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Study of p53, Pcna, Ki67 and Micronuclei Related to Genotoxic Damage for Risk Categorization in Pre-Malignant Oral Lesions

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Abstract In this prospective study, search for high risk cases of premalignant oral lesions were attempted through evaluation of oncoprotein expression, cell proliferation and micronuclei. *Materials and methods:* A total 50 cases of oral leukoplakia were diagnosed and adequate controls along with detailed history were included in this study. Study of tumour markers like p53, PCNA and Ki-67 were done immunohistochemically on tissue sections. Study of micronuclei was performed by feulgen staining method. *Results:* The mean age of the 50 cases were 44.48 ± 9.76 and median age was 50 years. There were 45 male cases (90%) and 5 female cases (10%). There were 26 non dysplastic (52%) and 24 dysplastic cases (48%). The smokers consist of 25 cases (50%) and other addictions (betel quid chewers, ghutka, khaini, alcohol etc.) had 24 cases (48%) and 1 case was non addict (2%). Out of 25 cases of smokers, 20 (80%) were positive in case of p53, 19 (76%) were positive in case of PCNA and 17 (68%) were positive in case of Ki67. Out of 24 cases of other addictions, 16 (66.67%) cases were positive in case of p53, 13 (54.17%) positive cases in case of PCNA and16 cases (66.67%) were positive in case of Ki67.The smokers had 0.51 ± 0.21 micronuclei frequency whereas other addiction groups had 0.24 ± 0.10. *Conclusion:* The histopathological risk assessment of these cases is inconclusive and thus p53, PCNA, Ki67 and micronuclei evaluation are needed for risk categorization.

Keywords: *oral leukoplakia, oral squamous cell carcinoma, immunohistochemistry, molecular-markers, p53, PCNA, Ki67, micronuclei, epithelial dysplasia*

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1. Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common human cancer and accounts for at least 90% of all oral malignancies [1]. There are approximately 650,000 new cases each year with no reported improvement in the survival rate in the last 30 years [2]. The 5-year survival rate has reached 80% in cases detected at the initial stage, 40% in cases of regional involvement, and less than 20% in cases with distant metastasis [3].

It is now accepted widely that most squamous cell carcinoma of the oral cavity arises from pre-neoplastic oral lesions, specially leukoplakia [4]. Leukoplakia is considered to be most frequent and important oral lesion with a very high potential for malignant transformation and 50% of the oral squamous cell carcinoma diagnosed clinicopathologically, along with oral leukoplakia [5]. Nearly one third of these lesions (31.4%) will eventually progress to malignancy within ten years [6]. Incidentally, no reliable histopathological parameters have been identified that can predict the risk of malignant transformation. However, Silverman et al, 1984 [7], found that histopathological evidence of moderate to severe dysplasia substantially increase the risk of oral cancer by 36%. Although histological evidence has been considered as the gold standard for cancer prediction, it is not always predictive of individual patient outcome. In other word, many patients with severe dysplasia do not at all progress to malignancy and remains stable for many years where as patients with hyperplasia and mild dysplasia carry significant risk and progress to malignancy within a short period of time [8].

Several clinical and etiological factors, especially personal habits like smoking, alcohol intake etc. and microbial infection have been evaluated to identify high risk groups for cancer prediction [9]. Common genetic mutation found in human cancer [10], is aberrant expression of p53 gene often resulting in the build up of the p53 oncoprotein accumulation within the affected cells and could be detected immunohistochemically. As well as, the expressions of PCNA and ki-67 have also been found to be associated with oral pre-neoplastic lesions among risk groups.

Exposure to mutagens such as smoking, alcohol, environmental pollution and microbial infections are also linked with chromosomal breakage and malfunction of spindle apparatus leading to formation of micronuclei, another very important biomarker for cancer risk prediction.

Therefore, in this study, we have tried to categorize risk groups among pre-neoplastic oral lesions by combining more than one marker, along with histopathological parameters and personal habits.

2. Materials and Methods

2.1. Sample Collection and Preparation

The samples were collected from 50 cases of oral leukoplakia reported to the Department of Cancer Detection Center (CDC) OPD of CNCI, Kolkata, India, along with adequate normal oral mucosa as control.

Demographic details for all patients including age, sex, socioeconomic status and personal history about smoking, alcohol consumption, tobacco chewing and other chewing habits were meticulously recorded. The age of the patients ranged from 24 to 54 years (mean age, 50 years). Among all 50 patients, 45 were male and only 5 were female. The smoking group had 25 cases, and other addictions like betel quid or ghutka / khaini chewing habits or alcohol consumption were present in rest 24 patients except one case had no addictions with leukoplakia. Normal controls were selected based on no history of smoking, alcohol, tobacco or betel quid use.

Only premalignant oral lesions were selected. All patients were healthy with no systemic disease except two cases (one had renal calculi and the other had systemic hypertension). The patients with disseminated disease, loco-regional recurrence, other serious illness, or with poor general condition were not included in the present study. All patients were from middle socioeconomic group except three cases who belonged to low socioeconomic group. A detailed clinical, radiological and histopathological data for each patient was also recorded.

All the 50 patients along with controls were subjected to excision or punch biopsy and the specimens were routinely fixed in 10% formalin (24-48 hours) and processed in the laboratory, in the Department of Pathology and Cancer Screening, CNCI, Kolkata, India. Before taking biopsies patients were asked to clean the oral cavity thoroughly with water and in all these cases additionally four slides of cytosmears were collected and fixed in 95% Ethyl alcohol for 20 minutes for micronuclei study. The histopathological diagnosis of each case was done by hematoxylin and eosin staining.

All the specimens were histopathologically categorized as non-dysplastic (26 cases) and dysplastic (24 cases). Oral epithelial dysplasia were categorized as mild (grade I), dysplasia demonstrates proliferation of atypia or immature basal cells above the parabasal region but not extending beyond the lower third of the epithelium. Moderate (grade II) dysplasia demonstrates a similar proliferation into the middle one-third of the epithelial cross section. The severe dysplasia (grade III) was considered for abnormal proliferation from the basal layer into the upper third of the epithelium. The normal controls were taken from buccal mucosa of age-matched healthy individuals without any deleterious smoking or chewing habit.

2.2. Haematoxylin and Eosin Staining

3-5 µm sections from formalin fixed paraffin embedded tissues were obtained from 50 cases and adequate controls. The sections were mounted on Poly-L-Lysine (Sigma, USA) coated glass slides. The sections containing sufficient epithelium or tissue were considered for the study which was evaluated under light microscope (Olympus CX 40, Japan) before immunohistochemistry.

Sections were de-paraffinized in xylene and dehydrated through graded alcohol. In each case, xylene 5 x 5 minutes and graded alcohol 5 x 5 minutes [11] followed by rinsing with phosphate buffered saline (PBS, pH-7.6) were done.

In each case a parallel of two slides stained for Haematoxylin and Eosin were studied after the immunohistochemical experiments (Figure 1).

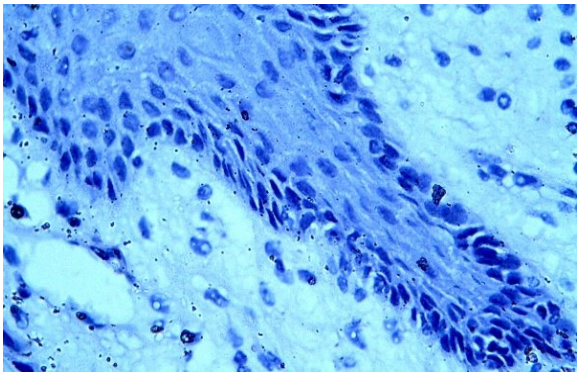

Figure 1. HE stains of leukoplakia tongue with intermediate grade keratinizing squamous lesion x 40X

2.3. Immunohistochemistry

The tissue sections were cut to 3-5 µ thickness and mounted on Poly-L-Lysine coated slides and the slides were marked.

The slides were left in hot air oven at 59°C for 30 minutes and later dipped and deparaffinized in xylene for 10 minutes (three changes).

Then slides were rehydrated through graded alcohols (5 x 5 min) and running tap water. The slides were not allowed to dry at any point.

For p53 and PCNA study: antigen retrieval was performed by placing the slides in a pressure cooker (Hawkins, India) containing 0.01M citrate buffer (pH-6.0) for 2 minutes and for Ki-67, retrieval was done by microwave oven (LG MS-2029UW) at 650 W for 2 minutes.

Then the slides were cooled to room temperature and next steps of staining were performed in a humid chamber.

After washing the slides with wash buffer (PBS-Tween 20, pH-7.6), endogenous peroxidase was blocked by 0.5% hydrogen peroxide in methanol and was applied for 10 minutes at room temperature [12]. The slides were then washed with wash buffer for 2 minutes (3 times).

The non-specific binding was blocked by incubating the sections with normal goat serum diluted (1 : 100) in 0.05 M filtered phosphate buffer containing human serum albumin (Sigma, USA) (2.5 mg / ml) for 10 minutes.

Primary antiserum from Sigma, USA (for p53, 1:200 dilutions, clone BP53-12; 1 : 400 dilutions for PCNA, clone PC10, and for Ki67, 1 : 400 dilutions, clone PP67) were added to the area marked by the diamond pen. During experiment two positive control slides were used in each case.

For negative control, additional two slides were omitted of primary antibody. In each slide 100-200 µl of serum was added and incubated overnight at 4°C in a humid chamber. The slides were washed with filtered wash buffer (PBS-Tween20, pH-7.6) for 2 minutes (five times). The sections were then incubated with secondary antibody (Biotin – SP-conjugated goat anti-mouse IgG (Sigma, USA); 1:500 dilutions for 20 minutes at room temperature.

Excess antibody was removed by washing with PBS for 2 minutes (three times). Then Streptavidin HRP conjugate (Sigma USA) (horse radish peroxidase enzyme polymer) reagent was applied (1 : 300) for 60 minutes at room temperature and the slides were washed in PBS for 2 minutes (three times).

The peroxidase was localized with the DAB (Sigma USA) hydrogen peroxide substrate solution with minor modification of the protocol as suggested by IHC world (http://www.ihcworld.com/_protocols/chromogen_substrates/POD_D).

Stock Solutions: 1% DAB (20X), 1% Nickel Ammonium Sulfate (20x), 1% Cobalt Chloride (20X), 0.3% H_2O_2 (20X).

Working solution: 5 drops of 1% DAB was added to 5 ml of PBS pH 7.2, mixed well and to it 5 drops of 1% Nickel Ammonium Sulfate and 5 drops of cobalt chloride were added. Finally, 5 drops of 0.3% H_2O_2 were added to the solution mixed well and layered on the sections and incubated for 1-3 minutes at room temperature and washed with PBS for 2 minutes (three times).

The slides were then counter stained with Delafield haematoxylin for 30 seconds and washed in running tap water for 6 minutes. Finally the slides were dehydrated in ascending grades of alcohol and then to xylene and mounted with DPX.

2.3.1. Evaluation of Staining

The pattern of expression was also analyzed semi quantitatively by counting 1000 cells at 40X under light microscope and only positive nuclear stains were considered for record (Figure 2, Figure 3, and Figure 4).

The immunoreactivity were categorized according to Raju et al [13], 2005; as mild (+, ≤ 10% cells positive), moderate (++, < ±10-49% cells positive), and strong (+++, ≥ 50% cells positive). Known positive immunostained slides were used as positive controls.

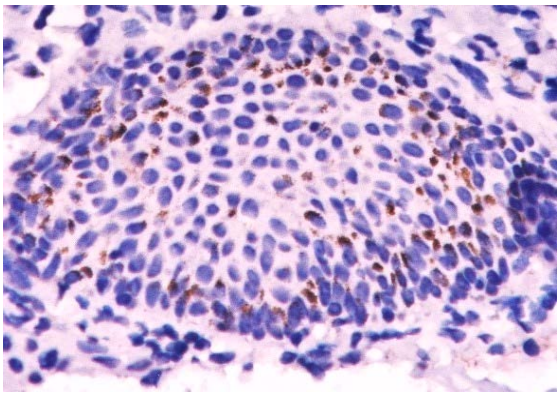

Figure 2. Leukoplakia tongue microscopically pseudoepitheliomatous hyperplasia with inflammatory mucosal changes expressing p53 x 40X

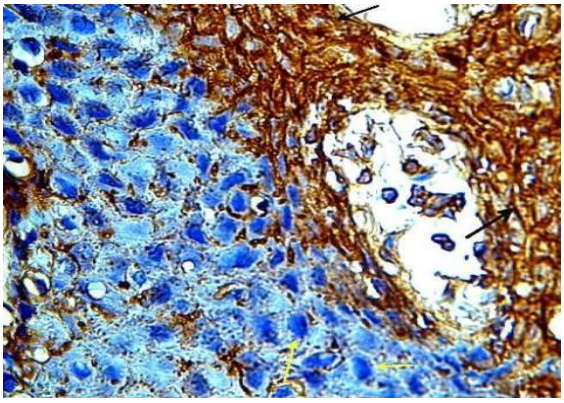

Figure 3. Leukoplakia buccal mucosa microscopically mild hyperkeratosis with nuclear atypia expressing PCNA x 40X

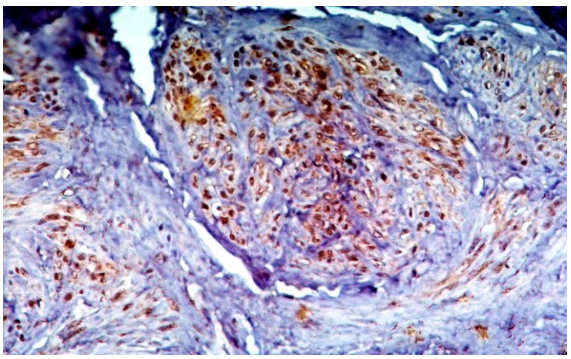

Figure 4. Leukoplakia buccal mucosa showing hyperkeratosis with pseudoepitheliomatous hyperplasia expressing Ki67 x 20X

2.4. Feulgen Stain for Micronuclei Assay

Study cases mentioned earlier along with two normal controls were also included and considered for evaluation of micronuclei status.

In each case patient was asked to wash oral cavity thoroughly twice with clean tap water before taking smear. A cytobrush (Famy care LTD, India) was used to collect smears from the lesion visible directly under bright light. In each case deep scrape was taken and smeared over the acid and alcohol cleaned glass slides and dipped immediately for 20 minutes in 95% ethyl alcohol to avoid drying artifact. Slides were marked with a patient code no and date of collection. For study of micronuclei slight modification of original Feulgen method [14] was done following the method of Millet [15].

Briefly, 95% alcohol fixed smears rinsed in cold 1N hydrochloric acid at room temperature (20°C) and immediately transferred to 5N hydrochloric acid and the slides were kept in it for 25 minutes at room temperature. Slides were then rinsed with cold 1N hydrochloric acid and washed in sterile deionized water.

Excess water was lightly shaken and the slides were blotted on sterile tissue paper and kept in Schiff's reagent for 1 hour at room temperature. Next the slides were taken out of schiff's reagent and kept in running tap water for 5-6 minutes and rinsed in double distilled water. Slides were then counter stained with 0.2% light green for 1 minute. Dehydration of slides was done through graded alcohol followed by cleaning with xylene and mounted with DPX.

All the slides were observed under light microscope (40X) (Figure 5).

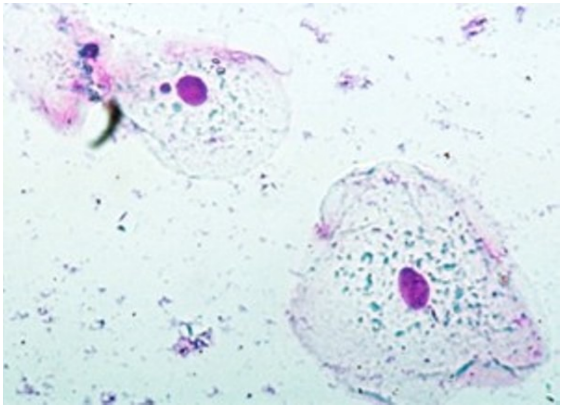

Figure 5. Buccal smear of leukoplakia showing micronuclei x 40X by Feulgen stain

At least 1000 cells were counted for micronuclei study and expressed as percentage [16,17].

2.4.1. Scoring Criteria:

The zigzag method was followed for screening of slides. One thousand cells with intact nuclei and structure were counted in each slide. The criteria for designating an extra-nuclear body as micronuclei were as follows:

a) diameter less than one third of the main nucleus, b) staining intensity similar to or slightly weaker than that of the main nucleus, c) round or oval in shape, d) texture same as that of the main nucleus, e) close proximity but no actual contact with the nucleus, f) plain of focus same as that of the main nucleus.

3. Statistical Analysis

At the end point of this cross sectional study, we report here, the reflective risk parameter for the cancer development in 50 cases of oral leukoplakia, randomly chosen at CNCI, OPD during the period of 2008 to 2012.

Statistical analysis was performed with the help of Epi info (TM) 3.5.3. EPIINFO is a trade mark of the Centres for Disease Control and Prevention (CDC), NIH, USA.

Z test was used to test the significant difference between two proportions or two means. Odds ratio with 95% confidence interval (CI) was calculated to measure the different risk factors. Significance level was set at 0.05 and confidence intervals were at 95% level. Pearson correlation coefficient was used to calculate the correlation between different variables. T-test was used to compare the means.

4. Results

50 cases of oral leukoplakia (including 45 males and 5 female cases) were included in this study. Test proportion showed that proportion of males were significantly higher than that of females ($p < 0.001$), proportion of Hindus was significantly higher than Muslims ($p < 0.001$), proportion of smokers and chewers were significantly higher than no habits ($p < 0.001$), frequency of smoking and chewing $\geq$ 10 per day was significantly higher and duration of smoking and chewing $\geq$ 15 years was significantly higher ($p < 0.001$).

The proportion of homogeneous leukoplakia was significantly higher than any other lesions ($p < 0.001$) and buccal mucosa as a site of lesion was significantly higher ($p < 0.001$) and proportion of NDL as microscopic findings was significantly higher ($p < 0.001$).

Under univariate analysis it has been found that risk of having p53 positivity was 3.69 [OR-3.69(1.05, 12.95); p = 0.03] time with a history of smoking and the same risk increased to 7.42 [OR-7.42(1.69, 79.96); p = 0.06] times for the cases with a history of more than 10 cigarette smoking per day. It is also found that risk of having PCNA positivity in cases of smokers is 4.75 times higher [OR-4.75(1.40, 16.05) p-0.009] than that of other addictions. It is also found that the risk of having Ki67 positivity in cases of smokers is 3.18 times higher [OR-3.18 (1.00, 10.17); p = 0.04] than that of other addictions. The risk increased to 7.20 times [OR-7.20 (1.06, 48.64); p = 0.03] for the cases with the history of more than 10 cigarettes per day. It has also been found that the p53, Ki67 and PCNA positivity are higher in the smoking group than other addictions (Figure 6).

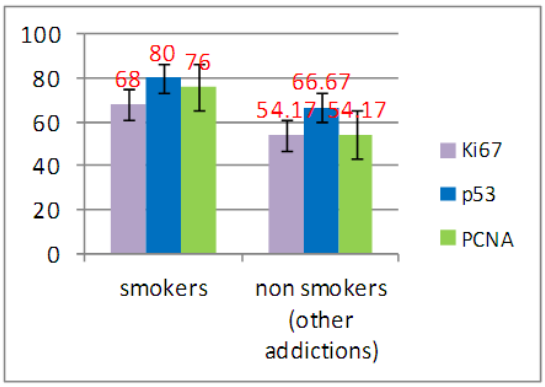

Figure 6. Histogram for comparative analysis of the Ki67, p53 and PCNA expression between smokers and other addictions

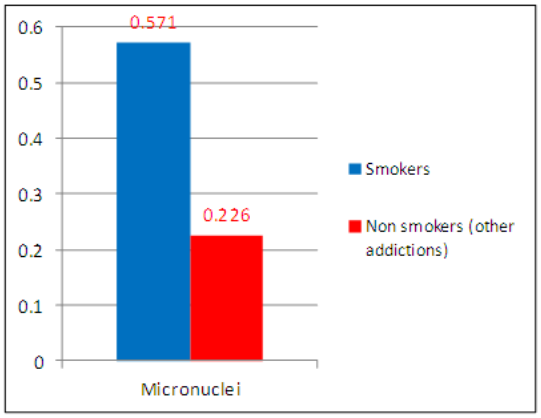

Figure 7. Histogram for comparative analysis of micronuclei Between smokers and other addictions

Similarly frequency distribution of micronuclei is higher among smokers than other addictions (Figure 7).

5. Discussion

Leukoplakia is a white patch or plaque and has been classified as homogenous and non homogenous leukoplakia. Homogenous leukoplakia rarely shows associated dysplasia whereas non-homogenous leukoplakia is usually associated with dysplasia and therefore, much more prone to become invasive [18].

Though histopathological finding is the gold standard for judging cancer potential of the lesion, dysplasia itself may not always be a reliable marker [7]. However, with regards to the clinical lesion it is apparent that fewer lesions actually progress further [19]. Today, no single marker or parameter has been found to detect the prognostic outcome of pre-malignant oral lesions.

Mutation of p53 tumour suppressor gene and subsequently p53 oncoprotein expression has been widely studied in different cancers but it is less frequently explored in oral leukoplakia at least in this part of the world.

The p53 gene, which encodes 53KD nuclear phosphoprotein, is a tumour suppressor gene located on chromosome 17 p13, activates and controls cell regulation [20].

Loss of function of wild type p53 tumour suppressor gene results in uncontrolled cell division leading to progressive genomic instability and cancer susceptibility [21].

Barring oral cancer and leukoplakia, other cancers like colon cancer [22], ovarian cancer [23], and brain cancer [24] has been found to express p53 oncoprotein in variable magnitude.

It is found that the expression of p53 in relation to oral leukoplakia is an important phenomenon. Further our study confirms that p53 expression in oral pre-malignant lesions with the history of smoking in any form carries significant risk, in cases where smoking frequency is more than 10 cigarettes per day. On the other hand, duration of smoking, which is also an important factor, does not carry any significant risk and it is also true in cases of addictions other than smoking.

Ki67 or MKI67 is a nuclear non-histone protein encoded by *MKI67* gene in humans. The protein is necessary for cellular proliferation and is also associated with ribosomal RNA transcription. Enhanced cellular proliferative process is clearly associated with the neoplastic process. The expression of Ki67 antigen is widely used in cancer research as a proliferative marker to measure the growth index of cells in human malignancies [25]. Risio et al [26] have found that in colon adenomas and adenocarcinomas have shown an increased number of proliferative cells compared to unaffected mucosa within the same individual. Inactivation of Ki-67 protein leads to inhibition of ribosomal RNA synthesis [27].

In relation to proliferative markers like Ki-67, smoking is a significant factor and people who smoke more than 10 cigarettes per day carries significant risk in relation to the duration of smoking (> 15years) and other addictions. However, in other addiction like chewing betel quid, gutka, khaini etc for more than 15 years, carries significant risk.

Many authors [28,29] have found important co-relation between p53 and PCNA expression in diagnosing malignancy. PCNA or proliferating cell nuclear antigen is a 36KD polypeptide encoded by the gene located on chromosome number 20. It is expressed in the late G to S phases of the cell cycle and is a cofactor of DNA polymerase delta in eukaryotic cells. It helps to increase the processivity of leading strand synthesis during DNA replication. It also plays a role in DNA repair and is regarded as a marker for cell proliferation.

Similarly PCNA expression is linked to frequency of smoking (> 10 cigarettes per day) rather than duration of smoking and carries significant risk for progression to cancer. Other addictions do not carry any significant risk for PCNA expression and progression to cancer.

Therefore, it is presumed that smoking, especially frequency of smoking (> 10 cigarettes per day) along with p53, Ki67 and PCNA expression carries important and significant risk for progression to cancer, in pre-malignant oral lesions.

Only 50% of biopsied leukoplakia shows dysplasia and overall the malignant transformation rate for leukoplakia is only about 0.1-2% per year [30].

Ironically rates are lower in the developing world where tobacco chewing habits are most prevalent [30].

Duration of smoking and histopathological finding has a direct relationship. Moreover, in our study the long duration (21 – 35 years) of addiction has more severe dysplastic lesions than minimal exposure to smoking (0 – 10 years). However no link can be established between quantity of smoking and histopathological outcome.

In conclusion, it can be said that histopathological or clinical assessment of oral leukoplakia for risk categorization is not convincing. However, from our study it has been clearly pointed out that a group of biomarkers assessment along with histopathological diagnosis and amount of exposure to addiction e.g. tobacco, as a whole can predict or categorize risk for the development of malignant transformation in these oral lesions.

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Statement of Competing Interests

'The authors have no competing interests'.

List of Abbreviations

CDC – Cancer Detection centre, CNCI-Chittaranjan National Cancer Institute, OPD – Out Patient Department, PBS-Phosphate Buffered Saline, DAB-3, 3'-Diaminobenzidine.

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