International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Micronuclei Evaluation in Oral Leukoplakia and Squamous Cell Carcinoma of Oral Cavity

Dr. Vanshika Garg[1], Dr. Arun Bhardwaj[2], Dr. Srishti Arora[3], Dr. Simran Rajpal[4], Dr. Rakesh Ranjan[5]

[1]Resident, Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Panchkula, Haryana, India
[2]Head, Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Panchkula, Haryana, India
[3]Resident, Department of Maxillofacial Pathology, Sudha Rustagi Dental College, Faridabad
[4]Resident, Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Panchkula, Haryana, India
[5]Resident, Department of Oral Pathology, Sudha Rustagi Dental College, Faridabad

ABSTRACT

Background: Oral cancer develops in a series of steps from pre-existing, possibly cancerous lesions. Several universities and scientific labs are currently working to create screening methods that identify specific diseases with specific biomarkers that aid in the identification of high-risk patients and serve as a tool for the prevention of individual tumors. Since the approach is highly reliable and inexpensive, Micronuclear accumulation is being progressively used as a bio-marker of genetic instability in both humans as well as a variety of species. It is also the best option for molecular epidemiological investigations.

Aims and Objectives: The aim of the study was to consider and contrast frequency of micronuclei (MN) using exfoliative cytology among healthy participants, individuals with Oral leukoplakia (OL) and Oral Squamous cell carcinoma (OSCC) using three different stains; PAP, Giemsa and Acridine orange fluorescent stain.

Material and Methods: Thirty healthy controls with no oral lesions or systemic disorders and forty-five individuals (30 for the OL and 15 for OSCC) each underwent three slides of buccal smears that were stained with Papanicolaou stain, Giemsa stain, and the effectiveness of stains in staining micronuclei is identified, measured, and compared using acridine orange fluorescent dye and examined at 40X magnification.

Results: According to the study's findings, PAP is a more effective stain than MGG for counting micronuclei, and the Mn frequency score decreased from OSCC patients to those with oral leukoplakia and controls. Additionally, the study found that using AO, a DNA-specific stain, reduced the Mn number and incidence in comparison to PAP which is a non-DNA-specific stain.

Conclusion: Micronuclei are extranuclear cytoplasmic bodies formed due to chromosomal damage from genotoxic substances. The micronucleus assay offers a simple, non-invasive, and cost-effective method to assess genotoxicity. A strong correlation exists between tobacco habits such as smoking and chewing gutkha or areca nut and increased micronuclei frequency, as seen in oral cancer and potentially malignant

disorders. Our findings support the use of exfoliated oral cells as an accessible and effective tool for detecting genetic damage.

Keywords: Micronuclei, Oral Cancer, Oral Leukoplakia, Exfoliative Cytology, Fluorescent Staining

INTRODUCTION

When combined, oral and pharyngeal cancer ranks sixth globally and in the top three in regions with high prevalence.[1] Around the world, oral cancer is becoming an increasingly serious problem. In high-risk nations like Bangladesh, India, and Sri Lanka, oral cancer is the highest predominant illness found more commonly in males as compared to females. Diagnosis requires early detection of mucosal changes that might be signs of disease but are not deviations from normal function. The survival and morbidity of individuals with premalignant or cancerous oral lesions are anticipated to improve with early identification.[2]

Leukoplakia is the most prevalent precancers, accounting for 85% of precancers. Oral cancer develops through a multi-step process that starts with pre-existing potentially malignant lesions. Researchers are working to create screening methods that identify specific malignancies using specific biomarkers, which serve as a tool for preventing individual tumors and identifying high-risk individuals.[1]

Disease development, including cancer, requires genomic damage in human cells, which can be caused by a variety of substances, including chemicals, radiation, environmental pollutants, and lifestyle choices like smoking, drinking, medications, stress, and genetics.[3]

MN accumulation has been used extensively as a biomarker of genetic instability and genotoxic stress in a variety of human and non-human animals.[4] The global success of genome damage has been attributed to the MN approach's excellent predictability as well as affordability allows it be used to detect carcinogenic effects in cells exposed to numerous carcinogenic chemicals at an early stage.[5]

Having gained widespread acceptance, exfoliative cytology is a diagnostic technique that is quickly becoming more significant as a tool for early cancer detection. Originally created to study gynecological cancer, the Papanicolaou technique has been widely used on material from a variety of sources.[6]

A histochemical fluorochrome known as acridine orange (AO) has a specific affinity for nucleic acids; at a pH of 6 and a concentration of 0.01%, the RNA fluoresces red and the DNA yellow to yellowish green. Comparative levels of DNA and RNA inside the cell can be determined thanks to the differential fluorescence, which is caused by various levels of nucleic acid polymerization. Because the malignant and normal cells present distinguished fluorescence resulting from the increased RNA of the malignant cell shining more brightly, it is easy to compare the overall concentration of nucleic acids in the various cells in the preparation. In addition, one may observe the morphological features of the cell.[6]

MATERIALS AND METHODS

The investigation was successfully completed at the Oral Pathology and Microbiology Department of SDDDC, Barwala. The samples were taken from patients who visited the outpatient department. As a control group, thirty healthy people without any systemic disorders or undesirable oral mucosal lesions were selected in the study. The study group contained thirty partakers each who had been previously diagnosed with OL and OSCC. Every participant had three cytosmears extracted from them. A detailed consent was received from each patient. The approval of Institutional Ethical Committee was received vide SDDHDC/IEC/March2022/001 before the initiation of the study.

A total of 75 subjects were enrolled in the present study and relevant demographic data including age, gender, and lesion site were recorded at the time of sample collection.. Group I comprised 30 healthy individuals who served as the control group. Group II included 30 patients with clinically and histopathologically diagnosed oral leukoplakia, further subdivided into three subgroups: Group IIa (10 patients with a history of tobacco smoking), Group IIb (10 patients with a history of tobacco chewing), and Group IIc (10 patients with a habit of both smoking and chewing tobacco). Group III consisted of 15 cases of oral squamous cell carcinoma (OSCC).

For cytological sampling, exfoliated buccal cells were collected from the lesion site (or corresponding site in controls) after cleaning with a saline-moistened cotton swab to remove debris and surface coatings. Using a wooden spatula, buccal mucosa was gently scraped, and the obtained exfoliated cells were immediately smeared on clean glass slides and fixed with 95% ethyl alcohol for Papanicolaou staining. The procedure for Papanicolaou (PAP) staining included fixation in 95% ethanol for 15 minutes, followed by rinsing in distilled water. Hematoxylin was applied for 1 to 3 minutes, followed by another rinse in water or Scott's tap water. After 10 dips in 95% ethanol, the smears were stained with OG-6 for 1.5 minutes, dehydrated again in 95% ethanol, and counterstained with EA-50 (or Modified EA-50/EA-65) for 2.5 minutes. Following two changes of 95% ethanol, the slides were treated with 100% ethanol for 1 minute, cleared in two changes of xylene (2 minutes each), and mounted with DPX.

For Giemsa staining, air-dried smears were fixed in methanol for 5–7 minutes. After air-drying, slides were stained with Giemsa diluted in deionized water at a 1:20 ratio for 15 to 60 minutes. Slides were then rinsed with deionized water, air-dried, and evaluated microscopically. Acridine orange (AO) staining was also employed for fluorescent analysis. Fixed smears were stained with freshly prepared AO solution and incubated on the slide surface for 2 minutes without drying. The slides were then rinsed with water, air-dried, and observed under a fluorescent microscope.

All stained slides were examined under a light microscope (for PAP and Giemsa stains) and a fluorescent microscope (for AO stain) at 40X magnification. Micronuclei were scored using the zigzag method. For each subject, 100 intact, non-overlapping cells with well-defined nuclear and cytoplasmic boundaries were evaluated. Micronuclei identification followed the criteria proposed by Tolbert et al., which include: (i) the presence of a smooth, rounded boundary indicative of a membrane; (ii) size less than one-third of the main nucleus; (iii) similar staining intensity and texture as the nucleus; (iv) same focal plane as the main nucleus; and (v) no connection or overlap with the main nucleus. Only structures fulfilling all criteria were considered as micronuclei. All evaluations were performed by a single blinded observer to minimize inter-observer variability.

Statistical Analysis

A significance level of $p<0.05$ was set after the data was evaluated using the statistical program SPSS 26.0 (SPSS Inc., Chicago, IL). Descriptive statistics were used to assess each group's mean and standard deviation. The data's normality was assessed using the Shapiro-Wilkinson test. The KRUSKAL WALLIS TEST and the BONFERRONI POSTHOC TEST were used as inferential statistics to determine the differences between and within the group.

RESULTS

This study comprised a total of 75 patients that included 30 subjects who had oral leukoplakia, 15 subjects who had OSCC and 30 subjects in the control group who had apparently normal oral mucosa. Clinically

healthy buccal mucosa was used to create the smears, which were subsequently fixed, dyed, and assessed in preparation for the photo shoot. In order to build tables and conduct statistical analysis, a master chart was created and a complete count of micronuclei was made. A graphical representation of the tabulated data was also provided.

TABLE 1: SHOWING GENDER DISTRIBUTION WITHIN THE STUDY GROUP

S. No	GROUPS	MALE	FEMALE	TOTAL
I.	CONTROL GROUP	16	14	30
II.	ORAL LEUKOPLAKIA IIa. Smokers IIb. Chewers IIc. Both	 9 6 8	 1 4 2	30 10 10 10
III.	ORAL SQUAMOUS CELL CARCINOMA	11	4	15

TABLE 2: SHOWING MEAN AGE DISTRIBUTION WITHIN THE STUDY GROUP

S. NO.	GROUPS	FEMALES	MALES	TOTAL
I.	CONTROL GROUP	40.22	48.43	44.32
II.	ORAL LEUKOPLAKIA IIa. Smokers IIb. Chewers IIc. Both	 40 55.25 57	 50.22 57.83 50.25	51.6 49.2 56.8 51.6
III.	ORAL SQUAMOUS CELL CARCINOMA	56	52.63	53.53

TABLE 3: COMPARISON OF MEAN MICRONUCLEI WITHIN STUDY GROUPS

S. NO.	GROUPS	PAP STAIN	GIEMSA STAIN	ACRIDINE ORANGE STAIN
I.	CONTROL GROUP	2.03+_1.18	1.21+_0.66	0.65+_0.47
II.	ORAL LEUKOPLAKIA	5.65+_1.53	3.97+_1.63	3.07+_1.26
III.	ORAL SQUAMOUS CELL CARCINOMA	11.8+_2.4	9.13+_1.89	6.33+_1.19

TABLE 4: Using PAP smears, the mean micronuclei counts for each of the three study groups were compared.

S. NO.	STUDY GROUPS	MICRONUCLEI COUNT		MEAN Mn COUNT	STANDARD DEVIATION
		Min.	Max.		
I.	CONTROL GROUP	0	5	2.03	1.18
II.	ORAL LEUKOPLAKIA	3	8	5.65	1.53

III.	ORAL SQUAMOUS CELL CARCINOMA	8	15	11.8	2.4

TABLE 5: Using Giemsa smears, the mean micronuclei counts for each of the three study groups were compared

S. NO.	STUDY GROUPS	MICRONUCLEI COUNT		MEAN Mn COUNT	STANDARD DEVIATION
		Min.	Max.		
I.	CONTROL GROUP	0	2	1.21	0.66
II.	ORAL LEUKOPLAKIA	0	7	3.97	1.63
III.	ORAL SQUAMOUS CELL CARCINOMA	5	12	9.13	1.89

TABLE 6: Using AO smears, the mean micronuclei counts for each of the three study groups were compared

S. NO.	STUDY GROUPS	MICRONUCLEI COUNT		MEAN Mn COUNT	STANDARD DEVIATION
		Min.	Max.		
I.	CONTROL GROUP	0	1	0.65	0.47
II.	ORAL LEUKOPLAKIA	0	5	3.07	1.26
III.	ORAL SQUAMOUS CELL CARCINOMA	4	8	6.33	1.19

DISCUSSION

The oral mucosa is a good indicator of overall health.[3] Exfoliated cells can reveal details about the living epithelium they are produced from when they are gathered and properly labeled. It is therefore possible to diagnose a number of clinical disorders by examining the patterns and morphological changes of the exfoliated cells.[12]

Micronuclei are tiny, extra nuclei created when chromosome fragments or entire chromosomes that are delayed at mitosis due to genotoxins or carcinogens are excluded. These are extranuclear cytoplasmic entities that harm the chromosomes and are produced in cells by a variety of genotoxic substances. The benefits of oral exfoliated cells as a superior method to investigate genotoxic damage are further enhanced by the ease of access and acquisition of smears.[3]

There is a substantial correlation between the micronuclei in exfoliated oral squamous cells and tobacco-related behaviors like smoking, chewing gutkha and areca nut, as well as oral cancer and potentially malignant illnesses.[3]

The current study assessed the prevalence of micronuclei in exfoliated cells from OSCC, OL, and normal oral mucosa. Leukoplakia and carcinomas showed higher average micronucleus frequencies than normal mucosa, indicating that micronuclei represent a genetic marker of cancerous development.

With all the slides that were evaluated, the variation in micronuclei frequency was not based on age or sex of the patient. The Mn count observed was higher in patients with OSCC (11.8+_ 2.4), followed by patients with OL (5.65+_ 1.53) and normal oral mucosa (2.03+_ 1.08).

Patel also performed a study to assess micronuclei and chromosomal aberrations in tobacco chewers and normal individuals in their peripheral blood lymphocyte and exfoliated oral mucosal cells and analyzed MN count being increased (p=0.01) in patients with the habit of chewing tobacco than the controls which was found in accordance with our study.

When using acridine orange stain, which is specific for DNA, instead of PAP stain, which is not, the current study likewise revealed a reduced MN count and frequency. Using non-DNA specific stains makes it difficult to distinguish between intercellular structures and micronuclei, which frequently results in an overestimation of the MN count. The DNA-specific stain, which is more specific since it only stains the nucleic acid component, cannot detect these intracellular structures.

The results of the current research suggested that PAP had superior staining quality when compared to Giemsa and Acridine Orange, having overall mean quality index values of 11.61, 8.23, and 5.83, accordingly. PAP was superior in regard to nuclear chromatin staining, strong differential cytoplasmic features, and decent cytoplasmic transparency.

The current study's findings indicate that additional comparable research on larger populations should be conducted in the future to confirm the MN assay's undeniable value in PMD diagnosis. However, the study also acknowledges its limitations in comparison to a biopsy, which is that a histological examination distinguishes between the various PMDs while this diagnostic test does not. It can only indicate whether a certain lesion is a PMD. When screening for suspicious lesions, which are often mistaken clinically as changes of normal mucosa, the MN assay plays a crucial role. Clinicians are frequently faced with the difficult decision of whether to perform a biopsy or continue to monitor the patient in cases of such lesions. A choice to proceed with biopsy for diagnosis confirmation can be made based on an elevated MN count seen in these lesions. Subjectivity in scoring and a few restrictions, such as an evaluation by a single examiner, necessitate additional research on the sensitivity and specificity of the PAP staining method for micronuclei detection.

CONCLUSION

In assessing the micronuclei count in individuals with OL and OSCC, our investigation found that Papanicolaou stain was more steadfast as compared to Giemsa and Acridine orange. The usefulness of the Mn count in diagnosing malignancy is demonstrated by the current study's observation that patients with OSCC had a greater micronucleated cells than patients with OL and the control group.

Accordingly, our study suggests that tobacco smoking may modify oral mucosal cells in addition to more serious cellular alterations, which may raise the risk of developing oral cancer. This work adds credence to the idea that tobacco-induced cytological alterations in the oral mucosa could serve as a helpful diagnostic method for the identification in regard to cancer as well as precancerous changes in the mouth. Additionally, these could serve as a teaching tool for population awareness campaigns to aid in the cessation of harmful oral habits.

REFERENCES:

1. Khanna, Shally & Bhalla, Saurabh & Anupam, Purwar & Singh, NarendraNath & Sreedhar, Gadiputi & Singh, Shailendra. (2014). Cytogenetic biomonitoring of premalignant and malignant oral lesions by micronuclei assessment: A screening evaluation. European Journal of General Dentistry. 3. 46. 10.4103/2278-9626.126211.

2. Grover, Sonal. (2014). Evaluation of diagnostic reliability of micronuclei in potentially malignant disorders of oral cavity. CHRISMED J Health Res. 1. 15-20. 10.4103/2348-3334.126776.
3. Jain, Anshi & Gupta, Pulkit & Khan, Aamna & Juneja, Saurabh & Gulati, Nikita. (2020). Micronucleus assay using DNA Specfic and non specific stains in exfoliated oral epithelial cells as a biomarker to assess cytogenetic damage in petrol station attendants. Journal of Dental Specialities. 7. 102-106. 10.18231/j.jds.2019.024.
4. Krupina K, Goginashvili A, Cleveland DW. Causes and consequences of micronuclei. Curr Opin Cell Biol. 2021;70:91-99. doi:10.1016/j.ceb.2021.01.004
5. Singh, Abhay. (2015). Buccal Micronucleus Cytome Assay- A Biomarker of Genotoxicity. Journal of Molecular Biomarkers & Diagnosis. 06. 10.4172/2155-9929.1000236.
6. Epstein JB, Zhang L, Rosin M. Advances in the diagnosis of oral premalignant and malignant lesions. J Can Dent Assoc. 2002;68(10):617-621.
7. Kumar M, Prasad UC, Chandolia B, Manjunath SM, Basu S, Verma S. Can Feulgen Stain be a Reliable Biomarker over PAP Stain for Estimation of Micronuclei Score?. J Clin Diagn Res. 2016;10(10):ZC07-ZC11. doi:10.7860/JCDR/2016/18859.8630
8. Patel U, Shah R, Patel A, Shah S, Patel D, Patel A. Effect of tobacco in human oral leukoplakia: a cytomorphometric analysis. Med Pharm Rep. 2020;93(3):273-279. doi:10.15386/mpr-1439
9. Sivakumar, Kokila & Prasad, Harikrishnan & Rajmohan, Muthusamy & Kenniyan Kumar, Srichinthu & Mahalakshmi, Loganathan & Shanmuganathan, Sivanandhan & Prema, Perumal. (2021). Evaluation of Micronuclei and Cytomorphometric Changes in Patients with Different Tobacco Related Habits Using Exfoliated Buccal Cells. Asian Pacific Journal of Cancer Prevention. 22. 1851-1855. 10.31557/APJCP.2021.22.6.1851.
10. Nersesyan A, Kundi M, Atefie K, Schulte-Hermann R, Knasmüller S. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. Cancer Epidemiol Biomarkers Prev. 2006 Oct;15(10):1835-40. doi: 10.1158/1055-9965.EPI-06-0248. PMID: 17035390.
11. Mhaske SP, Pattanshetti K, Jagtap K, Debta P, Misurya AL, Patel JH. Comparative Study using Papanicolaou Stain and Silver-stained Nucleolar Organizer Region Counts in Exfoliative Smear of Oral Mucosa in Bidi Smokers and Nonsmokers. J Int Soc Prev Community Dent. 2018;8(4):365-370. doi:10.4103/jispcd.JISPCD_65_18
12. Sidhu, SupreetK & Ramalingam, Karthikeyan & Goyal, Sandeep & Poonia, Monika & Rajawat, GauravS & Sharma, Nitish. (2018). Comparing the Efficacy of Leishman–Giemsa Cocktail Stain, Giemsa Stain, and Papanicolaou Stain in Potentially Malignant Oral Lesions: A Study on 540 Cytological Samples. Journal of Cytology. 35. 105. 10.4103/JOC.JOC_249_16.
13. Bansal H, Sandhu VS, Bhandari R, Sharma D. Evaluation of micronuclei in tobacco users: A study in Punjabi population. Contemp Clin Dent. 2012;3(2):184-187. doi:10.4103/0976-237X.96825
14. Ramaesh T, Mendis BR, Ratnatunga N, Thattil RO. Cytomorphometric analysis of squames obtained from normal oral mucosa and lesions of oral leukoplakia and squamous cell carcinoma. J Oral Pathol Med. 1998 Feb;27(2):83-6. doi: 10.1111/j.1600-0714.1998.tb02099.x. PMID: 9526735.