellular perimeter, nuclear area, nuclear perimeter and nuclear-to-cytoplasmic ratio of samples of normal gingival *mucosa* and various grades of oral squamous cell carcinoma (N = 51)

Sample group	Mean CA in mm² ± SD*	Mean CP in mm² ± SD*	Mean NA in mm² ± SD*	Mean NP in mm² ± SD*	Mean N/C ratio ± SD*	*P* value†
Normal *mucosa* (n = 15)	78.68 ± 7.96	30.27 ± 2.01	26.35 ± 20.07	20.07 ± 1.17	0.515 ± 0.132	
WD OSCC (n = 15)	123.03 ± 6.91	47.33 ± 4.81	60.32 ± 32.89	32.89 ± 3.34	0.965 ± 0.271	
MD OSCC (n = 15)	134.18 ± 8.45	54.45 ± 6.33	66.00 ± 39.45	39.45 ± 6.91	0.971 ± 0.164	<0.001
PD OSCC (n = 6)	156.02 ± 12.39	57.32 ± 7.58	74.66 ± 43.87	43.87 ± 7.17	1.018 ± 0.353	

CA = cellular area; SD = standard deviation; CP = cellular perimeter; NA = nuclear area; NP = nuclear perimeter; N/C = nuclear-to-cytoplasmic; WD = well-differentiated; OSCC = oral squamous cell carcinoma; MD = moderately-differentiated; PD = poorly-differentiated.
**Of both Feulgen- and 1% crystal violet-stained samples combined. †Using an analysis of variance test.*

Results

In terms of MF assessment, all four phases of *mitosis* were visible in the samples, with prophase being the most difficult to detect [Figures 1A and B]. The most commonly seen phases of *mitosis* were metaphase, anaphase and telophase [Figures 1C and D and 2A–D]. A few abnormal MFs, such as tripolar *nuclei*, were seen in various grades of OSCC using both stains [Figures 2E and F].

The frequency of MFs was significantly higher in the samples stained with Feulgen in comparison to 1% crystal violet (3.51 ± 2.93 per field versus 2.47 ± 2.47 per field; *P* = 0.050) [Table 1]. The CA, CP, NA, NP and N/C ratio of the cells increased steadily from the normal gingival *mucosa* samples to the well-differentiated and moderately-differentiated OSCC samples, before reaching their highest value in the poorly-differentiated OSCC samples, with statistically significant results (*P* <0.001 each) [Table 2].

Discussion

The Feulgen stain is a modified histochemical stain that is simple, cost-effective, reliable and allows for differentiation between apoptotic bodies, *karyorrhexis* and MFs, thereby excluding false-positive results.[2,5,10,11] Furthermore, the purple-coloured chromatin material against a green cytoplasm facilitates the identification of MFs, even at lower magnifications. Multiple studies have reported using Feulgen staining to study nuclear and cellular morphometry and chromatin frameworks in oral cytosmears or paraffin-embedded tissue sections of various potentially malignant lesions, such as oral hyperplasia, dysplasia and OSCC.[6–8,14–18]

In the present study, different mean mitotic counts were calculated using different stains, with a significantly greater frequency of MFs observed among samples stained with Feulgen stains compared to those stained with 1% crystal violet, regardless of OSCC grade. Similarly, Rao *et al*. also noted a significant increase in the number of MFs in Feulgen-stained sections as compared to 1% crystal violet-stained samples.[10] A similar study by Palaskar *et al*. evaluating the effectiveness of H&E, toluidine blue, Giemsa, crystal violet and Feulgen stains in oral epithelial dysplasia samples concluded that all of the aforementioned stains were useful in the identification of MFs, although Feulgen stains yielded superior results.[19] In a study evaluating oral epithelial dysplasia and OSCC, Nikita *et al*. reported that Feulgen stains provided a greater contrast when staining MFs as compared to H&E, toluidine blue, Giemsa and crystal violet stains; in addition, the MFs were easier to see at lower magnifications in comparison to the other stains.[20]

The current study also found a consistent increase in basal cellular and nuclear measurements beginning with the normal gingival *mucosa* samples and increasing with each grade of OSCC, thereby indicating heightened biological activity in the nucleus and cytoplasm. Nandini *et al*. proposed a new grading system for various nuclear features (NA, NP and nuclear form factor) based on their observations of a steady increase in all three features corresponding to increasing grades of OSCC in Feulgen-stained buccal *mucosa* samples.[8] Similarly, Smitha *et al*. studied the nuclear and cellular morphometry of oral *leukoplakia* and OSCC cells in the basal layer using computer-aided image analysis, concluding that the mean CA, CP, NA and NP steadily increased from normal oral buccal *mucosa* to *leukoplakia*, reaching their highest values in OSCC cells.[6]

An increased N/C ratio is a common feature of OSCC.[21] As with the MF count and cellular and nuclear measurements, an increase in the N/C ratio in the present study was also found to correlate with the OSCC grade. Shabana *et al*. likewise reported

an increase in N/C ratio to be consistent with the progression of oral lesions from a benign to malignant state.[22] Similarly, another cytomorphometric analysis of oral lichen *planus*, oral submucous fibrosis, OSCC and normal oral *mucosa* indicated that the N/C ratio was increased in potentially malignant lesions and OSCC cells.[23]

Based on the findings of the present study, it can be concluded that nuclear cytomorphometric parameters using computer-aided morphometry are a sophisticated tool to objectively assess and differentiate various grades of OSCC. As such, nuclear and cellular morphometric analysis can be utilised to quantitatively predict the rate of malignancy, thereby avoiding subjective errors and potentially informing treatment decisions. However, a prospective study involving a larger sample group should be performed in order to determine the validity and practicality of utilising such analyses for this purpose.

Conclusion

The authors strongly recommend the use of Feulgen stains for the distinct and selective staining of MFs. In addition, the authors further advise using computer-aided morphometry to evaluate a combination of nuclear and cellular variables as a method of determining tumour aggressiveness.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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