

## ORIGINAL ARTICLE

# Correlation between histological grading and ploidy status in potentially malignant disorders of the oral mucosa: A flow cytometric analysis

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**ABSTRACT**

**Background:** Histopathological grading of oral dysplastic lesions is the method of choice for evaluating malignant and potentially malignant disorders. Owing to inter- and intra-observer variability, determination of the DNA ploidy status of lesions may serve as an adjunct in the prediction of malignant transformation. **Aim:** To correlate histopathological grading and ploidy status in potentially malignant and malignant disorders of the oral mucosa. **Settings and Design:** A pilot study was done with 30 patients (10 patients with oral potentially malignant disorders predominantly leukoplakia, 10 patients with oral malignant lesions and 10 patients with normal mucosa). **Materials and Methods:** Incisional biopsy was done after isolating the biopsy site with 1% Toluidine blue staining. Two sections of the tissue were removed and sent for histopathological and Flow-cytometric analysis respectively. Histopathological diagnosis was obtained and compared with Flow-cytometric results which were graded as diploid and aneuploid. Further, the S – phase fraction, DNA index were also calculated to evaluate the severity of malignant transformation or malignancy. **Statistical Analysis:** The results were analyzed using Pearson Chi-Square Test. **Results:** There exists a significant correlation between histopathology and ploidy status in both potentially malignant and malignant group. ( $P = 0.002$ ). **Conclusion:** The data from this study has shown that DNA Ploidy analysis can be used as a valuable tool in assessing the carcinomatous progression of potentially malignant and malignant lesions.

**Key words:** Deoxyribonucleic acid index, flow cytometer, histopathology, oral cancer, potentially malignant disorder, S-phase fraction

**INTRODUCTION**

Cancer may be defined as an uncontrolled tissue growth in susceptible patients, which results from an imbalance between cell division and programmed cell death (apoptosis). The factors implicated as potential “initiators” and/or “promoters” of cancer are: tobacco, alcohol, solar radiation, ionizing radiation, occupational carcinogens, environmental pollutants, medications, infectious agents, and nutrients.<sup>[1]</sup> In India, tobacco is used in a variety of forms, which can be

broadly divided as smoked form (beedi, cigarette, cheroot, chutta, dhumti, hookli, chilum etc.) and smokeless form (paan, paan masala and snuff).<sup>[2]</sup> The age standardized incidence rate of oral cancer in India is 12.6/100,000 population. The prevalence of oral cancer in India is up to 4 times higher than in other countries.<sup>[2]</sup> Regardless of the accelerating factors, neoplasm arises clonally from transformed cells that have undergone specific genetic and epigenetic alterations in oncogenes or tumor-suppressor genes.<sup>[1]</sup> As a result of genotoxic insults from various etiological factors, the normal epithelia may undergo morphological changes with time, leading to lesions appearing as patches/plaques with white or red or mixed color.<sup>[3]</sup> Chromosomal aneuploidy at loci has been strongly implicated in the progression of leukoplakia to oral squamous cell carcinoma (OSCC).<sup>[3]</sup> Approximately two-thirds of OSCCs are diagnosed at Stage 3 or 4 disease with spread to adjacent tissues and regional lymph nodes, leading to an overall poor 5-year survival rate. Over 90% of oral and pharyngeal cancers are squamous cell carcinomas.

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Many OSCC'S are believed to develop from oral premalignant lesions. In India, about 80% of oral cancers were preceded by oral pre-cancerous lesions or conditions.<sup>[4]</sup> Leukoplakia is among the most common potentially malignant oral lesions.<sup>[5]</sup> The prevalence of leukoplakia in India varies from 0.2% to 5.2%.<sup>[6]</sup> Overall chance of malignant transformation in the world is 3.6%. Malignant transformation rates in leukoplakia varied from 0.13% to 10% in various Indian populations.<sup>[7,8]</sup> Among the subclinical types, at least 20% and up to 46% of nodular leukoplakia progress to cancer as compared to 0.5%. 1.7% of homogeneous leukoplakia.<sup>[8]</sup> The clinical form of the lesion is a poor indicator of the potential risk for such transformation. Therefore, identification of high-risk oral premalignant lesions and intervention at premalignant stages could help in reducing the mortality, morbidity and cost of treatment associated with OSCC. Histopathological grading of oral dysplastic lesions is currently the method of choice for evaluating the risk of oral cancer developing in potentially malignant leukoplakia [PML]. However, this method of grading is unreliable, owing to the lack of a validated grading system and due to the subjectivity of histopathological grading, with inter and intra-observer variability.<sup>[9]</sup> As a surrogate to individual molecular markers such as mutations in p53, loss of heterozygosity and chromosomal polysomy, measurement of gross genomic damage, in the form of aberrant deoxyribonucleic acid (DNA) content, could be a valuable method for prognostication of malignant and premalignant lesions.<sup>[10]</sup> If any correlation between DNA ploidy and the histological grading of dysplasia can be demonstrated, this might be used as an adjunctive aid for pathologists to arrive at a consensus in diagnosing the grade of epithelial dysplasia.<sup>[11]</sup> We have performed a preliminary study by taking advantage of high resolution DNA flow cytometry (FCM) to detect sub clones with DNA aneuploidy and then correlate the results with histopathological diagnosis.

## MATERIALS AND METHODS

A pilot case-control study was done and permission to conduct the study was obtained from Institutional Ethical Committee before the commencement of the study. Study proforma was prepared and only those patients, who gave a signed informed consent on an institutionally approved document, participated in the study. The study included a total of 30 patients (24 males and 6 females) belonging to age group of 42-62 years. Patients with clinically diagnosed potentially malignant lesions, clinically diagnosed cancerous lesions were included in this study. Patients with bleeding disorders, long-term anticoagulant therapy and history of severe systemic diseases such as cardio-vascular, hepatic, immunologic, renal, hematological, or other organ disorders were excluded.

The selected patients were divided into three groups based on clinical diagnosis as:

Group I: 10 patients with potentially malignant lesions

Group II: 10 patients with oral cancer

Group III: 10 patients with normal mucosa.

After obtaining complete blood examination report, toluidine blue (1%) staining of the lesion was done and areas of maximum staining were chosen for biopsy in order to specifically limit the incision to areas of dysplasia.<sup>[4]</sup> Two sections of tissue were removed and a section of tissue was transferred to a bottle containing 10% formalin and sent for histopathological examination. Grading of dysplasia was done by World Health Organization grading of dysplasia 2005 as mild dysplasia, moderate dysplasia and severe dysplasia. The other section of tissue was transferred to the FCM lab in a thermocool container with polar ice bag at -4°C and was processed immediately.

## Processing of tissue samples for DNA FCM analysis and sorting

The amount of DNA was measured by BD FACS Calibur (BD Biosciences, Sanjose, California). FCM was performed on nuclei obtained from the specimen by the modified technique of Hood *et al.*<sup>[12]</sup> Briefly, the fresh specimens were minced to approximately 1-mm slices with scissors and fixed overnight in 80% ethanol. The fixed specimens were minced again, filtered through an iron mesh with 10 µm pores and washed twice with phosphate-buffered saline. Suspensions of single nuclei were prepared by using pepsin (Sigma Aldrich Chemicals, St. Louis). Washed cells were incubated in the pepsin solution at 37°C for 20 min. Staining for FCM measurement was carried out by exposure to 50 µg/ml propidium iodide (PI) (Sigma Aldrich Chemicals, St. Louis) solution containing 0.1% RNase (Boehringer, Mannheim, Germany). The equipment was calibrated using PI labeled chicken red blood cell. Normal human peripheral blood lymphocytes were used to identify the normal diploid peak that served as reference peak for subsequent assays. The 488-nm line of a 200-mW argon laser was used for excitation. As many as 10,000 events were collected for each sample and the acquisition program was set to collect DNA fluorescence signal as the total area versus peak (signal height) in order to eliminate doublets and aggregates. Mean co-efficient of variation of the measurements were within the acceptable range (3-8%) in all the samples. Cell cycle analysis was done using Guava cell cycle pro software.

## RESULTS

Group I (PML) consisted of 10 patients (8 males and 2 females) within the age group of 42-57 years. Group II (cancer) consisted of 10 patients (8 males and 2 females) within the age group of 51-62 years. Group III (control) consisted of 10 patients (8 males and 2 females) with normal mucosa that was randomly selected. Subsequently, the tissue samples were processed simultaneously for both histopathology and FCM analysis. In order to eliminate the observer bias, the samples were double-blinded and assessed by two oral pathologists.

Using BD FACS Calibur the following parameters were assessed in each sample: Percentage - S-phase fraction (SPF) and DNA index (DI) values.

- Synthesis phase (S-phase) is the phase in cell cycle wherein, replicating cells are synthesizing DNA and progressively the cells will have diploid number of chromosomes in G2M phase. This is usually expressed as a percentage- SPF.<sup>[13]</sup> Malignant cells will have longer than normal SPF due to abnormal, excessive and uncoordinated increase in the quantity of DNA in the cells in S-phase. Normal ranges of SPF are usually determined using a control sample. In addition, SPF values can also be used as an independent prognostic marker during and after the treatment phase of oral cancer<sup>[14]</sup>
- DI is a value given to express the amount of DNA content relative to the normal and is calculated by the following equalities [Figure 1]<sup>[15]</sup>

The Clonal population is categorized depending on the values of DI as follows:

- A diploid population is given a DI of 1.00 by definition
- DNA aneuploid tumors are those tumors having more than 2N number of chromosomes
- Tetraploid tumors have 4N number of chromosomes
- Hypertetraploid tumors are those having more than 4N number of chromosomes
- Multiple aneuploid or DNA multiploid tumors may have more than one abnormal population.

The results of our study were based on the DI values as given by the DNA cytometry consensus committee in 1992 [Table 1].<sup>[15]</sup>

**Analysis of results**

The results of our study is tabulated in Table 2. *P* < 0.05 was considered as statistically significant. The mean SPF values and the DI of the three groups (Group I, II and III) were assessed using FCM and the values were 7.56, 7.80 and 6.68 and 1.19, 1.41, 1.00 respectively.

In our study, good correlation was obtained between histopathological diagnosis and ploidy status in both Group I (*P* = 0.002) and Group II (*P* = 0.002), when the results were analyzed using Pearson Chi-square test [Tables 3 and 4].

Between the Group I (PML) Group II (cancer) and Group III (control) patients, several comparisons were made based on age, habits, duration of habits and each of the correlations were statistically analyzed using analysis of variance test and *post-hoc* test (Tamhene’s) and the results were tabulated.

$DI = \frac{\text{Channel number of aneuploid Peak}}{\text{Channel number of diploid Peak}}$
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**Figure 1:** Deoxyribonucleic acid index calculation

In assessing the relationship between age and the mean SPF and DI values between the three groups, it showed that there is a significant difference between mean SPF values between PML group and control group (*P* = 0.002) and also between cancer and control group (*P* = 0.000) and also significant difference in the mean DI values between cancer and control group (*P* = 0.004).

In assessing the relationship between the habits (paan, smoking, and both) and the mean SPF and DI values of PML group, there is a significant difference in the mean values of SPF between patients who are either paan chewers (or) smokers and to those who use both forms of smoke and smokeless tobacco in PML group (*P* = 0.008).

The relationship between histopathology and the mean SPF and DI values, was found to be significant between mild and severe dysplasia cases and no significant difference exists between moderate and mild cases (*P* = 0.000 and *P* = 0.002) [Table 3].

There is no significant difference in the mean SPF values and DI between the histopathological diagnoses in cancer group. The mean SPF and DI values were calculated for each ploidy status in both Group I and Group II [Figures 2 and 3] and there is a significant difference seen in mean SPF values and DI values between aneuploid and other ploidy status in PML group. Significant difference is seen in mean SPF values between aneuploid and other ploidy status in cancer group, but interestingly, there is no significant difference in mean DI values between aneuploid and other ploidy status in cancer group.

**Table 1: DNA index values**

Diploid	1.0
Aneuploid	1.1–1.10
Hyperploid	1.10–1.90
Tetraploid	1.90–2.20
Hypertetraploid	>2.20

**Table 2: Results of the study**

Study Groups	Ploidy status			
	Diploid	Hyperdiploid	Aneuploid	Tetraploid
Group I(PML) <i>n</i> =10				
Mild Dysplasia ( <i>n</i> =4)	4		-	-
Moderate Dysplasia ( <i>n</i> =3)	2	-	1	
Severe Dysplasia ( <i>n</i> =3)	-	3		
GroupII (Cancer) <i>n</i> =10				
WDSCC( <i>n</i> =5)	-	4	1	
MDSCC( <i>n</i> =3)	-	1	2	1
PDSCC( <i>n</i> =1)	-	-	-	1

**Table 3: Correlation between histopathology and ploidy in PML group**

Histopathology	Histopathology*ploidy status cross tabulation: PML group				Total
	Ploidy status				
	Aneuploid	Diploid	Hyperdiploid	Tetraploid	
Mild					
Count		4			4
Row%		100.00			100.00
Col%		25.00			13.33
Moderate					
Count	1	2			3
Row%	33.33	66.67			100.00
Col%	25.00	12.50			10.00
Severe					
Count			3		3
Row%			100.00		100.00
Col%			37.50		10.00
<b>Chi-square Test</b>	<b>Value</b>		<b>df</b>		<b>Asymp. significant (2-sided)</b>
Pearson Chi-square	53.188		6		0.002

*P*<0.05 is significant, PML: Potentially Malignant Leukoplakia

**Table 4: Correlation between histopathology and ploidy status in cancer group**

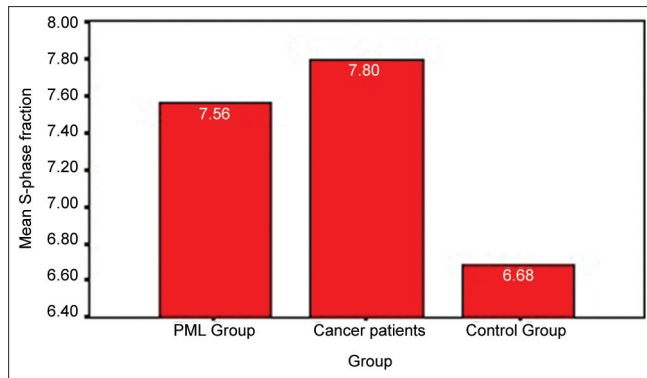
Histopathology	Histopathology*ploidy status cross tabulation: Cancer group				Total
	Ploidy status				
	Aneuploid	Diploid	Hyperdiploid	Tetraploid	
PDSCC					
Count				1	1
Row%				100.00	100.00
Col%				50.00	3.33
MDSCC					
Count	2		1	1	4
Row%	50.00		25.00	25.00	100.00
Col%	50.00		12.50	50.00	13.33
WDSCC					
Count	1		4		5
Row%	20.00		80.00		100.00
Col%	25.00		50.00		16.67
Total					
Count	3		5	2	10
<b>Chi-square test</b>	<b>Value</b>		<b>df</b>		<b>Significant (2-sided)</b>
Pearson Chi-square	53.188		6		0.002

*P*<0.05 is significant, PDSCC: Poorly differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, WDSCC: Well-differentiated squamous cell carcinoma

## DISCUSSION

The concept of a two-step process of cancer development in the oral mucosa, i.e., the initial presence of a precursor (potentially malignant) lesion subsequently developing into cancer, is well-established.<sup>[16]</sup> Oral leukoplakia is the best known precursor lesion. It is the most common potentially malignant lesion of the oral cavity.<sup>[16]</sup> It has been proved that certain clinical sub-types of leukoplakia such as the speckled leukoplakia or non-homogeneous leukoplakia are at a higher risk for malignant transformation than others.<sup>[16]</sup>

The risk of malignant transformation of leukoplakia with dysplasia has been reported to be as high as 43%.<sup>[16]</sup> Hence, the need for regular monitoring of these common potentially malignant lesions is of paramount importance. Increased malignant potential may be associated with certain clinical characteristics such as lesion type, size, site and dysplasia.<sup>[16]</sup> An early diagnosis of carcinomatous transformation of a potentially malignant lesion can improve the prognosis. The evidence that oral leukoplakia are potentially malignant is mainly derived from various follow-up studies which show that between <1 and 18% of oral pre-malignant lesions develop into oral cancer.<sup>[17]</sup>



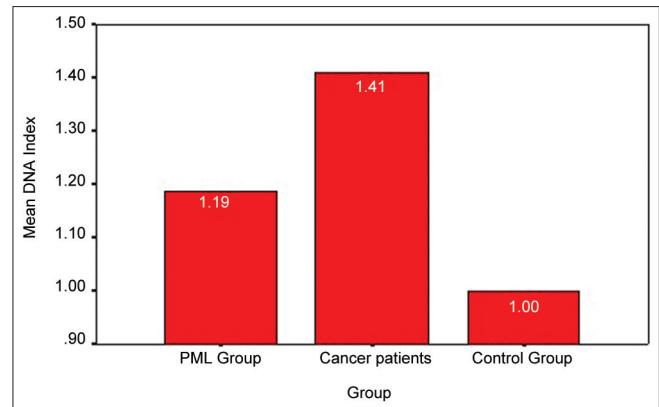
**Figure 2:** Mean S-phase fraction value

Histopathological assessment of leukoplakia and identifying the presence of epithelial dysplasia is the Gold standard for the prediction of carcinomatous transformation of potential malignant lesions.<sup>[18,19]</sup> Three major problems, however, are attached to the importance of epithelial dysplasia in predicting malignant development: (1) The diagnosis is essentially subjective and cannot be standardized; (2) it seems that not all lesions exhibiting dysplasia will eventually become malignant and some may even regress, and (3) carcinoma can develop from lesions in which epithelial dysplasia was not diagnosed in previous biopsies.<sup>[20-22]</sup> There is, therefore, a substantial need to improve the histological assessment of epithelial dysplasia. It may be necessary to develop other methods for predicting the malignant potential of potentially malignant lesions. As a consequence of these problems, numerous attempts have been made to relate biological characteristics to the malignant potential of leukoplakia.

Many authors believe that ploidy analysis is important in prediction of progression of carcinogenesis.<sup>[23]</sup> It has been shown that in some Potentially Malignant Disorders (PMDs) the epithelial cells exhibit changes from a diploid pattern to an aneuploid pattern preceding malignant transformation, which means that DNA alterations take place before transformation is apparent<sup>[24]</sup> and is seen as a surrogate marker of gross genetic damage.<sup>[25]</sup>

During recent years, FCM has established itself as a useful, quick novel method to determine efficiently, the relative nuclear DNA content and ploidy status. It has a potential to be used as an independent diagnostic tool in clinical pathology as well as a predictor of clinical behavior.<sup>[26]</sup> DNA ploidy analysis may also aid in compensating for intra- and inter-observer variability in the grading of epithelial dysplasia and may potentially aid in directing the management of the lesion, and probably suggest more aggressive treatment.<sup>[27]</sup>

The advantages of FCM over other molecular biological techniques are as follows: FCM helps in analyzing more cells (10000-50000 cells) than by conventional cytometry leading to statistically significant results. FCM helps in producing multiparametric measurements on several different



**Figure 3:** Mean deoxyribonucleic acid value

characteristics of each cell such as, size, protein content, DNA content, lipid content, antigenic properties, enzyme activity etc. Since FCM uses very sensitive electronic detectors to measure the intensity of scattered light or fluorescence at a given wavelength, different intensities of light scatter/fluorescence can be distinguished and therefore by calibrating the instrument with samples of known size or fluorescent intensity, it is possible to obtain quantitative measurements of sample heterogeneity.<sup>[28]</sup>

In oral cancer and pre-cancer, FCM provides “fingerprint-type” information of an individual patient’s tumor which, if proven prognostically relevant, may provide the basis for treatment selection in the future. Among 10 samples of potentially malignant lesions, eight lesions were of erythroleukoplakic type and among them three were with moderate dysplasia and three were with severe dysplasia. DNA ploidy of the same sample using high resolution FCM revealed that all the four cases in mild dysplasia category were diploid, whereas among the three moderate cases, two were diploid and one was aneuploid. All the severe dysplasia cases were hyperdiploid in the potentially malignant group.

All moderate and severe dysplastic lesions had an elevated SPF and DI ( $P = 0.001$ ). The result of our study is in correlation with a study on 27 leukoplakia specimens by Saito *et al.*, in 1995.<sup>[29]</sup>

All Severe dysplastic lesions had an elevated SPF and DI values when compared with lesions with moderate dysplasia, the fact which is in correlation with the study by Khanna *et al.*, in 2010<sup>[30]</sup> the more severe the carcinomatous transformation in a potentially malignant lesion, the higher will be the proliferating cells in S-phase and thereby the DI will also be  $>1.1$ .

Among the 10 samples of clinically diagnosed oral cancer five were histologically diagnosed as well-differentiated squamous cell carcinoma, four were moderately differentiated squamous cell carcinoma and one was poorly differentiated squamous cell carcinoma. DNA ploidy analysis revealed that among five

well-differentiated squamous cell carcinoma cases, four were hyperdiploid and one was aneuploid, whereas among four moderately differentiated squamous cell carcinoma cases, one was hyperdiploid, two were aneuploid and one was tetraploid and a one poorly differentiated squamous cell carcinoma case was tetraploid.

The increase of aneuploid lesions with age is associated with generalized malnutrition, generalized deficiency states and increased occurrence of immunologically mediated chronic systemic illness leading to decreased host immune response to carcinogens.<sup>[30,31]</sup>

Interestingly we observed that the synergistic effects of both smoke and smokeless form of tobacco, which is a common practice in India, results in an increased percentage of cells in S-phase, which is regarded as an independent prognostic marker of progression of potentially malignant lesions in contrast to independent forms of consumption ( $P = 0.008$ ).

The result of our study is consistent with the findings of Saito *et al.*, in 1995<sup>[29]</sup> and Grassel-Pietrusky *et al.*,<sup>[23]</sup> in 1982.

## CONCLUSION

In this study, DNA ploidy correlated significantly with the degree of epithelial dysplasia in both potentially malignant lesions and malignant lesions with 100% sensitivity in severe dysplasia cases and malignant lesions. Hence, in our study it is interpreted that, DNA ploidy analysis can be used as a valuable tool in assessing the carcinomatous progression of potentially malignant lesions, since, there are chances of inter observer variability in histopathological diagnosis of potentially malignant Lesion.

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