Original Article

Correlation Between Histological Grading and Ploidy Status in Oral Leukoplakia, Oral Submucous Fibrosis, and Oral Squamous Cell Carcinoma: A Flow Cytometric Analysis

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ABSTRACT

**Background:** Methods to analyze progression of carcinogenesis and stage of cancer are vital nowadays due to the high prevalence of these lesions. DNA ploidy analysis is one such important method in early diagnosis and improving prognosis. **Aims and Objectives:** The aim of this study was to correlate histopathological grading and DNA ploidy in oral leukoplakia, oral submucous fibrosis (OSMF), and oral squamous cell carcinoma (OSCC) cases. **Materials and Methods:** Our study included 80 subjects, grouped into 4 groups of 20 each of OSCC, leukoplakia, OSMF, and healthy individuals. Histopathological grading was carried out in study cases, DNA ploidy was estimated using flow cytometry, and both the findings were correlated. **Results:** Among the 20 cases of leukoplakia group, 6 cases showed aneuploidy and 14 showed diploidy. In the 20 cases of OSMF group, 2 cases showed aneuploidy and 18 showed diploidy, and in the 20 cases of OSCC group, 10 showed aneuploidy and 10 showed diploidy. Most of the aneuploidy cases showed severe dysplasia. **Conclusion:** Analysis of DNA ploidy status can serve as a diagnostic tool for early detection of malignancies owing to the subjective nature of traditional histopathological grading.

**KEYWORDS:** Aneuploidy, diploid, DNA ploidy, flow cytometry, leukoplakia, oral cancer

INTRODUCTION

Oral cancer accounts for about 270,000 new cases per annum worldwide, of which majority are oral squamous cell carcinomas (OSCCs).[1] The morbidity and mortality associated with OSCC can be reduced by early diagnosis and treatment. OSCC is usually but not always preceded by oral potentially malignant disorders (OPMDs) such as oral leukoplakia and oral submucous fibrosis (OSMF). The risk and prognosis of these lesions is currently based on the histopathological grading.[2,3]

As there is deficiency of authenticated grading system and the histopathological grading varies a lot between the intra- and interobserver individuals, the reliability on grading system is questionable.[2,3]

The exact malignant potentiality of OPMDs is variable in different studies, usually in a range of 0.5%–3.2%, with oral leukoplakia showing higher malignant potentiality than OSMF. The valuable prognostic factors in both OPMD and OSCC are age, site, and size of the tumor; status of lymph nodes; histological grade; and tumor ploidy.[4,5]

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DNA ploidy is the number of chromosomes in a cell. For predicting the malignant transformation of an OPMD, many tools are there, but in the latest advancement technique, estimation of DNA ploidy status has gain lot of importance. Abnormalities in nuclear DNA content and DNA aneuploidy indicates chromosomal changes and is often a critical step in carcinogenesis. Huang stated that assessment of gross genomic aberration (DNA aneuploidy) holds the answer to develop diagnostic tools and grading of OPMD. [3-5]

Flow cytometry (FCM) is a fairly instant method and a greater number of cells (10,000–30,000) may be measured in 3–15 min for analyzing DNA content of solid tumors. Vijayavel and Aswath [1] showed that DNA ploidy assessment may play role as a worthwhile adjunct in evaluation OPMD to carcinomatous progression and also staging of neoplastic lesions. Earlier studies showed that DNA index reveals a good correlation with the histological features of OSCC.

Earlier studies focused on correlating the histopathological grading and DNA ploidy status in OSCC by means of image cytometrical analysis, but very few studies were carried on correlation of the histopathological grading and DNA ploidy status in OPMD and OSCC using flow cytometric analysis. Hence, we carried this study to correlate the histopathological grading and DNA ploidy status in leukoplakia, OSMF, and OSCC using FCM.

MATERIALS AND METHODS

Our longitudinal cross-sectional study included 80 subjects, obtained from the archives of Department of Oral and Maxillofacial Pathology of our college. The sample was categorized into 4 groups of 20 samples in each group. Group I included 20 cases of OSCC, group II included leukoplakia cases, group III included OSMF cases, and group IV (control group) included 20 healthy individuals. An institutional ethical committee board clearance was obtained and an informed consent was obtained from both study and control groups.

The parameters recorded were age, gender, site, and histopathological diagnosis. Two senior oral pathologists graded the sample, based on 2005 World Health Organization (WHO) classification of epithelial dysplasia for OSCC and oral leukoplakia, and grading of epithelial dysplasia of OSMF was according to the classification given by Utsunomiya et al. [6]

Flow cytometry

In this histological technique, preparation of sections was carried out according to the Hedley method. From the paraffin wax block, 4–6 sections of 40 μm were sectioned out, enrolled in 50 μm mesh of nylon, placed in a cassette, manually dewaxed, and hydrated in distilled water. Later the wrapped sections were digested in subtilisin Carlsberg solution at a temperature of 37°C in a centrifuge tube for 120 min and then agitated for 20 min. After this the Carlsberg solution was subjected to filtration method with the help of 50 μm mesh of nylon, and DAPI (4′,6-diamidino-2-phenylindole) staining solution was summated with the help of PAS II Flow Cytometry tool (Partec, Munster, Germany); Equipped with a 100 W mercury lamp [Figure 1] and FCM procedure was carried out. DNA histograms of at least 1000 cells were plotted. For the referencing purpose, the diploid cell population was considered for the marking of the aneuploid cells. The sampling of the tissues was performed and divided as diploid or aneuploid. Lesion was classified as diploid if there was only one peak (2c) at the G₀ or G₁ phase. A lesion was defined as aneuploid if there were aneuploid peaks [Figures 2–5]. The main result of the study was subjected to statistical correlation of DNA ploidy with histopathological grading of OSCC, leukoplakia, and OSMF.

Statistical analysis

Data categorized were analyzed using the Pearson’s chi-square test. Results obtained were conceived as significant at a P value of <0.05, and confidence intervals and relative risks were reported. For the statistics of the study and its analysis, services of an eminent clinical epidemiologist were taken.

RESULTS

Among the leukoplakia group, eight cases were mild dysplasia of which one showed aneuploidy and seven were diploid. In seven cases of moderate dysplasia, two showed aneuploidy and five showed diploidy. In five cases of severe dysplasia, three cases were aneuploid.
and two were diploid. Analysis revealed that there was increase in the frequency of aneuploidy as the sample moved from the mild to severe degrees of dysplasia. This association of increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$).

In the OSMF group, nine cases were early stage of OSMF, of which all the nine cases were diploid. Among seven cases of intermediate stage of OSMF, one showed aneuploidy and six showed diploidy. In four cases of advanced stage of OSMF, one case showed aneuploidy. Analysis revealed that there was increase in the frequency of aneuploidy as the sample moved from the early to advanced stages of OSMF. This association of increase in aneuploidy along with severity of stage of OSMF was, however, found “not” significant statistically ($P > 0.05$).

In the OSCC group, 10 cases were well differentiated OSCC, of which four showed aneuploidy and six were diploid. Among the six cases of moderately differentiated OSCC, three showed aneuploidy and three showed diploidy. Three of the four poorly differentiated OSCC samples showed severe aneuploidy. Analysis revealed that there was increase in the frequency of aneuploidy as the sample moved from the mild to severe degrees of dysplasia. This association of increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$).

**Histological grading of sections and DNA ploidy**

Pearson’s chi-square was computed to evaluate the association between the grade of dysplasia and ploidy status. Analysis revealed that there was increase in the frequency of aneuploidy as the sample moved from the mild to severe degrees of dysplasia. This association of
increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$), indicating that degree of dysplasia and aneuploidy were associated positively [Tables 1 and 2].

Analysis revealed that there was increase in the frequency of aneuploidy as the sample moved from the mild to severe degrees of dysplasia. This association of increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$), indicating that degree of dysplasia and aneuploidy in OSCC were associated positively [Table 3].

Pearson’s chi-square was computed to assess the association between the condition and ploidy status. Analysis revealed that the least frequency of aneuploidy was observed among OSMF group, and OSCC exhibited the highest frequency of aneuploidy. This association of increase in aneuploidy along with severity of condition was found to be significant statistically ($P < 0.05$), indicating that worsening of condition from control to OSCC had a positive relationship with aneuploidy status [Table 4].

**DISCUSSION**

Diploid oral dysplasia is characterized by a prominent peak representing the cells in the $G_0/G_1$ phase of the cell cycle. The much smaller peak with exactly double the DNA content of the first one contains cells in the $G_2$ phase and mitosis. The counts plotted between both peaks reflect cells in S-phase characterized by intermediate DNA values. The width of the $G_0/G_1$ peak is expressed as the coefficient of variation.$^{[3-9]}$

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*Figure 4: Moderately differentiated epithelium of oral squamous cell carcinoma and diploidy and aneuploidy demonstrated by peaks*

*Figure 5: Poorly differentiated epithelium of oral squamous cell carcinoma and diploidy and aneuploidy demonstrated by peaks*
Malignant transformation potential of OPMDs necessitates regular monitoring. The factors that influence malignant potentiality are lesion type, size, site, and so on. Epithelial dysplasia of the squamous epithelium of the mucosa develops due to variations in cellular proliferation and maturation. It has been proved that oral epithelial dysplasia is related with an increase in risk for malignant progression to OSCC, with the more increase in severity of the dysplasia, the more likelihood of malignant progression.[7]

| Table 1: Analysis of overall ploidy distribution among groups |
|-------------------------------------------------------------|
| **Condition** | **Leukoplakia** | **OSMF** | **OSCC** | **Control** |
| Frequency (%) | Frequency (%) | Frequency (%) | Frequency (%) | Frequency (%) |
| Aneuploid | 6 | 2 | 10 | 0 | 0 |
| Diploid | 14 | 18 | 10 | 20 | 100.0 |
| Total | 20 | 20 | 20 | 20 | 100.0 |

| Table 2: Analysis of association of the degree of dysplasia with ploidy status in leukoplakia |
|--------------------------------------------------------------------------------------------|
| **Dysplasia and ploidy cross-tabulation** |
| **Ploidy** | Aneuploid | Diploid | Total | **Chi-square** | |
| **Dysplasia** | | | | |
| Mild dysplasia | 1 | 7 | 8 | $\chi^2 = 3.316$ | $P = 0.019$ |
| Moderate dysplasia | 2 | 5 | 7 | |
| Severe dysplasia | 3 | 2 | 5 | |
| Total | 6 | 14 | 20 | |

| Table 3: Analysis of association of the degree of dysplasia with ploidy status in OSCC |
|---------------------------------------------------------------------------------------|
| **Dysplasia and ploidy cross-tabulation** |
| **Ploidy** | Aneuploid | Diploid | Total | **Chi-square** | |
| **Dysplasia** | | | | |
| Well differentiated | 4 | 6 | 10 | $\chi^2 = 1.886$ | $P = 0.028$ |
| Moderately differentiated | 3 | 3 | 6 | |
| Poorly differentiated | 3 | 1 | 4 | |
| Total | 9 | 11 | 20 | |

| Table 4: Analysis of association of the condition with ploidy status |
|-------------------------------------------------------------------|
| **Condition and ploidy cross-tabulation** |
| **Ploidy** | Aneuploid | Diploid | Total | **Chi-square** | |
| **Condition** | | | | |
| Leukoplakia | 6 | 14 | 20 | $\chi^2 = 14.566$ | $P = 0.002$ |
| OSMF | 2 | 18 | 20 | |
| OSCC | 10 | 10 | 20 | |
| Control | 0 | 20 | 20 | |
| Total | 17 | 63 | 80 | |

| Frequency (%) | 30.0% | 70.0% | 100.0% | |
| 10.0% | 90.0% | 100.0% | |
| 50.0% | 50.0% | 100.0% | |
| 0.0% | 100.0% | 100.0% |
WHO classification of histological grading of epithelial dysplasia (2005) laid down certain criteria: architectural and cytological. Architecture criteria included irregular epithelial stratification, loss of basal cell polarity, drop-shaped rete ridges, abnormal superficial mitoses, increased number of mitotic figures, premature keratinization in individual cells (dyskeratosis), and keratin pearls within rete ridges. Whereas, cytology criteria include abnormal variation in nuclear size (anisonucleosis), nuclear shape (nuclear pleomorphism), cell size (anisocytosis), and cell shape (cellular pleomorphism); increased nuclear size, number, and size of nucleoli and nuclear cytoplasmic ratio; atypical mitotic figures; and hyperchromatism.[5,6]

Even though histopathological assessment of OPMD in identifying the presence of epithelial dysplasia is considered as the gold standard for predicting malignant transformation of OPMDs,[7] the limitations include the subjective nature of diagnosis, not all dysplastic lesions transforming to OSCC, and all carcinomas do not develop from OPMDS. Hence, there is a need to develop other methods for predicting the malignant potential of OPMDs. One such method is ploidy analysis.[5] Studies showed that in some OPMDs, the epithelial cells exhibit changes to an aneuploid pattern from a diploid pattern prior to malignant transformation, suggesting occurrence of alterations in DNA before transformation is apparent and hence act as a surrogate marker of gross genetic damage.[7,8]

During cell division if fragments of chromosomes are missing, leading to unbalanced DNA content, it is known as aneuploidy. Aneuploidy is usually noticed in human cancers, and studies reported that mutations in genes that control chromosome segregation during mitosis and abnormalities in centrosome play a role in cause of genetic instability found in cancer cells. As aneuploidy indicates genetic instability, few authors assumed that aneuploidy may predict the malignant potential of OPMDs.[5,6]

FCM has advantages over other molecular biological techniques such as analyzing more cells (10,000–50,000 cells); multiparametric measurements of each cell such as size, protein content, DNA content, lipid content, antigenic properties, and enzyme activity; and quantitative measurements of sample heterogeneity.[9]

High-resolution DNA FCM was performed in OSCC cases, and studies showed that about 80% of OSCC cases were found to be aneuploid and 20% diploid. FCM measures particle’s relative size, relative granularity, and relative fluorescence intensity using optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. FCM DNA ploidy analysis may aid in compensating for intra- and interobserver variability in epithelial dysplasia grading and also may aid in the management of these lesions.[9-11]

The aim of our study was to evaluate the ploidy status in oral leukoplakia, OSMF, and OSCC with high-resolution FCM. An attempt was made to correlate the results with histopathological grading of oral leukoplakia, OSMF, and OSCC. Analysis revealed that the least frequency of aneuploidy was observed among OSMF group and OSCC exhibited the highest frequency of aneuploidy. This association of increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$), indicating that worsening of condition from control to OSCC had a positive relationship with aneuploidy status.

Among leukoplakia group, there was an association of increase in aneuploidy along with severity of dysplasia ($P < 0.05$), indicating that degree of dysplasia and aneuploidy were associated positively. In OSMF group, the association of increase in aneuploidy along with severity of stage of OSMF was not significant statistically ($P > 0.05$), indicating no particular statistical relationship between progression of OSMF and ploidy status. In OSCC group, an association of increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$), indicating that degree of dysplasia and aneuploidy in OSCC were associated positively.

We found an increase in the frequency of aneuploidy from mild to severe dysplasia cases. Aneuploidy was shown by 10% of OSMF, 30% of leukoplakia, and 50% of OSCC, and 90% of OSMF, 70% of leukoplakia, and 50% of OSCC showed diploidy, which shows carcinoma and leukoplakia show more number of aneuploidy than OSMF, and also the 60% of severe dysplasia cases of the leukoplakia show aneuploidy and are more prone to malignancy.

The correlation of leukoplakia, OSMF, and OSCC in mild dysplasia cases showed that 12.5% of mild cases of leukoplakia showed aneuploidy and 40% of well-differentiated cases show aneuploidy. Aneuploidy was not seen in early stages of OSMF. This association of increase in aneuploidy was found to be significant statistically ($P < 0.05$), indicating that aneuploidy was more significantly observed in mild dysplasia samples of OSCC as compared to other groups.

The correlation of leukoplakia, OSMF, and OSCC in moderate dysplasia cases showed that 28.6% of moderate cases of leukoplakia show aneuploidy, 14.3%
of intermediate stage shows aneuploidy, and 50% of moderately differentiated cases show aneuploidy. This association of increase in aneuploidy was, however, not found significant statistically ($P > 0.05$), indicating that aneuploidy was not significantly distributed in between the moderate dysplasia samples of these conditions.

The correlation of leukoplakia, OSMF, and OSCC in severe dysplasia cases shows that 60% of severe cases of leukoplakia show aneuploidy, 25 of advanced stage shows aneuploidy, and 75% of poorly differentiated cases show aneuploidy. This association of increase in aneuploidy with condition was, however, not found significant statistically ($P > 0.05$), indicating that aneuploidy was not significantly distributed in between the moderate dysplasia samples of these conditions.

Seoane et al. performed flow cytometric analysis on leukoplakia cases and found that there were three patterns of aneuploid (9.7%) and 20 patterns of diploid (90.3%), showing any statistically significant difference between ploidy and epithelial dysplasia the presence or absence. They came to a conclusion that DNA analysis was not of diagnostic value for the differentiation of dysplastic leukoplakias from the leukoplakia not showing any statistically significant difference ($P > 0.05$), indicating that aneuploidy was not significantly distributed in between the moderate dysplasia samples of these conditions.

Bradley et al. found an association with increased aneuploidy in leukoplakia cases with severe dysplasias and suggested that terminal degrees of epithelial dysplasia were noticed with help of FCM.

Seoane et al. performed Epics Profile II flow cytometric analysis of OSCC samples according to Hedley’s method and observed DNA aneuploidy in 15 tumors (41%). There was a strong correlation between histological malignancy and DNA index and concluded that DNA index can be used as a complement to histological grading analysis diminishing the subjective nature of grading, even though ploidy status was not statistically associated with the differentiation of tumors.

Kahn et al. observed populations of aneuploid cell in 6 of the 24 dysplastic leukoplakia lesions, and arrived at a conclusion that DNA analysis did not demonstrate as an effective tool in segregating varied grades of dysplasias.

Cooke et al. concluded that ploidy status was related neither to patient factors (age and sex) nor to tumor factors (T-stage or nodal status) and also ploidy did not affect overall survival. Feichter et al. in their study on S-phase fractions and DNA ploidy of oropharyngeal squamous carcinomas found aneuploid DNA distribution of the nuclei in the G0 phase (76.4%) and the remaining 13 cases (23.6%) were DNA-diploid and concluded that grading of the tumors can be improved by measuring the S-phase fractions by FCM.

Hemmer and Kreidler found that the portion of aneuploid tumors increased with decreasing degree of histological differentiation from $G_1$ (38.1%) to $G_2$ (76.6%) and $G_3$ (92.0%). They stated that aneuploidy obviously represents a marker of malignancy progression in oral carcinoma, and ploidy status by flow cytometric analysis may act as an additional factor to improve the definition of risk groups in OSCC.

The results of this study are consistent with the study conducted by Abou-Elhamd et al. who reported the existence of a multiplicity of genetic changes in head and neck squamous cell carcinoma (SCC). They concluded that flow cytometric analysis allows better understanding of the molecular basis of head and neck SCC and also enables the development of new tools for the early identification of the oral white patches that will develop into SCC through close and regular follow-up and appropriate treatment.

van Zyl et al. conducted a flow cytometric analysis on 110 cases of oral leukoplakia and found increased number of aneuploidy in the severe dysplasia and less aneuploidy in mild dysplasia. Vijayavel and Aswath reported that DNA ploidy correlated significantly with the degree of epithelial dysplasia in both OPMD and OSCC.

**Limitations of the study**

1. Lack of follow-up of the cases
2. Less sample size.

The results of this study are consistent with the previous studies with increase in the frequency of aneuploidy as the sample moved from the mild to severe degrees of dysplasia. This association of increase in aneuploidy along with severity of dysplasia was found to be significant statistically ($P < 0.05$), indicating that degree of dysplasia and aneuploidy were associated positively. To the best of our knowledge, ours is the first study that correlates DNA ploidy status by FCM in OPMDs and OSCC with histopathological grading.

**Conclusion**

This study was carried to confirm the correlation of DNA ploidy status with histopathological grading of OPMDs and OSCC. There was an increase in aneuploidy status with severity of dysplasia. We suggest that analysis of DNA ploidy by FCM can serve as a diagnostic tool for early detection of malignancies.
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Conflicts of interest
There are no conflicts of interest.

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