

# Quantitative DNA Assay (Ploidy), Koilocytotic Changes and AgNOR Expression for Risk Estimation in Oral Leukoplakia

Asoke Roy<sup>1,\*</sup>, Satyendra Prasad Bhatnagar<sup>2</sup>, Malay Chatterjee<sup>3</sup>, Goutam Mandal<sup>4</sup>, Dipanwita Ghosh<sup>1</sup>

<sup>1</sup>Department of Pathology and Cancer Screening, Chittaranjan National Cancer Institute, Kolkata, India

<sup>2</sup>Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi, India

<sup>3</sup>Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

<sup>4</sup>Department of Pathology, Hospital Wing, Chittaranjan National Cancer Institute, Kolkata, India

\*Corresponding author: [asokeroy\\_cnci@yahoo.co.in](mailto:asokeroy_cnci@yahoo.co.in)

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**Abstract** In this study, quantitative DNA estimation (DNA ploidy), AgNORs and koilocytotic changes were assessed in oral leukoplakia for risk categorization. *Materials and methods:* 50 cases of oral leukoplakias along with adequate controls were selected for the study. Quantitative DNA analysis was done by FACS scan. AgNORs were studied by silver staining method, koilocytotic changes and histopathology were studied in HE stained tissue sections. *Results:* Out of 50 cases, 45 cases (90%) were linked to traditional addiction of various forms and 5 cases (10%) were non-addicts. Histologically, there were 52% non dysplastic and 48% dysplastic cases. Out of these 50 cases, there were diploid, tetraploid and aneuploid population including hypodiploid DNA content. The DNA index (DI), among these cases, were ranged from 0.22 (hypodiploid) to 2.12 (tetraploid) (co-efficient of variation ranged from 0.39 to 18.43). The mean AgNOR count for smokers were  $3.5 \pm 2.058$  whereas the mean AgNOR count for the betel quid group was  $2.85 \pm 1.47$  and for khaini- gutka group was  $3.67 \pm 2.21$ . Less than 50% cases showed positivity for koilocytotic changes (20 out of 50 cases). *Conclusion:* The quantitative DNA (ploidy) study along with AgNORs can be considered dually as a prospective prognostic marker for cancer risk prediction in cases of oral leukoplakia. But koilocytotic changes for the HPV association as a marker for risk prediction in cases of oral leukoplakia is controversial and needs to be investigated on a large scale basis and follow up for a longer duration.

**Keywords:** koilocytosis, AgNORs, DNA ploidy, diploid, tetraploid, aneuploid, hypodiploid, dysplasia, FACS, HPV

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## 1. Introduction

Head and neck malignancies have been considered as one of the most common cancer worldwide [1]. According to Choudhury et al. [2] in northern India the head and neck cancers account for about 30-40% of all cancer types in men [2]. Oral squamous cell carcinoma (OSCC) is the most common human cancer that accounts for at least 90% of all oral malignancies. Leukoplakia is the most common pre-cancerous lesions, which is a clinical diagnosis, and cannot be defined by any other means, but microscopically, it may range from benign hyperkeratosis to invasive Squamous Cell Carcinoma [3].

From the previous studies it has been now widely accepted that the most squamous cell carcinoma of the oral cavity develops from preneoplastic oral lesions like leukoplakia [4]. Nearly 50% of OSCC clinicopathologically presents with oral leukoplakia [5]. Almost one third of these lesions (31.4%) will eventually progress to

malignancy [6]. Presently there is no single prognostic marker available that can predict individual risk of malignant transformation. Moreover, in many cases, even histopathological assessment, considered as gold standard, does not correlate accurately with clinical outcome and may not reveal all possible markers of prognostic importance [7].

OSCC is a multistep process and personal habits like smoking, Khaini, gutka, alcohol intake, etc. and microbial infection have been linked to its development.

The deoxyribonucleic acid (DNA) content of cells of oral leukoplakia can predict the outcome of the disease process and the risk of cancer development. The quantitative measurement of DNA has been shown to be prognostically useful in a number of tumors. A group of research workers has shown that the presence of aneuploidy, as detected by DNA image cytometry, was found to be indicative of high risk of oral squamous cell carcinoma [4]. Out of many techniques used for this purpose, cytogenetic analysis, Feulgen micro densitometry, and more recently flow cytometry has been considered as

one of the most reliable procedure for quantitative analysis of DNA estimation thus for risk categorization [8,9].

Of the various markers used for assessing the risk of malignant transformation, silver binding Nucleolar Organizer Regions (AgNORs) has become popular for its simplicity, low cost and good correlation with other proliferative markers [7].

Nucleolar Organizer Regions (NORs) are chromosomal segments containing loops of DNA in which ribosomal RNA is encoded [10], and located in the cell nucleoli during interphase. NORs also contain a set of acidic, non histone proteins that bind silver ions and are selectively visualized by silver staining methods in routinely processed cyto-histological samples [11].

They are located in the acrocentric chromosomes 13, 14, 15, 21 and 22 [12]. Their number per nucleus has been shown to be correlated with the rate of ribosomal RNA transcription, cell proliferation and DNA ploidy [10]. Silver staining of exfoliative cytology smears was first used by Howell et al. [11] 1975. Subsequently, several research workers have used this method both in exfoliative cytology as well as histopathological slides for the study of different lesions. It is found that the cases with a history of smoking show higher quantity of AgNOR expression than nonsmokers signifying a higher rate of cellular replication in smokers than nonsmokers [13].

'Human Papilloma Virus (HPV) has been considered as one of the risk factors for the development of head and neck cancers [14]. About 20 to 40% Head and Neck squamous cell carcinoma has been related to type 16 and 18 HPV, justifies its link to HPV induced squamous cell carcinoma of head and neck [14]. However, another study [15]; has linked only 22% HPV 16 DNA and 14% HPV 18 DNA with squamous cell carcinoma of the Head and Neck. Therefore, its' relationship with oral cancer and pre-neoplastic lesions are yet to be established conclusively.

## 2. Materials and Methods

A total of 50 cases of oral leukoplakia reported to the Department of Cancer Detection Center (CDC) OPD of Chittaranjan National Cancer Institute (CNCI), Kolkata, India, along with adequate controls were selected for this study. In each case detailed clinical history, including history of personal hygiene, duration of the lesion, the history of addiction, including history of smoking and use of any other form of tobacco etc. were taken. All the 50 cases along with the controls were subjected to punch biopsy. Oral smears and exfoliated cells were also taken for the study. Before taking samples patients were asked to clean the oral cavity thoroughly with water.

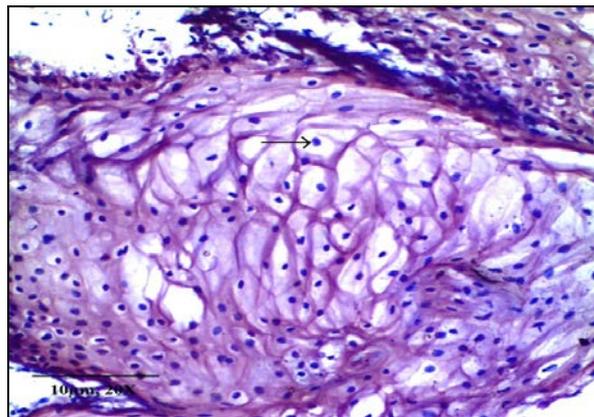
### 2.1. Materials and Methods of Koilocytosis

Koilocytotic changes were seen in 3 to 5µm paraffin sections obtained from biopsy samples. In short, for deparaffinization the sections were heated to 56°C for 30 minutes and immediately the tissue sections were passed through xylene 5 x 5 minutes, followed by graded alcohol (100%, 90%, 80%, 70%, and 60%) 5 x 5 minutes. The slides were rehydrated in de-ionized water and blotted. The sections were used for haematoxylin and eosin stain (H & E stain) to see the koilocytotic changes along with histopathological identification of each case.

In brief, the sections were kept in 'Harris haematoxylin' for 20 seconds and washed well with running tap water for 5 minutes or until the sections turned blue. Sections were then treated with 1% HCl in 70% Alcohol (for differentiation) for 8 seconds.

In the next step, sections were washed well with tap water until sections were again blue (12 minute treatment in tap water was done) and sections were stained with 1 % eosin Y for 10 minutes.

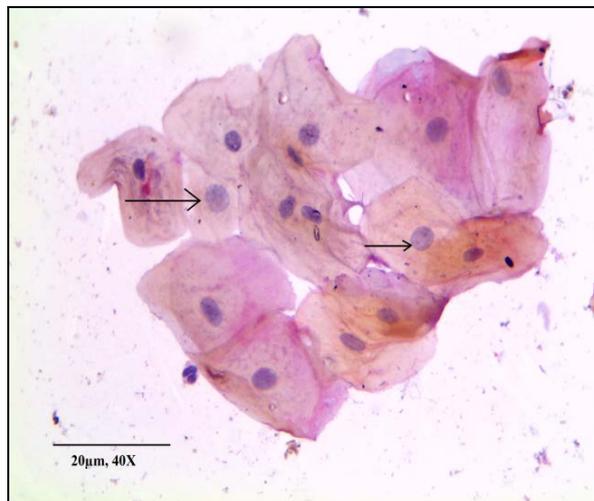
The Sections were then washed with running tap water for 5 minutes and dehydrated through graded alcohols, cleared in xylene and mounted with DPX. In the next step the H&E stained slides were observed under light microscope in 20X and 40X (Figure 1).



**Figure 1.** H&E stained tissue sections (20X) showing koilocytotic changes characterized by perinuclear halo, hyperchromasia and irregular nuclear membrane

### 2.2. Materials and Methods for AgNORs

The oral smears were collected on the acid/alcohol cleaned slides with the help of cyto brush and fixed in 95% alcohol at room temperature. Out of which in each case two slides were used for AgNOR staining, one slide was used for PAP staining for cytopathological evaluation (Figure 2) and parallel smears of normal cases were used as control.



**Figure 2.** PAP stained smear showing mild dysplastic changes in a case of 50 years old male patient clinically diagnosed as homogenous leukoplakia tongue (40X)

In brief, cyto smears were washed with deionized water and blotted on clean tissue paper.

The slides were then incubated in freshly prepared working solution for 45 minutes at room temperature [16]. The working solutions were made as follows.

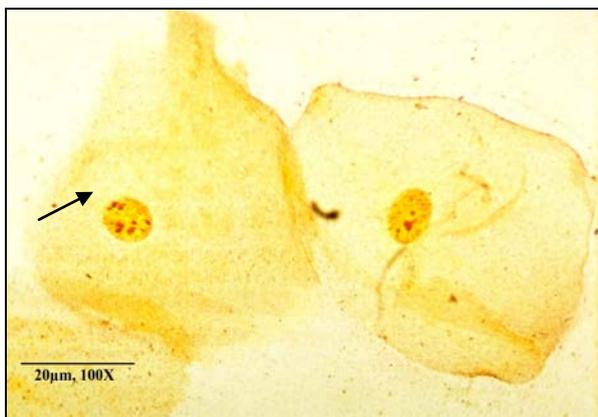
#### A. 50% silver nitrate solution

- Silver nitrate: 0.005kg
- Deionized water: 0.01 Lt.

#### B. Gelatin solution

- Gelatin: 0.002 kg
- Formic Acid: 0.0001 Lt
- Deionized water: 0.01 Lt

The working solutions were made by mixing solution A; two parts warmed at 37°C with one part solution B. Solution A and B were mixed just immediately before use, and layered on the smears and kept for 45 minutes as mentioned before. The reaction was stopped by dipping the slides in deionized water for 1 minute and blotted with clean tissue paper, dried and cleared in xylene and mounted by DPX mountant. All slides were studied under 40X magnification and in oil immersion lens at a magnification of 100X and a total of 1000 cells were counted for AgNORs study (Figure 3). Only clear black dots were considered for the study. In negative control slides, working solution was replaced by deionized water.



**Figure 3.** Expression of AgNORs, as black dots (100X), in a case of homogeneous leukoplakia with Silver Staining Method

### 2.3. Materials and method for DNA Ploidy

Cytobrush was used to collect exfoliated cells from oral lesions. Carefully cells were collected from the visible lesions (clinically diagnosed as leukoplakia). The cytobrush was rubbed on the lesion gently and dipped into 5 ml of phosphate buffered saline pH, 7.6. The cells were washed twice with PBS to clean the cellular debris and other contaminants.

The DNA content for cell cycle analysis on fixed cells was done according to the protocol of the University of Virginia [17] last modified on November 25, 2003. 70% ethanol was prepared freshly and cells were made ready for the experiment. PBS of pH- 7.6 was prepared according to international protocol.

#### Preparation of propidium iodide (PI) solution

Freshly, propidium iodide solution was made by adding 2 mg of DNase free RNase A (Sigma, USA) in 10 ml of PBS containing 0.1% (v/v) Triton X100 (Sigma USA) and 200 ml of 1 mg/ml PI (Sigma USA) added to it.

#### Staining cells with PI

The 12 X 75 mm tubes were filled with 4.5 ml of 70% ethanol and kept on ice. The cells collected in PBS from

cases were centrifuged for 6 minutes at 300x g. A total of  $10^6$  to  $10^7$  cells were taken in 5 ml PBS suspension. After centrifugation the supernatant was discarded, the cells were resuspended in 0.5 ml PBS. It was ensured that the cells were lying in single cell suspension form by gentle pipetting with 'Pasteur pipette'. The cells were then transferred into the test tube containing chilled 70% ethanol kept for a minimum period of 2 hours.

The ethanol fixed cells were centrifuged for 5 minutes at 300x g and the ethanol was decanted thoroughly. The cell pellet was suspended in 5 ml PBS for 1 minute and then centrifuged for 5 minutes at 200x g. The supernatant was decanted and the pellet was suspended in 1 ml of PI solution and kept for 30 minutes at room temperature in dark and the cells with PI solution were then brought to a flow lab for analysis.

#### Flow cytometry

Flow cytometry was carried out with a Becton Dickinson Facsallibur flow cytometer using an argon laser, wavelength 488nm, run at 15mW. Calibration was carried out using latex beads.

#### Histogram interpretation [18]

##### Image analysis:

The DNA Index (D.I) between 0.8-1.2 was considered as diploid population, DNA Index between 1.8-2.2 was considered as tetraploid population and the DNA Index outside the above range is considered as aneuploid population.

The maximum permitted coefficient of variation of the G0/G1 peaks was 10. An aneuploid population must be >10% of the total cell count and a tetraploid population must exceed 20%.

*Interpretation of the peaks (DNA Ploidy):* By convention the first (left most) peak represents the diploid peak. A tetraploid peak is identified when 20% or more of nuclear events occur within the D. I range of G2M peaks (i.e. 1.8-2.2). Aneuploidy indicated by the presence of an abnormal peak.

## 3. Results

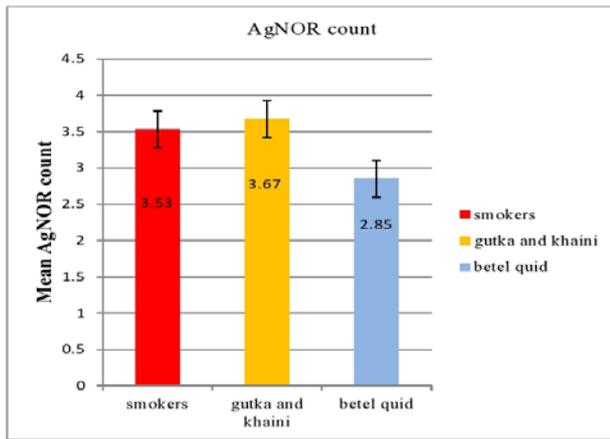
There were 50 cases of pre-malignant lesions and adequate controls in this study. The cases consisted of males and females; and the average ages of males were  $44.71 \pm 9.8$  & in female cases, it was  $34.4 \pm 9.7$ . Out of 50 cases, 45 cases (90%) were linked to traditional addictions of various forms and 5 cases (10%) were non addicts. The average age of smokers was  $47.12 \pm 8.66$ , betel quid chewers were  $46.54 \pm 8.03$  & Khaini & gutka chewers were  $41.2 \pm 8.98$ . Normal cases had an average age of  $36.5 \pm 14.17$ .

### 3.1. Statistical Analysis

Statistical Analysis was performed with the help of Epi Info (TM) 3.5.3. EPI INFO is a trademark of the CDC. Both univariate and multivariate analyses have been done to find the Odds Ratio with 95% confidence interval and corresponding p-values. Also Anova Graphpad 3.6.1 was used to evaluate AgNORs for risk prediction of oral leukoplakia.

### 3.2. Results of AgNORs

The non-addictive 5 cases showed a mean (average) AgNOR count  $2.05 \pm 0.576$ . The AgNOR distributions among addicted cases were as follows. The smoking group of 25 cases (50%) had a mean average AgNOR count of  $3.53 \pm 2.058$ . The 11 betel quid group (22%) mean (average) AgNOR count was  $2.85 \pm 1.47$  and khaini and gutka group (18%) had AgNOR distribution of  $3.67 \pm 2.21$  (Figure 4).



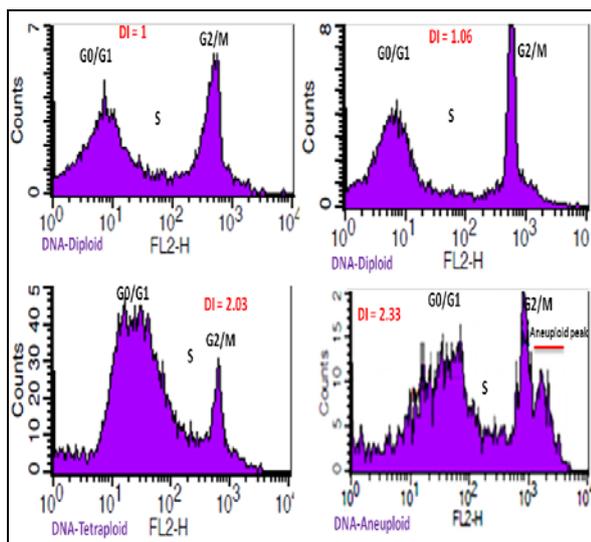
**Figure 4.** Histogram for comparative analysis of the mean AgNOR counts among smokers, gutka-khaini group and betel quid chewer group

Histopathologically, there were dysplastic cases and non-dysplastic cases among this study.

The mean (average) AgNOR count in dysplastic lesions was  $3.29 \pm 1.789$  & non dysplastic lesions were  $3.19 \pm 2.05$ .

### 3.3. Result of DNA Ploidy

All the cases of premalignant lesions were included in this study and successfully analyzed by flow cytometer. In our study, it was shown that there were diploid, tetraploid and aneuploid cases, including hypodiploid DNA content as indicated in Figure 5.



**Figure 5.** Representative histograms showing DNA ploidy status

The DNA index ranged from 0.22 (hypodiploid) to 2.12 (tetraploid) and coefficient of variation ranged from 0.39 to 18.43.

In this study, 26 % (n = 13) cases showed diploid DNA content, the aneuploid barring tetraploid population was

28% cases and the rest tetraploid DNA containing cases was 46%.

There were 52% non-dysplastic cases and 48% dysplastic pre malignant oral lesions. There was also no significant relationship between dysplasia and DNA content of the cell population.

Though there was a definite history of addiction in each of these cases, no significant correlation was found between DNA ploidy status and addictions related to quantity, duration and type of addictions.

### 3.4. Result of Koilocytosis

Out of 50 cases of pre-malignant lesions studied for koilocytotic changes it is found that 20 cases (40%) were positive for koilocytotic change and rest 30 cases did not show any koilocytotic change. Considering the non dysplastic or dysplastic lesions out of 20 positive cases 13 non-dysplastic and 7 dysplastic cases showed koilocytotic changes (i.e. 65% and 35%). In the dysplastic lesions 3 cases showed mild dysplasia, 1 case showed moderate dysplasia and 3 cases showed severe dysplasia (i.e. 43%, 14% and 43% +ve). The koilocytotic change was mostly found in the parabasal layers diagnosed by a distinct perinuclear halo and condensed pyknotic or angulated nuclei. During this study a serious look was given on the addiction status of each individual in relation to koilocytotic change. There were smokers 25 cases (50%) among them 13 were negative for koilocytosis (52.1%) and 12 cases (47.9) showed positive for HPV (koilocyte), among Khaini and gutka users, 9 were negative (90%) and 1 case of (10%) was positive for koilocytosis. Among betel quid chewers; out of 11 cases, 5 were positive for HPV (koilocytotic change (45.45%) and out of normal 5 cases, 3 were negative for koilocytotic changes and 2 was positive (10%).

## 4. Discussion

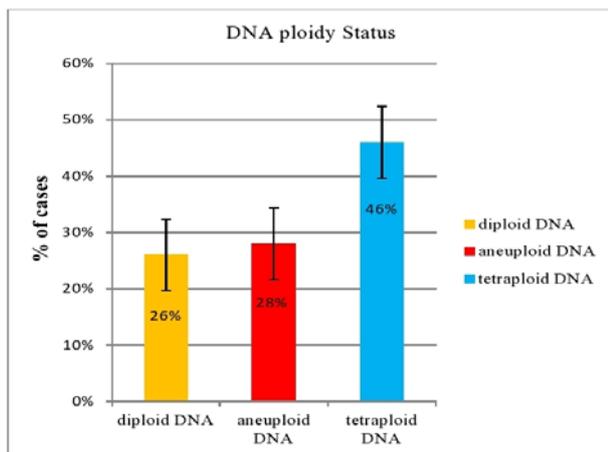
From our result, it is found that out of 50 cases of oral leukoplakia there were 26 cases (52%) of non dysplastic lesions and 24 cases (48%) were dysplastic of various grades, whereas Sudbo et al. 2001 [19] has found that out of 150 cases of leukoplakia all are dysplastic. The histopathological diagnosis of our study justifies the universally accepted definition of leukoplakia considering the diversified outcome of the histopathological findings. In our study only leukoplakias are included barring erythroleukoplakia or lichenplanus. As many of the oral cancers are represented with leukoplakia [5,20] or on the other hand actually many leukoplakias are histopathologically diagnosed as oral cancer have been excluded from this study.

The validity of oral cytology for analyzing the number of keratinized cells and the nuclear activity (AgNORs) in smokers has recently been demonstrated [21]. Remmerbach [22] reported on an AgNOR analysis in oral cytology and concluded that this may be used as a routine method for diagnosing oral cancer [22]. In our study, AgNOR count was significantly higher in the smoker group than non smokers and non addict groups. In another study by Fontes et al. 2008 [23] and Paiva et al. 2004 [12] have found a similar result in other population and different geographical area.

In this study, betel quid chewers had comparatively lower AgNOR counts, but it was higher than the group having no addiction. There was no significant difference of AgNOR counts between dysplastic and non dysplastic lesions having leukoplakia, but both had higher AgNOR counts than normal control. The gutka and Khaini groups had the highest AgNOR count. There was no significant difference of AgNOR count per nucleus in the smoker subgroups with variable number of cigarettes/ bidi consumed per day. Similar results were observed by Cancado et al. [13] in their study. In this study risk of cancer progression was observed using statistical methods (Epi Info (TM) 3.5.3 and Anova Graph Pad 3.6.1). The smoker group had 3.5 times and the betel quid chewers group had 1.56 times higher risk than the normal control group. The gutka and khaini groups had  $\pm 5.5$  times higher risk than the control group.

In conclusion, it is observed that traditional addictions like smoking of bidi and cigarette, betel quid and gutka chewing, use of khaini etc. all have a close relationship with the development of leukoplakia and oral cancer. There is no significant relationship between HPV infection and oral leukoplakia. However, AgNOR expression assessment as prognostic markers is significantly linked to leukoplakia and oral cancer with previous history of addictions.

In another study by Sudbo et al. 2001 [19] found that the smoking has no significant effect on ploidy status. In our observation, it is found that the aneuploidy is not only linked to severe dysplasia, but also found in cases with other dysplasias and non-dysplastic cases, substantially justifies the statement that more than half of the cancers arises from the normal cell population [24]. In a follow up study by Maraki et al. 2001 [25], they found that out of 150 cases of histologically proven dysplasia, 36 cases developed squamous cell carcinoma. There were 105 diploid lesions out of 150 cases of dysplasia. From these 105 diploid lesions only, 3 developed squamous cell carcinoma, whereas out of 25 cases of aneuploid lesions 21 progressed to squamous cell carcinoma. Similarly, in our study, out of 50 cases 13 (26 %) cases showed diploid DNA content, 14 (28%) cases showed aneuploid population and the rest 23 cases (46%) were tetraploid DNA containing cases (Figure 6).



**Figure 6.** Histogram for showing different DNA ploidy

In our study, within 2years of follow up only one case was converted to malignancy from aneuploid population.

Therefore, a close follow up for a longer period of time is necessary to evaluate the effect of quantitative DNA changes and development of oral cancer. Since there is an important place for AgNOR as prognostic marker, both the DNA ploidy and AgNORs could be used for risk assessment for cancer progression in oral leukoplakia.

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## Conflicts of Interest:

'The authors have no competing interests'.

## References

- [1] Flohr, J.K, Lee, J.H, " Identical but different mechanism of cancer development and response to treatment for human papilloma virus-related and non related squamous cell cancer of the head and neck," *S D Med*, 6,453-5, 2008.
- [2] Choudhury, A.K, Singh, M., Sudaram, S., et al., "Role of human Papiloma virus and its detection in potentially malignant head and neck lesions; updated review," *Head Neck Oncol*, 1, 22, 2009.
- [3] Nooshin, M., Nasrollah, S., Noorieh, K., Mohammad, T.S., Shima, A.G., Setareh, S., "Evaluation of P53 and PCNA Expression in Oral Leukoplakic Lesions with and without Epithelial Dysplasia by Immunohistochemistry Technique," *J Mash Dent Sch*, 33(2), 169-76, 2009.
- [4] Van Zyl, A.W., Van Heerden, M.B., Langenegger, E., Van Heerden, W.F.P., "Correlation between Dysplasia and Ploidy Status in Oral Leukoplakia," *Head and Neck Pathol*, 6, 322-327, 2012.
- [5] Schepman, K., Van der, M.E.H., Smeele, L., et al., "Concomitant leukoplakia in patients with oral squamous cell carcinoma," *Oral Dis*, 5, 206-9, 1999.
- [6] Lee, J.J., Hong, W.K., Hittelman, W.N., Lotan, L.R., Shin, D.M., et al., "Predicting cancer development in oral leukoplakia :ten years of translational research," *Clin Cancer Res*, 6, 1702, 2000.
- [7] Manu, V., Rajaram, T., Rai, R., et al., "Value of Silver binding Nuclear Organizer Regions (AgNOR) in squamous cell of carcinoma of upper aero digestive tract," *MJAFI*, 62, 123-8, 2006.
- [8] Pierre, R.V., "Cytogenetics in malignant lymphomas," *Virchows Arch B cell Pathol*, 29, 107-12, 1978.
- [9] Seoane, J., Bascones, A., Asenjo, J.A., et al., "Flow cytometric analysis of nuclear DNA content in oral leukoplakia," *Clin Otolaryngol*, 23, 136-40, 1998.
- [10] Costa, A.L., de Araujo, N.S., Pinto, D., et al., "PCNA / AgNOR and Ki 67/AgNOR double staining in Oral squamous cell carcinoma," *J Oral Pathol Med*, 28, 438-4, 1999.
- [11] Howell, W.M., *Selective staining of nucleolus organizer regions (NORs)*. In: Busch H and Rothblum L, Editors,1982, The Cell Nucleus Academic Press, New York, pp. 89-143.
- [12] Paiva, R.L., Sant' Ana Filho, M., Bohrer, P.L., Lauxen Ida, S., Rados, P.V., "AgNOR quantification in cells of normal oral mucosa exposed to smoking and alcohol. A cytopathologic study," *Anal Quant Cytol Histol*, 26,175-80, 2004.
- [13] Cancado, R.P., Yursel, L.S., Santanna, F., "Evaluation of the nuclear organizer regions associated proteins in exfoliative cytology of normal vocal misuse. Effect of smoking," *Oral Oncol*, 37,446-54, 2001.
- [14] Shindoh, M., Chiba, I., Yasuda, M. et al., "Detection of human papillomavirus DNA sequence in oral squamous cell carcinomas and their relation to p53 and proliferating cell nuclear antigen expression," *Cancer*, 76, 1513-1521, 1995.
- [15] Sugeran, P.B., Shillitoe, E.J., "The high risk human papillomaviruses and oral cancer: Evidence for and against a causal relationship," *Oral Dis*, 3, 130-47, 1997.
- [16] Ploton, D., Menager, M., Jeannesson, P., Himber, G., Pigeon, F., Adnet, J.J., "Improvement in the staining and in the visualization

- of the argyrophilic proteins of the nucleolar organizer region at the optical level," *Histochem J*, 18, 5-14, 1986.
- [17] Protocol of the University of Virginia last modified on November 25, 2003 [cited 2003 Nov 25] Available from: <http://hsc.virginia.edu/intrnet/cytometry/protocols/dnacontent.efm>.
- [18] McDermott, N., Suliman, Y., Bary Walsh, C., Kay, E.W., Curran, B., Milburn, C., et al., "Ploidy profile of morphologically normal squamous epithelium adjacent to high grade cervical intraepithelial neoplasia," *British Journal of Obstetrics and Gynaecology*, 104, 623-625, 1997.
- [19] Sudbo, J., Kildal, W., Risberg, B., Koppang, H.S., Danielsen, H.E., Reith, A., "DNA content as a prognostic marker in patients with oral leukoplakia," *N Engl J Med*, 344, 1270-8, 2001.
- [20] Fernandez, M.H., Bagan, J.V., Cortes, J.M., Roda, R.P., Calabuig, C., "The prevalence of oral leukoplakia in 138 patients with oral squamous cell carcinoma," *Oral Disease*, 10, 346-348, 2004.
- [21] Orellana-Bustos, A.I., Espinoza-Santander, I.L., Franco-Martinez, M.E., Lobos-James-Freyre, N., Ortega-Pinto, A.V., "Evaluation of keratinisation and AgNORs count in exfoliative cytology of normal oral mucosa from smokers and non-smokers," *Med Oral*, 9, 197-203, 2004.
- [22] Remmerbach, T.W., Weidenbach, H., Muller, C., et al., "Diagnostic value of nuclear organization (AgNORs) in brush biopsies of suspicious lesion of oral cavity," *Anal. Cell Pathol*, 25,139-46, 2003.
- [23] Fontes, P.C., Correa, G.H.M., Issa, J.S., et al., "Comparison of exfoliative PAP stain and AgNOR counts of the tongue in smokers and non-smokers," *Head and Neck*, 2, 157-162, 2008.
- [24] Kim, J.M., Adel-el-Naggar, D., Lee, J.S., Corrale, C., Lippman, S.S., Hong, W.K., et al., "Chromosome polysomy and Histological Characteristics in oral premalignant Lesions," *Cancer epidemiology, Biomarkers and Prevention*, 10, 319-325, 2001.
- [25] Makari, D., Becker, J., Boecking, A., "Cytologic and DNA – cytometric very early diagnosis of oral cancer," *Anal Cell Pathol*, 22, 211-21, 2001.