Research Article

# Marking the Micro Details in Potentially Malignant Disorders of Oral Cavity

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**ABSTRACT:**

**Background**- The malignancy of the oral cavity constitutes the most important group of malignancies in South and Southeast Asia.The risk increases with the use of tobacco products.

**Materials & Methods-** Micronuclei frequency scoring was used as a biomarker to identify different potentially malignant disorders.

**Results**- Mean micronuclei index was found higher using Hematoxylin and Eosin stain than Papanicolaou's stainand May Grunwald's stain.

**Conclusion**- We concluded that the micronuclei frequencies in oral exfoliated epithelial cells using three different stains- Hematoxylin and Eosin stain, Papanicolaou's stain, May Grunwald's stain may be useful in predicting the malignant potential of premalignant lesions.

**KEYWORDS**: papanicolaou's stain; may grunwald's stain; micronuclei

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## INTRODUCTION:

Oral cancer is still a major global health concern even after major advance in understanding of carcinogenesis.[1] Tobacco consumption is the most common preventable attribute for oral cancer disease. Tobacco-related disease kills approximately 6 million people in world; the figure is estimated to reach around 8 million by 2030. India being one of highest tobacco producer and consumer of tobacco-related products is at a verge of risk of being the country with high oral cancer patients. Tobacco paan masala, and tobacco with pan and betel quid are the most consumed form of SmokelessTobacco (SLT) in India.[2] SLT is strongly associated with precursor lesions of oral cavity and esophageal cancers.[3] Tobacco contains nicotine and carcinogens, including nitrosamines (*i.e*., NNK and NNN). Nitrosamines cause epithelial cells' division leading to focal growth and morphologic changes in the early stages in cell transformation.[3] Accumulation of genetic alterations within oral epithelial cells/mucosa, induced by the genotoxins present in tobacco-related products often lead to oral potentially malignant disorders (PMDs).

These PMDs includes leukoplakia, erythroplakia, lichen planus and oral submucous fibrosis, are known to have an increased risk of Oral squamous cell carcinoma (OSCC). These lesions harbor genomic abnormalities in the form of micronuclei indicating enotoxity in oral epithelium.The micronuclei index has become one of the standard cytogenetic biomarkers used in cancer biology. Many studies have indicated that there is an increased micronuclei index in PMD which are procurers of OSCC.[4] Identifying the presence of

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micronuclei in oral epithelial cells in early stage can help clinicians in the initiation of preventive measures.

Exfoliative cytological study of oral cells is a non-aggressive technique and its application in the early diagnosis of PMDs is well-established. The assay is reliable and technically easy to perform. Early detection and early intervention of oral cancer can prolong life expectancy and reduce the years of life lost, indicating the importance of proactive screening and oral hygiene.

Numerous DNA specific stains available are used to study the micronuclei and chromosomal abnormalities within the cells. Many investigators investigated a variety of stains including DAPI, acridine orange (AO), Hoechst, and propidium iodide, Feulgen-Fast Green (FFG), May-Grunwald Giemsa (Giemsa) and Papanicolaou (Pap). Feulgen-Fast Green (FFG) which enable easy identification of Micronuclei, making it clearly stand among other stains. However, relatively lengthy procedures may result in theunder-scoring of Micronuclei. Many comparative studies of micronuclei using stains were performed in the past. Casartelli et al. evaluated Hoechst, PI, and Giemsa, and found Hoechst was the more reliable for staining and identifying micronuclei.Giemsa, FFG, DAPI, and acridine orange stains' comparison show that Giemsa is more associated with falsification of micronuclei and FFG is better than other stains.The Papanicolaou (Pap) stain was the preferred method of detecting micronuclei in oral epithelial cells when compared to Giemsa stain. The fluorescent staining (acridine orange) was more sensitive for micronuclei detection than FFG in oral exfoliated cells in subjects of leukoplakia and squamous cell carcinoma. Studies are needed to determine whether some micronuclei and nuclei may lose DNA through karyolysis while maintaining the protein structure of chromatin and the nuclear envelope, so that they would still be detectable by stains that are not DNA-specific.

Looking at the present scenario, we decided to evaluate micronuclei index in exfoliated cells using Hematoxylin and Eosin stain (H&E), Papanicolaou's stain (PAP), May Grunwald's stain (MGG) among the OPMD's cell samples. The objective of study was: (a)to determine micronuclei index among OPMD's and OSCC, (b)to determine efficacy of H & E, PAP, MGG stains for staining and identifying micronuclei, (c) comparisons of H&E, PAP, MGG in OPMD's.

## MATERIALS &METHODS:

The study was approved by the Research ethical committee, PCDS & RC and study was conducted at Department of Oral Pathology & Microbiology. The subjects were pooled from the outpatient department based on criteria and divided into four groups as follows: (a) subjects withoral submucous fibrosis and later confirmed histopathologically as dysplastic lesions, (b) subjects with clinical leukoplakia and later confirmed histopathologically as dysplastic lesions, (c) subjects with clinical lichen planus and later confirmed histopathologically as dysplastic lesions(d) healthy subjects without history or habit of SLT chewing or any other form of tobacco consumption. Each group was comprised of 20 subjects. Written consent was obtained from subjects after explaining the purpose of study.

***Collection of sample and processing*:**

Subjects were asked to rinse the oral cavity with saline before obtaining the cytology sample. Standard exfoliative cytology procedure was used to collect samples from site. The scrapes were taken with the help of a sterile metal spatula. The sample was transferred to a clean glass slide and fixed with alcohol. These slides were dried for 5-10 minutes and stained with H&E, PAP and MGG. All stained slides were examined at 1000X magnification using oil immersion objective under Binocular research Microscope (Olympus CH 20i, Olympus, India**).** Minimum 5 slides were prepared and screened for cell yield. Those with greater cell yield were selected for evaluation and counting of micronuclei. Minimum two slides for each stain per subject were counted. Micronuclei were counted as per Tolbert's criteria: (a) Rounded smooth perimeter suggestive of a membrane. (b) Less than a third the diameter of the associated nucleus, but large enough to discern shape and color, (c) Staining intensity like that of the nucleus, texture like that of nucleus, (d) Same focal plane as nucleus and absence of overlap with, or bridge to, the nucleus. Only those structures fulfilling the above-mentioned criteria were recorded as MNi (Figure 1). 720 slides were prepared, and 1000 cell/slide were counted for micronuclei. 7, 20,000 cells were counted in total. The gathered data was analyzed using unpaired t- test and chi-square test.

## RESULTS:

The mean number of micronuclei were observed using H&E, PAP and MGG stain in subjects with OSMF. Mean micronuclei index was found high ($p$<0.001) using H &E stain (12.20) than PAP (8.20) and MGG (5.40)[Graph 1].The mean number of micronuclei were observed using H&E, PAP and MGG stain in subject with leukoplakia. Mean micronuclei index was found high ($p$<0.001) using H &E stain

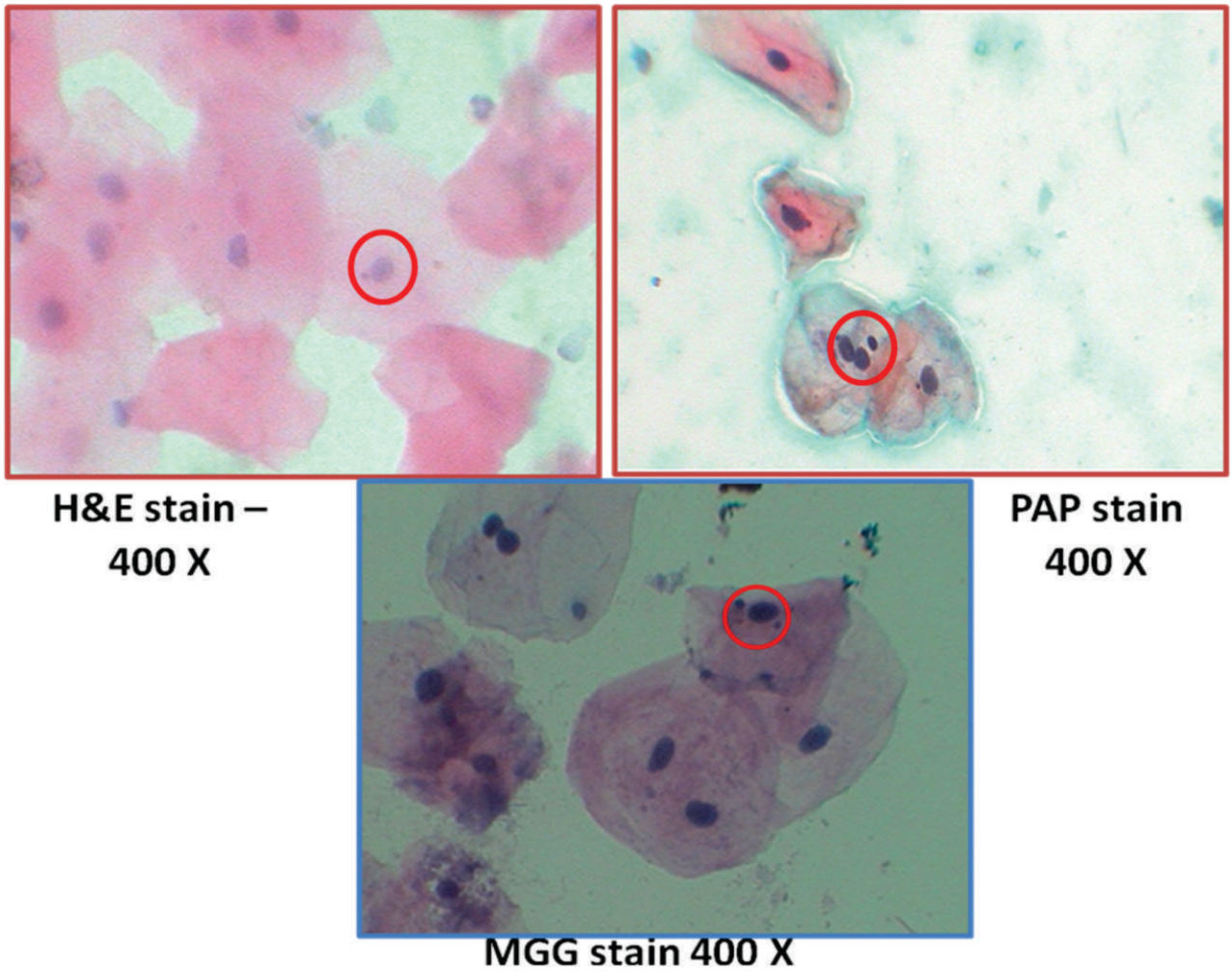
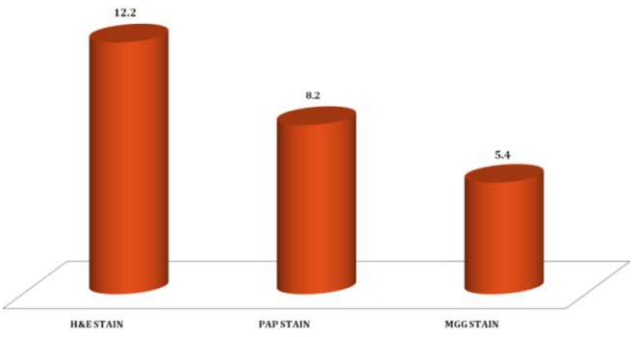



**Figure 1:** Photomicrographs showing micronuclei (circle) in oral exfoliated epithelial cells (a: H and E, b: Pap, c: MGG, ×1000)

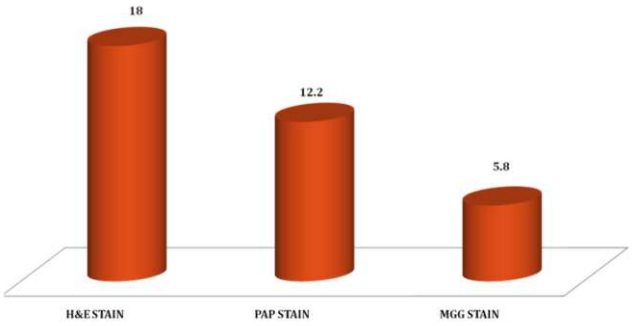
(18.00) than PAP (12.20) and MGG (5.80)[ Graph 2]. The mean numbers of micronuclei were observed using H&E, PAP and MGG stain in subject with Lichen planus. Mean micronuclei index was found high ($p$<0.001) using H &E stain (8.0) than PAP (5) and MGG (2.2)[ Graph 3].The mean number of micronuclei were observed using H&E, PAP and MGG stain in subjects with Control subjects. Mean micronuclei index was found high ($p$<0.001) using H &E stain (2.40) than PAP (1.60) andMGG (0.40)[ Graph 4].The mean number of micronuclei were observed using H&E stain in subjects with OSMF, Leukoplakia, Lichen planus. Control Mean micronuclei index was found high ($p$<0.001) in Leukoplakia (12.20) when observed using H &E stain [Graph 5].



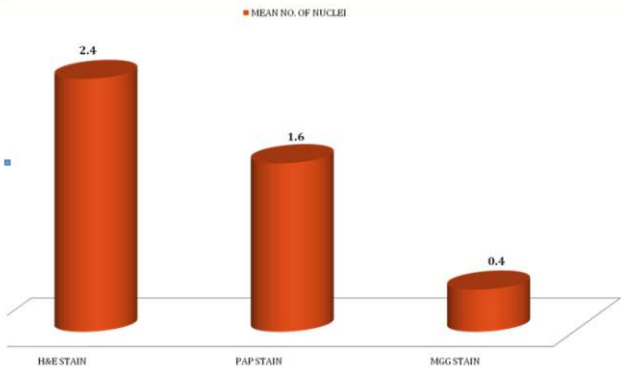

**Graph 1**: Mean no. of nuclei among three stains in OSMF patients.

## DISCUSSION:

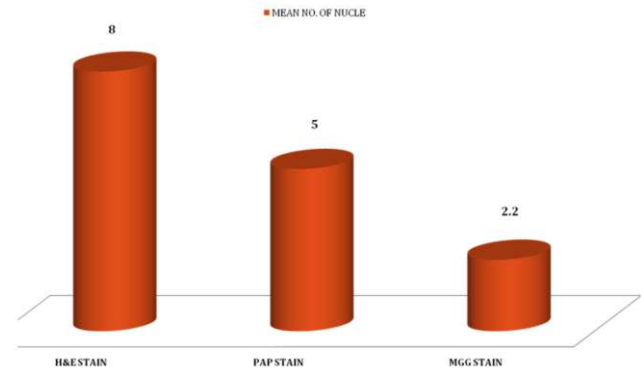
Oral cancer is still a health burden in the world even in the 20th century. Even though there are many advances in detection and treatment, mortality and morbidity rates are high in many body cancers. Prevention is still the most effective way to reduce mortality and morbidity rate in the world. However, to detect and predict early premalignant changes in the tissue is still a dilemma among the pathologists, clinicians, and oncologists. The Etiology factor-based



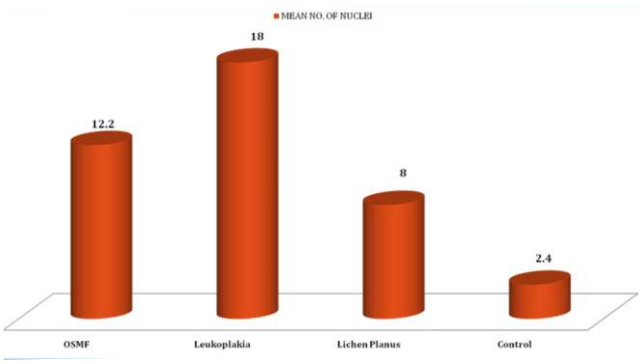

**Graph 2:** Mean no. of nuclie among all three stains in LEUKOPLAKIA Patients.




**Graph 4**: Mean no. of nuclei among all three stains in CONTROL Patients.




**Graph 3**: Mean no. of nuclei among all three stains in LICHEN PLANUS Patients.




**Graph 5:** Mean no. of nuclei among all four lesion by H & E STAINING.

approach is vague since carcinogenesis is a multifactorial and continuous evolving process.

Oral cancer is preceded by OPMD's and has variable rate of malignant transformation. Oral Leukoplakia is a common potentially malignant disorder affecting oral mucosa. The annual malignant transformation ranges of leukoplakia, Lichen planus and OSMF are 2% to 3%, 0.5%, 7.6 % respectively % .[5,6]

Currently, it's difficult to anticipate transformation of OPMD's that can be implemented to prevent such transformation.Cytology has been reliable, convenient, low cost, effective primary diagnostic testutilized all over the world. Cytological changes such as cytoplasmic granulation, cellular enlargement, vacuolization, pyknosis, binucleation, karyorrhexis, karyolitic, micronucleation, nuclear budding, nuclear enlargement is easily identified within exfoliated cell samples. Recently, the Micronuclei index has been suggested to be effective in cancer risk prediction, screening, diagnosis, and monitoring treatment progress[7].

Cytology for micronuclei index has been used before to determine the genotoxicity due to SLT among PMD's. Various environmental, chemical, occupational factors, lifestyle habits, can produce these changes among the oral mucosal cells.[8] Among them tobacco-specific nitrosamines have been reported to be potent mutagenic agents which are thought to be responsible for the induction of chromosomal aberrations resulting in production of micronuclei.

Tobacco users have high micronuclei index [9], which also plays role in development of OSCC.[4] The effect of tobacco on buccal cells shows that there is chromosomal breakage and increase in DNA damage. Buccal micronucleus cytome assay has been suggested as a tool for biomonitoringDNA damage under HUMN project.[10]

Our study has tried to resolve the issue of micronuclei counting among OPMD's, viz. OSMF, Leukoplakia, and Lichen planus. The present study found that leukoplakia has higher ($p$<0.001) micronuclei index as compared to the others. The results were significant. This is in accordance with what was observed by other researchers previously. [11, 12] The tobacco-related DNA damage, which was more closely correlated with OSCC than OSMF and lichen planus, is due to tobacco smoking.[13]

Areca nut, autoimmunity is usually associated with OSMF and lichen planus respectively. Our observations are like Mahimkar et al.[14], indicating increased DNA damage in leukoplakia.

Various specific stains and non-specific DNA stains were used in evaluation of micronuclei in exfoliated buccal cells.[11,15] Our results were in accordance with Grover et al and Katarkar et al that OPDM's show higher micronuclei counts than normal subjects as well as higher micronuclei count was seen with H &E stain. The micronuclei count in OPMD's was 5-fold as compared to control whereas Bloching et al.[16] and Katarkar et al[11] showed there was a 2.2 and 3.6-fold increase in the micronuclei frequency in leukoplakia as compared to normal subjects.

## CONCLUSION:

The micronuclei count assay has potential to predict genotoxicity as well as malignant potential among the premalignancies since there are no other parameters available as of now. Our study concludes that Micronuclei cont. index can be helpful in predicting the malignant potential among the subjects even with the help of Non-specific H & E stain. H&E stain is routinely used, available and have easy practicability. However, one needs to train themselves in micronuclei counting procedure and acquire skills for accurate identification.

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**Conflicts of interest**
There are no conflicts of interest.

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