# AN ALLELIC LOSS AT CHROMOSOME 9p21 TO DETERMINE ORAL POTENTIALLY MALIGNANT DISORDERS-A NON INVASIVE GENETIC SCREENING TECHNIQUE

**Dr Prahalad Hunsigi[1],Dr DNSV Ramesh[2] ,Dr Dhanu G[3],Dr Shivanand Aspalli[4], Dr Shiny Raj R[5],Dr Pradeep M R[6]**

**[1] Prof and Head,Department of Oral pathology and microbiology ,Ames Dental college and hospital Raichur**

**[2]HOD, Department of Oral medicine ,Ames Dental college Raichur**

**[3]Professor,Department of Pedodontics, Subbaiah dental college ,Shimoga**

**[4]Principal, Ames dental college ,Raichur**

**[5]Sr lecturer, Dept of Pedodontics, AME's Dental college and hospital, Raichur**

**[6] Reader, Dept of Oral Pathology, AME's Dental college and Hospital, Raichur**

## ABSTRACT

**Background & Objectives**: Tumorigenesis is a multistage process in which multiple genetic abnormalities lead to malignancy. In individuals with precancerous oral lesions, allelic loss at 3p and 9p appears important in predicting who will advance to invasive oral cancer. The aim of the present study is to detect the presence of allelic imbalance at chromosome locus 9p21 in oral potentially malignant disorders though non-invasive screening method.

**Methodology:**The study comprised of three - control group with no history of any habit ,moderate risk group with tobacco related habits over a duration of less than 2yrs and 15 patients were of high risk group with tobacco related habits over a duration of more than 2yrs.Genomic DNA was extracted from buccal epithelial cells. Al was assessed using 8 microsatellite markers located at chromosomes 9p21. Al analysis was done by PCR using primers with fluorochromes.

**Results:** The analysis of all of the data indicated that allelic imbalance was present in exfoliated cells of the group who had oral lesions studied. None of the samples of control showed Al, confirming the apparent high specificity of the assay (100%).

**Conclusion**: The present study, therefore, demonstrates that Allelic Imbalance (AI) on chromosome 9p is found in the oral potentially a malignant disorder at a relatively high frequency is probably an early event in oral carcinogenesis.

**Key Words**: Allelic Imbalance (AI); Loss of Heterozygosity (LOH); Oral Potentially Malignant Disorders (OPMD); Oral Squamous cell carcinoma (OSCC)

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**INTRODUCTION:**

The incidence of oral and oropharyngeal cancer worldwide is around 500,000 new cases every year, accounting for approximately 3% of all malignancies, thus creating a significant worldwide health problem[1]. The American Cancer Society estimated 40250 new cases of these cancers for 2012 in the United States alone. The most common form of oral cancer is squamous cell carcinoma (SCC), which accounts for 96% of all cancers of the oral cavity. Tobacco use and alcohol consumption are regarded as the main risk factors for oral squamous cell carcinoma (OSCC), while human papilloma virus (HPV) infection is emerging as the leading risk factor in cancers of the oropharynx[2]. Other risk factors associated with this disease include dietary deficiencies, poor oral hygiene, asbestos and indoor air pollution from fossil fuel combustion. Understanding the evolving molecular pathophysiology of head and neck tumorigenesis is vital for the development of more effective preventative, diagnostic, and treatment strategies[3]. Many recent studies have addressed the molecular basis of the process of cancer development, and genetic progression models have been proposed for various tumor types. It is now well established that an accumulation of genetic alterations forms the basis for the progression from a normal cell to a cancer cell, referred to as the process of multistep carcinogenesis[4]. The development of HNSCC(head and neck squamous cell carcinoma) is a multistep process involving the accumulation of genetic and epigenetic alterations in key regulatory genes[5,6].

Recent studies examining oral squamous cell carcinomas (SCC) have identified frequent deletion or mutation affecting one allele of the p53, RB and DCC tumor suppressor genes. However, few studies have interrogated both copies of these sequences although homozygous deletions of p16 have been reported. Several other chromosomal areas that are likely to harbor other suppressor sequences likely to play a role in the development of these tumors have been identified at chromosomes 3p, 8p and 9p by cytogenetics and loss of heterozygosity studies; however, the critical sequences within these candidate suppressor areas are currently unknown. Although only preliminary data about the genetic aberrations associated with tumorigenesis is available, a genetic model has been proposed with specific gene alterations indicated as early, intermediate and late events. Study of head and neck and of other solid tumors has suggested that AI at specific chromosomal regions, and the number of aberrations detected, can help predict outcome. This new knowledge means that it is now possible to apply molecular studies to tumors in vitro and incorporate information about the key genetic abnormalities for each tumor into conventional staging systems. This will enable clinicians to identify a tumor's potential for progression more accurately and use this information to modify existing treatment protocols to improve clinical course and outcome[7].



Detection and monitoring of such nonvisible precancerous fields by histology would require multiple biopsies surrounding the treated area, and a noninvasive screeningtool would be a much more attractive alternative. Such an assay would also be of relevance for leukoplakia patients, as it has been shown that these patients can develop oral squamous cell carcinomas outside the visible lesions[8]. Therefore, it seems important to screen and monitor leukoplakia patients not only for precancerous changes in the visible lesion(s), but throughout the whole oral cavity[9].Therefore a non-invasive genetic screening assay might be of large value for identifying people of high risk group. The aim of the present study was to determine the cancerous potential of patient with oral potentially malignant disorder, assess the potential value of a non-invasive screening technique to detect precancerous fields and grade visible and possibly non visible oral potentially malignant disorders genetically

**METHODOLOGY**

The study was conducted in Department of Oral Pathology, A.M.E's Dental College and Hospital, Raichur city, Karnataka, India. The study comprised of three groups in which 20 patients were kept as control group with no history of any habit and 15 patients were of moderate risk group with tobacco related habits over a duration of less than 2yrs and 15 patients were of high risk group with tobacco related habits over a duration of more than 2yrs.

Study sample was divided into three groups asGroup 1: 20 patients as control, Group II : 15 moderate atypia and Group III : 15 severe atypia. Inclusion criteria were;Patients willing for the study voluntarily, Individuals who are systemically healthy, Clinically diagnosed cases of oral potentially malignant disorders, patients of age group 18-50 yrs,patients with habits of tobacco chewing, smoking for duration of more than 2yrs depending on the frequency, quantity and quality of the tobacco related habit are considered under the high risk group,patients who have previously not taken any treatment for Oral potentially malignant disorder. Exclusion criteria werepatients not willing for the study, Geriatric Patients with debilitating diseases, patients suffering from any systemic diseases like diabetes mellitus,hypertension, cardiovascular disease, renal dysfunction, liver disorders etc and medically compromised patients.

Patients were made to sit comfortably on a dental chair. General history and thorough clinical examination was carried out. A structured Performa was used to collect relevant information from each patient regarding his demographic, medical and lifestyle history including tobacco and alcohol use. A written informed consent was obtained from all the patients. Ethical clearance was seeked from the ethical board of A.M.E's Dental college and hospital, Raichur.

In the preset study we have used covid box for collecting the samples keepin in mind the welfare of patients and it was mandated for each patient to be prevented from propogation of corona virus.In the present study cytological smears are prepared by cyto or tooth brush.

Sites of 5* 10 mm were brushed repeatedly five times at six anatomical sites in the oral cavity in healthy controls or at the site of oral potentially malignant disorders(clinically diagnose). Brush samples were taken from the lesions as well as from different oral mucosal sites with relatively high incidence of oral squamous cell carcinoma: border of tongue (left and right),floor of mouth (left and ridge/retromolar trigone (left and right).The cytology brush is rotated 360° on the surface of the lesion and placed on a clean glassslide. The slide is immediately sprayed with a cytofixative spray and allowed to dry. Each brush was immediately placed into a 2ml vial containing phosphate-buffered saline (PBS). The vials were named according to the site of brush biopsy and the samples were then transported to the Sandor Lifesciences Pvt. Ltd, Hyderabad, India, for DNAextraction.

AI was assessed using microsatellite markers located at chromosome 9p. The following markers is used: D9s171.AI analysis was done by PCR using primers with fluorochromes. PCR products were run on an applied biosystems 3730x1 sequence analyser (Applied Biosystems BV). Data processing and quality control.



**Statistical Analysis**

Statistical Product and Service Solution (SPSS) version 21 for Windows (Armonk,NY:IBMcorp) software was used to analyse the data.Statistical analysis was done by using tools of descriptive statistics such as Mean, and SD for representing quantitative data.Probability $p<0.05$, considered as significant as alpha error set at 5% with confidence interval of 95% set in the study. Power of the study was set at 80% with beta error set at 20%.One way Analysis of variance (ANOVA) test was used for comparison between three study groups in relation to mean age, tobacco usage duration and frequency.Chi square test was used for comparison between three study groups in relation to presence/absence of allelic imbalance on chromosome 9p

**Results:**

Mean age in Group A (High Risk group) was found to be 34.73 (6.06) years, Group B was 32.8 (5.32) years and in Control group was 27.05 (6.79) respectively. On comparison of mean gae between three study groups using One way Anova F test , it was observed that statistical significant difference ($p<0.05$) exist in relation to mean age.

Mean frequency of tobacco units consumption /day in Group A (High Risk group) was found to be 12.46 (2.38) years, Group B was 4.46 (2.03) years and in Control group was nil respectively. On comparison of mean frequency between three study groups using One way Anova F test , it was observed that highly statistical significant difference ($p<0.001$) exist with highest consumption in Group A (High Rish Group) [Table1]

Mean duration of tobacco units consumption in Group A (High Risk group) was found to be 8.26 (1.48) years, Group B was 3.06 (1.09) years and in Control group was nil respectively. On comparison of mean frequency between three study groups using One way Anova F test , it was observed that highly statistical significant difference ($p<0.001$) exist with highest years of tobacco consumption in Group A (High Risk Group) [Table 2]

On Comparison of presence/absence of allelic imbalance on chromosome 9p respectively using Chi square test, highest alleleic imbalance (93.3%) was present in Group A (High Risk ) followed by moderate allelic imbalance (13.3%) and nil in Group C (Control). Highly statistical significant difference ($p<0.001$) exist overall between three study groups. On pairwise comparison , highly statistical significant difference ($p< 0.001$) exist among the three study groups.[Table 3]

**Discussion**

The development of Oral Squamous Cell Carcinoma (OSCC) is characterized by a multistep row of events governed by the early detectable, gradual accumulation of genetic changes in genes transcribing key upstream and downstream regulatory proteins. Despite recent updates in our knowledge of OSCC developing process and therapeutic improvements, the prognosis of this malignancy still remains poor[10].

It is noteworthy that many oral squamous cell carcinomas develop from potentially malignant disorders (PMDs)[11,12,13]. Correct diagnosis and timely treatment of PMDs 18 may help prevent malignant transformation in oral lesions[12].The term "potentially malignant disorders" was defined by World Health Organization (WHO) as the risk of malignancy being present in a lesion or condition either during the time of initial diagnosis or at a future date[82,85].

Clinically, oral precancerous lesions may appear as a white or red lesion (leukoplakia or erythroplakia, respectively[15]. Histopathologic grading requires taking a biopsy, and to monitor the progression of a lesion, repeated biopsies need to be taken, which is a large burden and reluctant to go for biopsy and it is a hereculonous task in backward remote areas,for the patient. Furthermore, histopathologic grading may largely depend on the precise location of the biopsy, given the heterogeneity of some lesions. Hence, screening and monitoring oral precancer by histopathologic examination of tissue biopsies does not seem to be feasible, except for the visible lesions[9].

Notwithstanding, a noninvasive genetic screening assay might be of large value for populations at high risk for developing oral cancer such as treated oral cancer patients. Leukoplakia patients, genetically predisposed subjects such as Fanconi anemia patients, and individuals frequently exposed to environmental carcinogens[9].

Recent advances in molecular techniques has deepened our understanding of certain signaling networks enhancing our ability to synthesize biomarkers for diagnosing cancerous lesions and creating individualized treatment options, improving the survival rate of oral cancer patients[16]. The identification of specific biomarkers appointed, respectively, to

the early, intermediate and late stages of oral tumorigenesis would prove very helpful in the management of OSCC[17].

Genetic alterations are the hallmark of human cancer. A cell, whose genome has undergone mutations, may encompass loss of genomic material ranging from a few hundred nucleotides to a whole chromosome. As a result, numerous alleles are silenced, while their function is being sustained by the remaining active alleles (the 89 cell is named heterozygous for that mutation)[18]. Loss of heterozygosity represents the loss of normal function of one allele of a gene in which the other allele was already inactivated and marks a suppressor phenotype that is characterized by a wide variety in chromosomal numbers (aneuploidy) and extensive loss of genetic material (allelic imbalance-AI)[19,20]

In a previous study conducted by Bremmer JF, various genetic assays were developed and evaluated that might allow the detection of oral precancerous fields in small brushed samples. They found that measurement of Al, commonly known as allelic loss or loss of heterozygosity, seems most suitable. A marker panel of microsatellite markers, located at chromosomes 3p. 9p. 11q, and 17p, was selected based on the following criteria: high percentage of loss of heterozygosity in HNSCC, frequent loss of heterozygosity in precancerous fields indicating that these occur early 6,56,96 in carcinogenesis," high percentage of informativity of these particular microsatellites, amplimer lengths that can be combined for multiplex sequence runs and known to be associated with malignant transformation of leukoplakia[6,22,23],

The present study was undertaken to show that progressing lesions and controls had significantly different LOH profiles, supporting the hypothesis that LOH patterns could be used as cancer risk markers to identify genetic profiles that have predictive value for early premalignant lesions.

In the present study we explored Al analysis on noninvasive brushed samples of the oral cavity as a method for identification of precancerous changes. This would allow identification and monitoring of precancerous changes in the oral mucosa without the burden of taking biopsies. Al was absent in all samples of the 20 control subjects, yielding a specificity of 100% Allelic imbalance was present in exfoliated cells in 80% of the leukoplakia lesions studied. Out of 30 patients evaluated for the markers D9S157, D95161, D9S162, D9S168, D9S171, D9S199, D9S741, D9S1748, allelic imbalance was detected at chromosomal arm 9p21 in patients with oral lesions indicating the possibility of loss of tumour suppressor genes in this loci and clonal outgrowth in these lesions.This present study was in accordance with Mao et al. (1996), who by studying 84 cases of oral leukoplakia (OPL) demonstrated that losses of the 9p21 and 3p14 regions may be related to early processes of tumorigenesis in HNSCC and their existence in premalignant tissues may serve as a marker for cancer risk assessment[24].

The present study was similar to the study conducted by Partridge et al. (1998) who wed a microsatellite assay to screen 31 potentially malignant oral lesions for genetic abnormalities and found Al in 24 (77%) of them. They also observed that the presence of lesions with Al at two or more (2) relevant loci is correlated with high risk for them evolving into squamous cell carcinoma. The same researchers (Partridge et al., 1999) later proposed that allelic imbalance at 3p22-26, 3p14.2, should be considered a better predictor of outcome than the TNM system[25].

The present study was in accordance with the study conducted by JantineF.Bremmer (2009), in which exfoliated cells were removed by a brush from multiple small areas of the oral mucosa, including the leukoplakia. Brushed samples were investigated for allelic imbalance (AI) at chromosomes 3p. 9p, 11q, and 17p using microsatellite markers known to show frequent alterations in oral precancer[9]. Al was absent in all (137) of the samples of the 20 control subjects, yielding a specificity of 100%. Al was detected in exfoliated cell samples of 40% (10 of 25) of the leukoplakia lesions studied.

Genetic changes were also found outside the leukoplakia lesions. Most frequent was Al at 9p (9 of 10). The noninvasive assay was validated against the biopsy results of the leukoplakia lesions yielding an estimate of sensitivity of 78% (7 of 9) and a positive predictive value of 100% (7 of 7). Altogether, these results show the feasibility of a noninvasive genetic screening approach for the detection and monitoring of oral precancer[9].Deletion of 21 region on chromosome 9 has been found in several other tumor types implying the presence of a tumor suppressor gene at this locus. The inactivation of a tumor suppressor gene on chromosome 9p may represent the most commonly described genetic alteration in HNSCC.

The non invasive nature of collecting exfoliative cells means that multiple samples can be taken easily, which allows investigation of whether different genetic clones are emerging in different sites of the oral cavity or in different areas of a large lesion. In the present study exfoliative cells were taken from two different sites in two large diffuse oral lesions. The results showed different patterns of allelic loss in the two sample regions. This suggests that it may be possible to map the position of genetic clones within lesions by collecting exfoliative cells from specific sites. It is generally recognized that cases presenting with a single dysplastic lesion may develop an invasive tumor within a relatively short time frame, because the dysplasia is a precursor lesion at the margin of a developing tumor. Alternatively, malignancy mayarise at other site years later, presumably because the entire mucosa is exposed to the same carcinogens.

Following this regimen should minimize an individual's risk of developing a tumor at a distant site, or locally when molecular aberrations are present in histologically normal mucosa[26]. However, it may also be prudent to carry out biopsy for molecular and histological analysis every year, to judge the effect of this preventative regimen in this high-risk group. In addition, based on the findings from this study, it seems desirable to screen clinically normal mucosa as part of the routine work-up for head and neck cancer patients by histological examination and, by using the molecular test whenever possible, to establish whether there is molecular evidence of field cancerization, to help with the overall risk assessment for these patients[27].

The molecular assessment of exfoliated cells from oral rinsing/swabbing must now be tested in a prospective, blinded fashion in clinical settings requiring actual cancer detection. The fact that no healthy control subject had any DNA alteration is encouraging, indicating the excellent specificity of the microsatellite analysis. Normal appearing mucosa in patients with early cancer or premalignant lesions has beenshown to harbor occult microsatellite alterations. It is precisely in these patients thatthe value of detecting asymptomatic cancers by microsatellite analysis must beprospectively assessed, a setting requiring a highly specific test. A saliva test could beadministered by non specialists in remote locations as a screening tool to selectpatients for referral for careful evaluation of the upper aerodigestive tract. Findingearly stage, previously undetected disease and prompt identification of persistentdisease after therapy using sensitive microsatellite analysis may ultimately savelives[28] .



**Conclusion**

Early diagnosis of oral precancerous but clinically nonvisible fields might be of importance for clinical management, particularly in high-risk populations such as patients with leukoplakia, patients with treated oral cancer, and even healthy individuals frequently exposed to environmental carcinogens. The noninvasive genetic assay presented here seems to be a valuable screening tool to detect these nonvisible precancerous fields.

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**Table 1: Comparison of mean tobacco units consumption per day (frequency) between threegroupsrespectively**

| Frequency/Day | Mean | SD | ayAnova Ftest | P value,Significance |
|---|---|---|---|---|
| **Group A(HighRiskGroup) (n=15)** | **12.46** | **2.38** | | |
| **Group B(LowRiskGroup) (n=15)** | **4.46** | **2.03** | **F=229.11** | **P<0.001**** |

| | | | | |
|---|---|---|---|---|
| **Group C(Control)(n=20)** | **0.0** | **0.0** | | |

****p<0.001–highlystatisticalsignificantdifference**

**Table 2: Comparison of mean years of tobacco units' consumption per day (duration) betweenthreegroupsrespectively**

| Duration(in years) | Mean | SD | ayAnova Ftest | P value,Significance |
|---|---|---|---|---|
| **Group A(HighRiskGroup) (n=15)** | **8.26** | **1.48** | | |
| **Group B(LowRiskGroup) (n=15)** | **3.06** | **1.09** | **F=288.742** | **p<0.001**** |
| **Group C(Control)(n=20)** | **0.0** | **0.0** | | |

****p<0.001–highlystatisticalsignificantdifference**

**Table3:Comparisonofpresence/absenceofallelicimbalanceonchromosome9prespectively**

| AllelicImbalance | Present | Absent | Chisquaretest | P value,Significance |
|---|---|---|---|---|
| **Group A(HighRiskGroup) (n=15)** | **14(93.3%)** | **1(6.7%)** | | |

| | | | | |
|---|---|---|---|---|
| **Group B(LowRiskGroup) (n=15)** | **2(13.3%)** | **13(86.7%)** | **Chi=37.745** | **p<0.001**** |
| **Group C(Control)(n=20)** | **0 (0%)** | **20(100%)** | | |
| **Group A(HighRiskGroup) vsGroupB (LowRiskGroup)** | **Chisquaretest=23.86,p<0.001**** | | | |
| **Group A(HighRiskGroup) vsGroup C(Control)** | **Chisquaretest=48.5,p<0.001**** | | | |
| **Group B(LowRiskGroup) vsGroup C(Control)** | **Chisquaretest=6.51,p =0.09(NS)** | | | |

**p>0.05–nosignificantdifference(NS) *p<0.05– significant **p<0.001–highlystatistical**



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