https://doi.org/10.48047/AFJBS.6.8.2024.293-312

African Journal of Biological Sciences

Research Paper Open Access

Evaluation of correlation between DNA methylation and tobacco use in oral potentially malignant disorders

1.Dr. Mimansha Patel, 2. Dr. Pankaj Gupta, 3. Dr. Koyel Jana, 4. Dr. Ketki K Thakare, 5. Dr. Shilpa Wasu, 6. Dr. Kriti Sao

[1]Professor & HOD, Department of Oral and Maxillofacial Pathology and Microbiology, Triveni Dental College, Bodri, Bilaspur, Chhattisgarh

[2]Assistant Professor, Amaltas Institute of Medical Sciences, Dewas M.P.

[3]PG Student, Department of Oral and Maxillofacial Pathology and Microbiology, Triveni Dental College, Bodri, Bilaspur, Chhattisgarh

[4]PG student, Department Oral and Maxillofacial Pathology, Maitri Dental College, Durg, Chhattisgarh

[5]Lecturer, Department of Periodontology and Implantology, VYWS dental college and Hospital, Amravati – 444602

[6]Senior Lecturer, Department of Oral and Maxillofacial Pathology

New Horizon Dental College and Research Institute, Sakri, Bilaspur, Chhattisgarh.

Corresponding author

Dr. Mimansha Patel, Professor & HOD, Department of Oral and Maxillofacial Pathology and Microbiology, Triveni Dental College, Bodri, Bilaspur, Chhattisgarh

mimanshapatel_24@yahoo.com

Article History

Volume 6,Issue 8, 2024

Received:09 Mar 2024

Accepted : 12 Apr 2024

doi: 10.33472/AFJBS.6.8.2024.293-312

Abstract

Background:. DNA methylation is a potential approach for "cancer therapeutics" and can help researchers truly understand the genomic mechanism that leads to cancer. Tobacco consumption is considered as most important risk factor for development of oral potentially malignant disorders (OPMDs). Tobacco either in the smokeless form, smoked form may contribute to development of OPMDs. It is not clear whether DNA methylation is associated with tobacco use in patients with OPMDs.

Aim: This study was conducted to identify, correlate and compare "DNA methylation" among healthy people, tobacco users having premalignant disease, and tobacco users donot having premalignant disease.

Methods and Materials: Individuals would be enrolled in three group at random using random table procedures. Each group would have 16 people in it. Individuals in Group A were in good health. Group B: Study participants using tobacco and suffering from disease Group C: Study participants who were not suffering from disease. Full case history of tobacco use and analysis of quantitative variables."DNA methylation" — methylation-specific quantitative real-time PCR (qMSP) was carried out.

Results: Methylation of all the genes i.e p16, DAP-K and MGMT among tobacco users with no oral premalignant lesions was observed in 9.8% cases. 31.2 cases were found to have methylation of two genes. 10.14% cases were found to associated with methylation of p16 and DAP-K, 09.43% cases were found to have methylation in the p16 and MGMT and 11.27% cases showed methylation of DAP-K and MGMT. The difference was significant statistically. ($p \leq 0.05$). Methylation of all the genes i.e p16, DAP-K and MGMT among normal healthy individuals was observed in 1.7% cases. 2.11 % cases were found to associated with methylation of p16 and DAP-K, 1.21% cases were found to have methylation in the p16 and MGMT and 2.38 % cases showed methylation of DAP-K and MGMT. The difference was significant statistically. ($p \leq 0.05$). Methylation of p16, MGMT and DAP-K was quite significant in tobacco users having diseases. Further it was also observed that methylation of p16, MGMT and DAP-K was also quite significant in tobacco users having no diseases

Conclusion: DNA methylation" in tobacco abusers without any existing oral disease elevates the probability of development of "oral potentially malignant disorders.

Keywords: DNA methylation, tobacco use, OPMDs

Introduction

Cancer of oral cavity is one of the most serious issues of community based health around the globe. public health concern around the world.[1-3]One-third of the cases (37.5 percent) were found only in Asia.[1] Victor Babes (1875) coined the phrase "premalignant lesion" to describe a disease that, if left untreated, could grow causing "cancer." Premalignant lesions of oral region Oral premalignant lesions (OPMLs), which affect about three percent percent of total human population, are a specific target for prevention of cancer of oral cavity. The importance of precancer lesion like oral leukoplakia, oral erythroplakia, or oral erythro leukoplakia arises from the large number of patients where a biopsy reveals dysplasia or similar "frank cancer."[4]

There is a progressive histopathological sequence which can be graded as regular, hyperplastic as well as carcinoma in situ during the progression of premalignant lesion to the malignant lesion.[6-10] Oral pre-malignant lesions can be identified and managed with a visual inspection and are conveniently accessible for further testing including microscopy and biopsies.[11]Early diagnosis of abnormalities reduces mortality and morbidity, but prolonged identification, particularly in places with the highest incidence rate, lowers the chance of survival, despite modern treatment procedures.[12-13]

Pre-cancerous lesions of the oral cavity include leukoplakia of oral cavity, erythroplakia of oral cavity, oral submucous fibrosis, condition of actinic keratosis, condition of discoid lupus erythematosus, condition of lichen planus of oral cavity, and condition of reverse smokers, all of which are referred to as "potentially malignant illnesses (PMDs)."[14] It has been documented that around 13% of these abnormalities of oral cavity get transformed into cancer of oral cavity.[10]

The contemporary process for analysing progression of cancer is determined by the process based on morphological changes observed clinically. However there is some issue of diagnosing and treating pre-cancerous lesions of the oral cavity early. Patients experience severe "morbidity" in both overtreated and undertreated cases. Therefore under such circumstances when evaluation based on clinical and histopathological examination are not successful in identification of pre-malignant condition then the invention of biochemical markers can make the process of early identification of these lesions quite effective. It will help in better diagnosis and management.[7,10]

Exploration on promoter epigenetic modifications of genes involved in tumor-suppressor in the setting of "OPMD" appears relevant, given the enormous occurrence of that kind of epigenetic modification in oral squamous epithelium abnormalities" Initial genetic alterations could subject tissues to more genetic problems, enabling the "oncogenic activity" to advance.[1] Therefore, genomic methylation being used as sensitive indicator for identifying "OPMD" could be a viable option. DNA methylation is a potential approach for "cancer therapeutics" and can help researchers truly understand the genomic mechanism that leads to cancer. Tobacco consumption is considered as most important risk factor for development of OPMDs. Tobacco either in the smokeless form, smoked form may contribute to development of OPMDs. It is not clear whether DNA methylation is associated with tobacco use in patients with OPMDs. Therefore this study

was conducted to identify, correlate and compare "DNA methylation" among healthy people, tobacco users having premalignant disease, and tobacco users donot having premalignant disease.

Methods and Materials

Type or Nature of study

It was a cross-sectional research carried out in association with Sterling Accuris Diagnostic Human Genetic Unit, Ahemadabad, at Outpatient Department of Oral Pathology and Microbiology. SDPC.

Sample Size Commensurate with the Study design

The "purposive sampling technique" is used to determine sample size.

Participants

Criteria for inclusion

• Individuals who use tobacco in any form, with or without sickness.
• Cases of oral possibly malignant conditions that have been histopathologically diagnosed.

Criteria for exclusion

• Presence of other condition inside mouth producing inflammatio , like tooth having sharp cusp, inflammation of gingiva, or inflammation of periodontium in non-smokers.
• Those who refused to provide their consent.

The following are three groups.

Individuals would be enrolled in each group at random using random table procedures. As a result, the trial would cover a total of 48 patients. As a result, each group would have 16 people in it.
Individuals in Group A are in good health.
Group B: Study participants using tobacco and suffering from disease
Group C: Study participants who are not suffering from disease.
Tobacco use – full case history – quantitative variables
"DNA methylation" — methylation-specific quantitative real-time PCR (qMSP)

Variables are the things that can change.

• **Result –** "possibly malignant oral diseases"
• **Confounding factors** – These include history of habit other than tobacco, hereditary history of disease, presence of condition in mouth that can cause inflammatory response.
• **Dependent variable** – "DNA methylation"
• **Independent variable** – cigarette usag

METHODOLOGY

Sources of data and measurements –

• Sociodemographic information, such as age, gender, socioeconomic position, education, and occupation.

• Tobacco use – comprehensive history, including duration, smoked/non-smoked form, frequency/per day, placement, quadrant/overall, chewing duration, and any other habits.

• "Oral Potentially Malignant Disorders" — cases of "OMPDS" that have been histopathologically diagnosed.

• "DNA methylation" during clinical evaluation if a patient provisionally diagnosed with "oral potentially malignant disorders (OPMDS)" and a habit of tobacco smoking in any form is enrolled in the study with their informed agreement. They'd be included in the study if histopathology results revealed "oral possibly malignant diseases." Individuals who would come to the OPD for minor surgical treatments such as "disimpaction," "crown lengthening," and other procedures other than biopsy for precancerous or cancerous tumours. They would be categorized into two groups – one with a smoking habit in any form and the other without any habit or disease. Their informed consent would also be obtained. Approximately 1 mL of saliva was collected from each individual. They were instructed to rinse their mouth five times with water (to remove food debris) and to scrap their buccal mucosa with a new set of toothbrush so, as to get a quality saliva sample containing buccal epithelial cells. The saliva samples were collected in sterile containers. The samples collected outside the laboratory were placed in a sealed plastic bag and transported in dry ice to the laboratory. Tissue samples would also be obtained from healthy individuals & individuals with smoking habit but without disease.

DNA isolation

The "HiPurA Mammalian Genomic DNA Purification Kit (HiMedia Labs.)" would then be used to isolate DNA from saliva. Nanodrop 2000 would test the purity and aggregation of DNA (ThermoScietific). The EZ DNA methylation Gold Kit (Zymo Research, USA) would select around 500 ng of genomic DNA from each individualspecimen for sodium bisulfitetranslation as per the manufacturer's instructions. The Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc, USA) would be used to assess genome-wide DNA methylation according to the manufacturer's instructions.

"Bisulfite sequencing PCR and quantitative real-time PCR for methylation analysis"

Bisulfite sequencing PCR (BSP) was done with bisulfite conversion-specific primers produced by MethPrimer. The BSP compounds will be quantified on a one percent agarose gel. A fraction of the products obtained in BSP were then analysed with the help of qMSP. This qMSP related reactions would be carried out utilizing an ABI 7900HT Fast Real-Time PCR System Instrument (USA) with the help of primers specific for methylation and Rox Fast Start Universal SYBR Green Master Mix (Roche, Switzerland).[6]

BSP cloning and sequencing

The products of BSP were then choosen for the purpose of sequencing and cloning. Another portion of the products of BSP products would be purified with the help of device namely MiniElute Gel Extraction Kit (USA). Nearly one hundred sixty five ng of real product of BSP was attached to the TA based vector namely PTZ57R/T with the help of T4 Deoxyribonucleic acid

Ligase (Japan). To confirm the likely positive clones, colony PCR will be conducted under normal conditions with universal M13 forward and reverse primers. The positive clones will next be sequenced using the 3100 Genetic Analyzer (ABI, USA). With the help of chromatogram collected and the percentage of methylation calculated, the methylation status of each clone was established.[6]

Gender bias and age bias are two examples of bias (would be removed by regression analysis)

Statistical Analysis

SPSS software 2021 was used for statistical analysis. Chi square test and one way ANOVA was used for statistical analysis. P value ≤ 0.05 was considered as statistically significant.

Results

66.66% of study participants were found to use tobacco in various forms while 33.33% of study participants were found to have no history of tobacco use (table 1, graph 1). One third of the study participants were tobacco abusers with no disease, one third of the study participants were tobacco users having OMPD and one third of the study participants were healthy individuals with no history of tobacco use and oral potential malignant disorders (table 2, graph 2)

60% of tobacco abusers were found to use tobacco in smoked form while 40% of tobacco users were found to have history of smokeless tobacco use (table 3, graph 3). 20% of the tobacco users each had frequency of 5 to 8 times each day, 6 to 10 times each day, 6 to 11 times each day, 9 to 12 times each day and 8 to 10 times each day (table 4, graph 4). 20% each of tobacco users were using tobacco since 8 to 10 years, 11 to 12 years, 16 to 18 years while 40% of tobacco users were using tobacco since 13 to 15 years. (table 5, graph 5).

50% of the smokeless tobacco users placed tobacco in buccal vestibule while 50% of the smokeless tobacco users placed tobacco in labial vestibule.(table 6, graph 6). There was involvement of buccal mucosa of third and fourth quadrant in 25% study participants using smoked tobacco while there was involvement of all quadrants in 75% of study participants having smoked tobacco. (table 7, graph 7). 33.33% study participants using smokeless tobacco chewed tobacco for 20 to 30 minutes while 66.66% study participants using smokeless tobacco chewed tobacco for 30 to 40 minutes (table 8, graph 8)

33.33% of study participants used alcohol while 66.66% of the study particants had no habit other than tobacco (table 9, graph 9). Methylation of all the genes i.e p16, DAP-K and MGMT was observed in 11.9% of oral premalignant lesions. 38.51% of oral premalignant lesions were found to have methylation of two genes. 12.23% cases were found to associated with methylation of p16 and DAP-K, 12.12% cases were found to have methylation in the p16 and MGMT and 14.16% cases of oral premalignant lesions showed methylation of DAP-K and MGMT. The difference was non significant statistically. ($p \geq 0.05$) (table 10, graph 10).

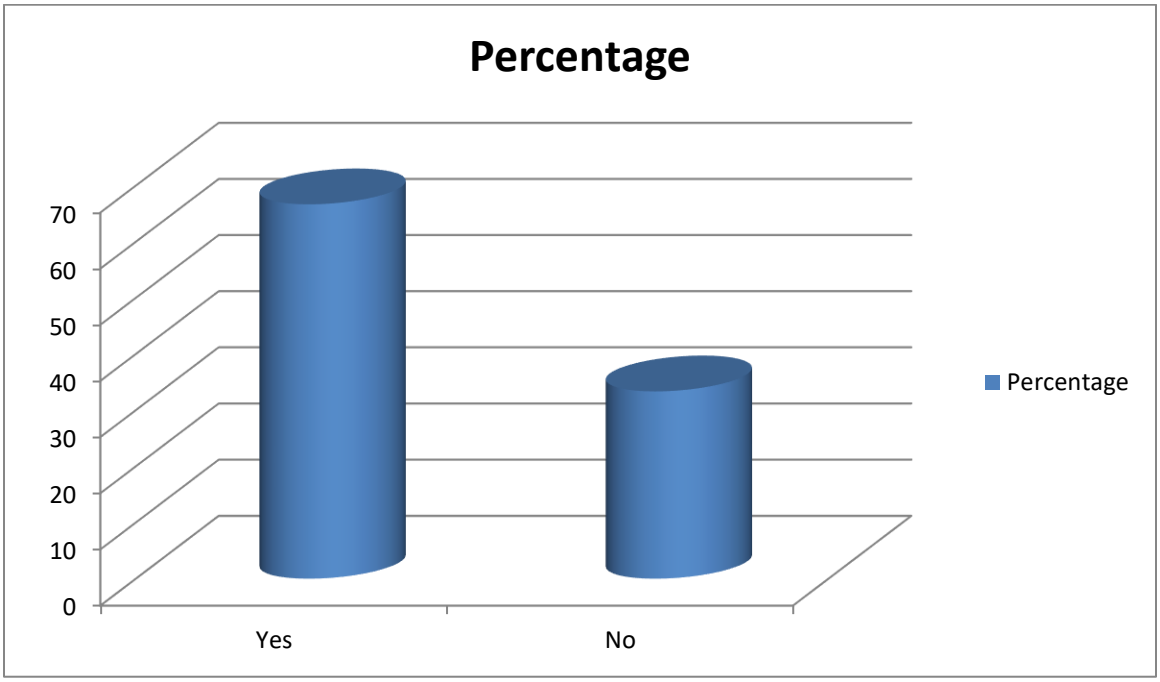
Methylation of all the genes i.e p16, DAP-K and MGMT among tobacco users with no oral premalignant lesions was observed in 9.8% cases. 31.2 cases were found to have methylation of two genes. 10.14% cases were found to associated with methylation of p16 and DAP-K, 09.43%

cases were found to have methylation in the p16 and MGMT and 11.27% cases showed methylation of DAP-K and MGMT. The difference was significant statistically. ($p \leq 0.05$). (table 11, graph 11)

Methylation of all the genes i.e p16, DAP-K and MGMT among normal healthy individuals was observed in 1.7% cases. 2.11 % cases were found to associated with methylation of p16 and DAP-K, 1.21% cases were found to have methylation in the p16 and MGMT and 2.38 % cases showed methylation of DAP-K and MGMT. The difference was significant statistically. ($p \leq 0.05$).(table 12, graph 12)

Table 1 : Details about tobacco abuse

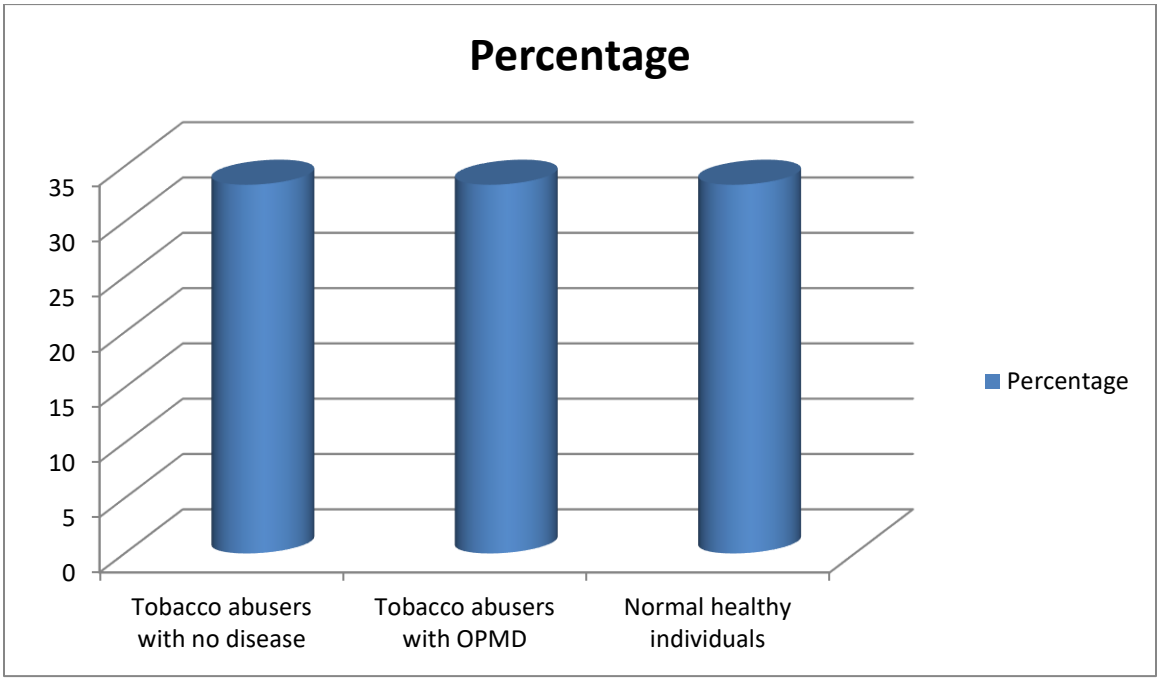
Tobacco usage	**Percentage**
Yes	66.66%
No	33.33%



Graph 1: Details about tobacco abuse

Table 2: Distribution of study subjects according to tobacco use and presence of OPMD.

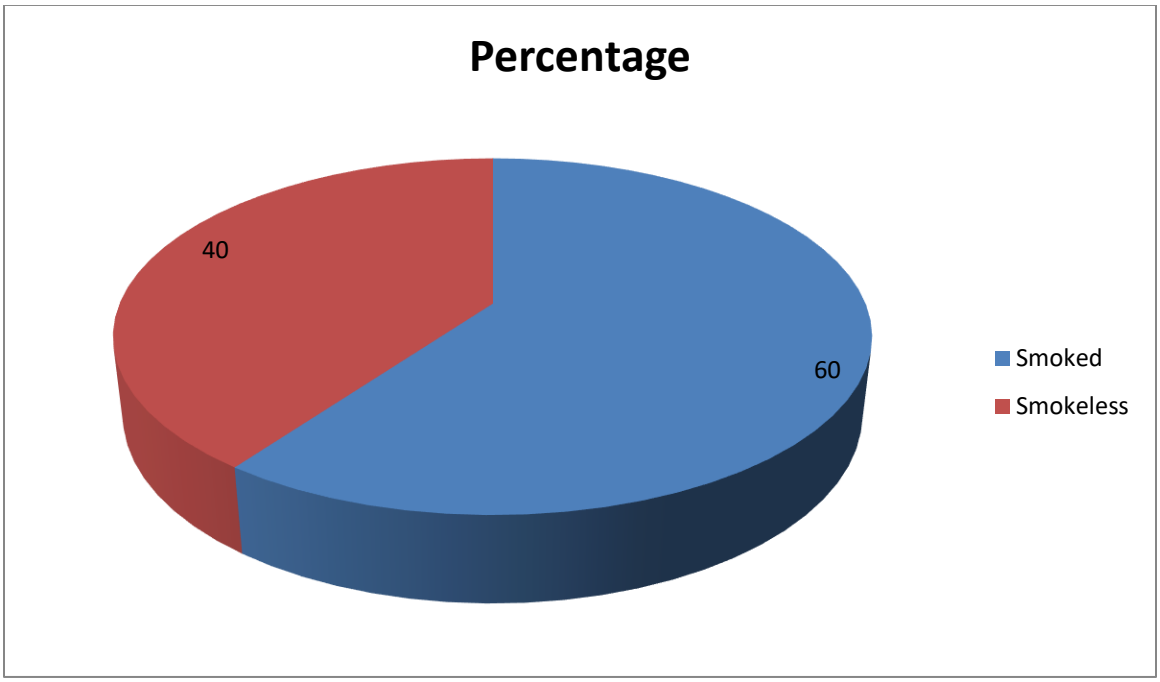
Subgroup	**Percentage**
Tobacco abusers with no disease	33.33
Tobacco abusers with OPMD	33.33
Normal healthy individuals	33.33



Graph 2: Distribution of study subjects according to tobacco use and presence of OPMD.

Table 3: Form of tobacco used

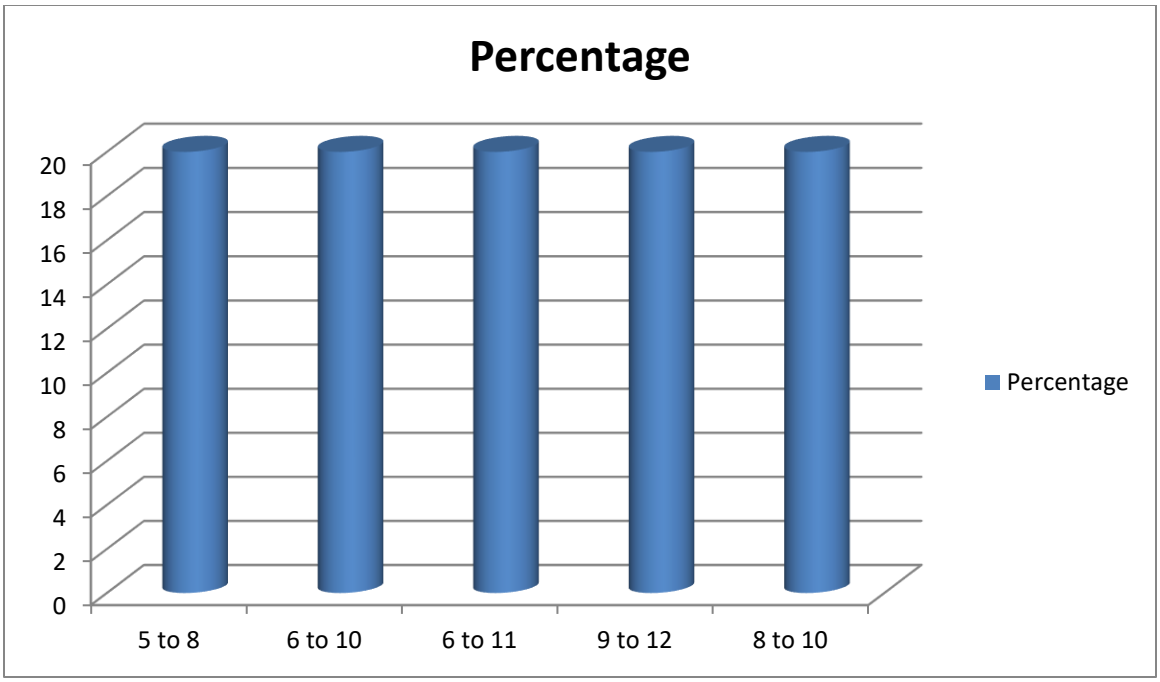
Tobacco usage	**Percentage**
Smoked	60
Smokeless	40



Graph 3: Form of tobacco used

Table 4: Frequency of the tobacco use in different forms (No/ Day)

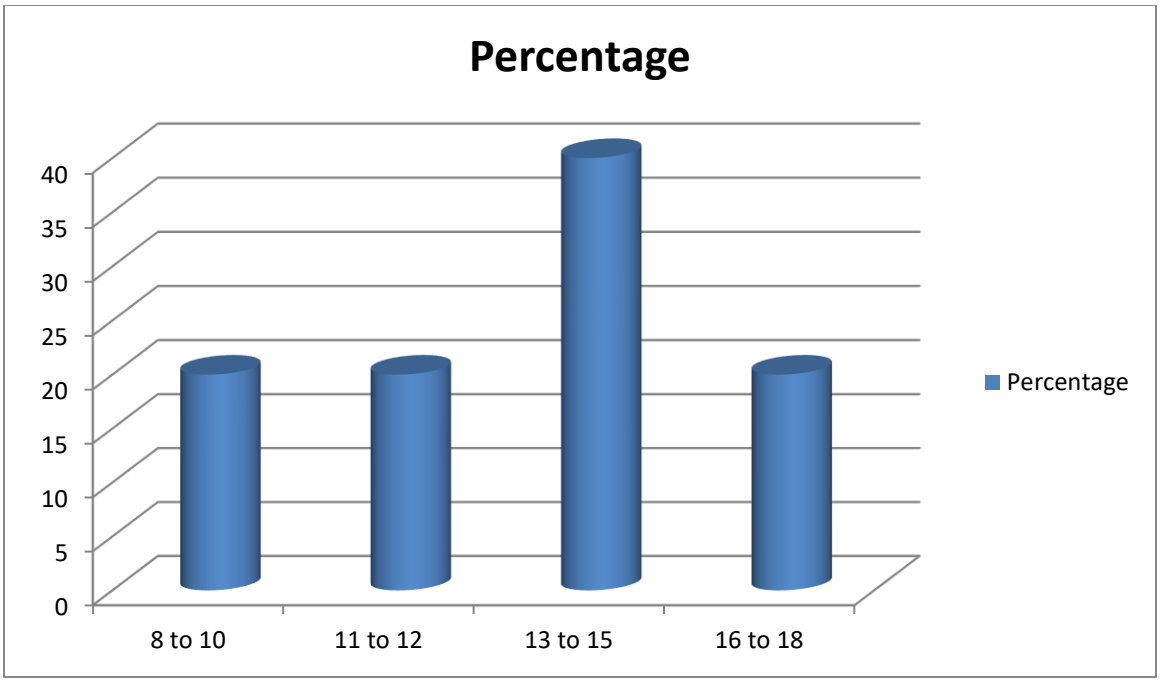
Frequency (No/Day)	**Percentage**
5 to 8	20
6 to 10	20
6 to 11	20
9 to 12	20
8 to 10	20



Graph 4: Frequency of the tobacco use in different forms (No/ Day)

Table 5: Years of tobacco usage

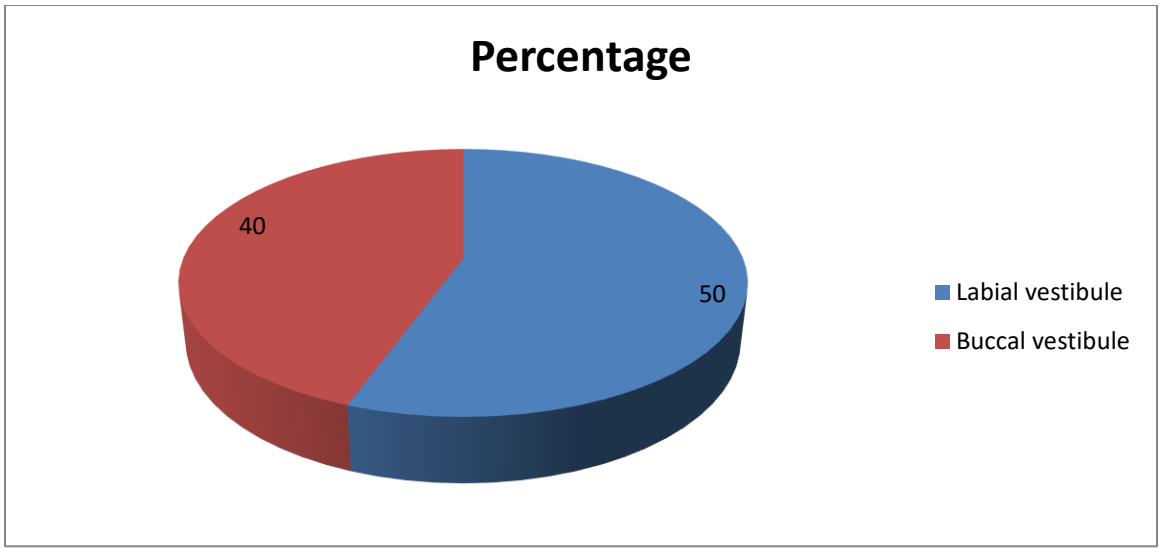
Years of tobacco use	**Percentage**
8-10	20
11-12	20
13-15	40
16-18	20



Graph 5: Years of tobacco usage

Table 6: Location of placement of smokeless tobacco

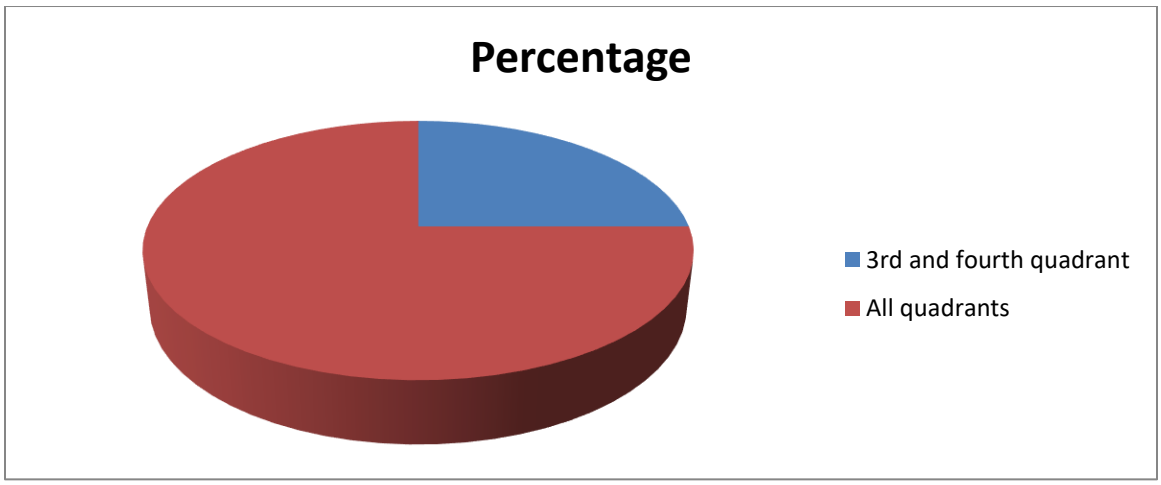
Location	**Percentage**
Labial vestibule	50
Buccal vestibule	50



Graph 6: Location of placement of smokeless tobacco

Table 7 : Involvement of the different quadrants in smoked tobacco

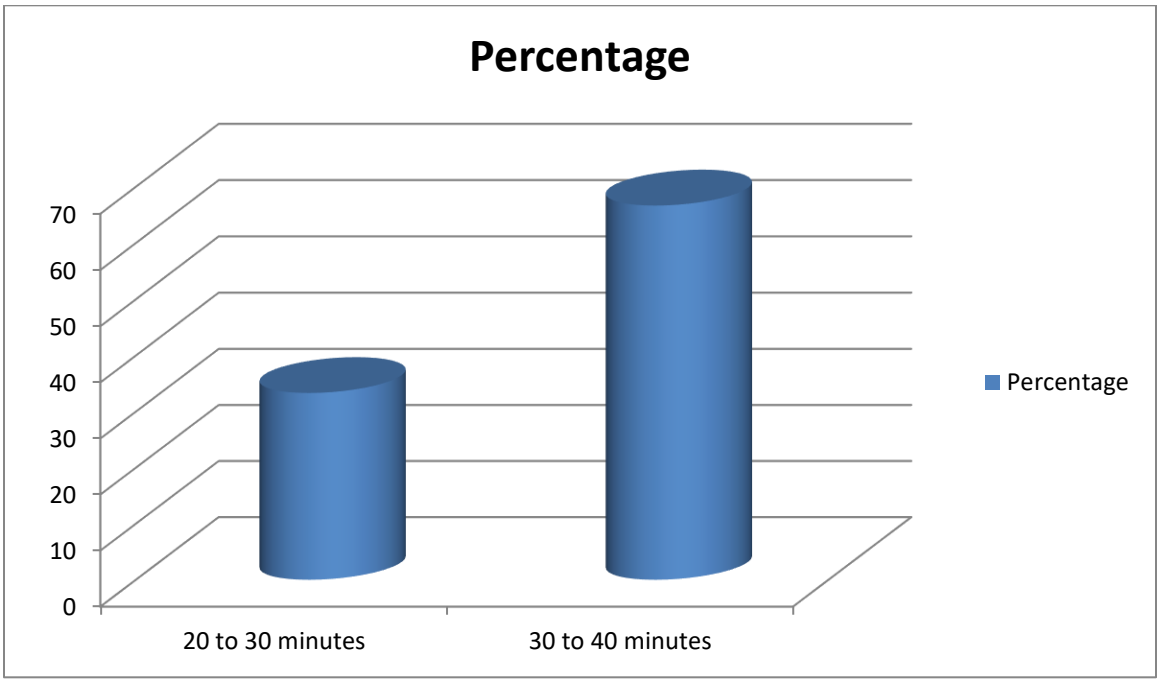
Location	**Percentage**
3rd quadrant and 4th quadrant	25%
All quadrants	75%



Graph 7 : Involvement of the different quadrants in smoked tobacco

Table 8: Duration of chewing

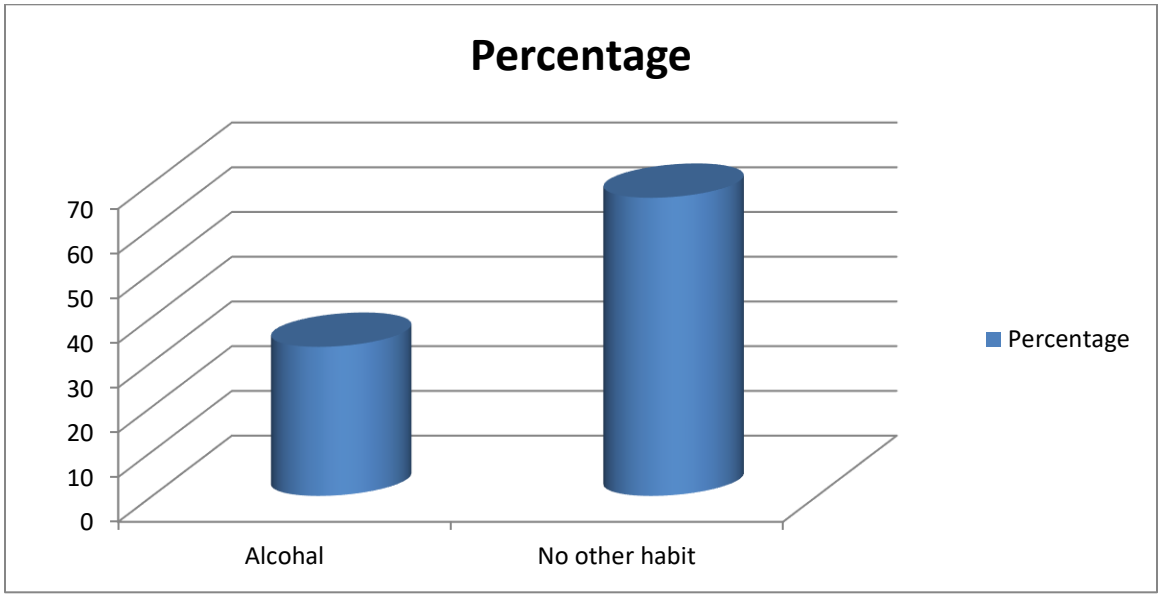
Location	**Percentage**
20 to 30 minutes	33.33
30 to 40 minutes	66.66



Graph 8: Duration of chewing

Table 9 : Presence of habit other than tobacco

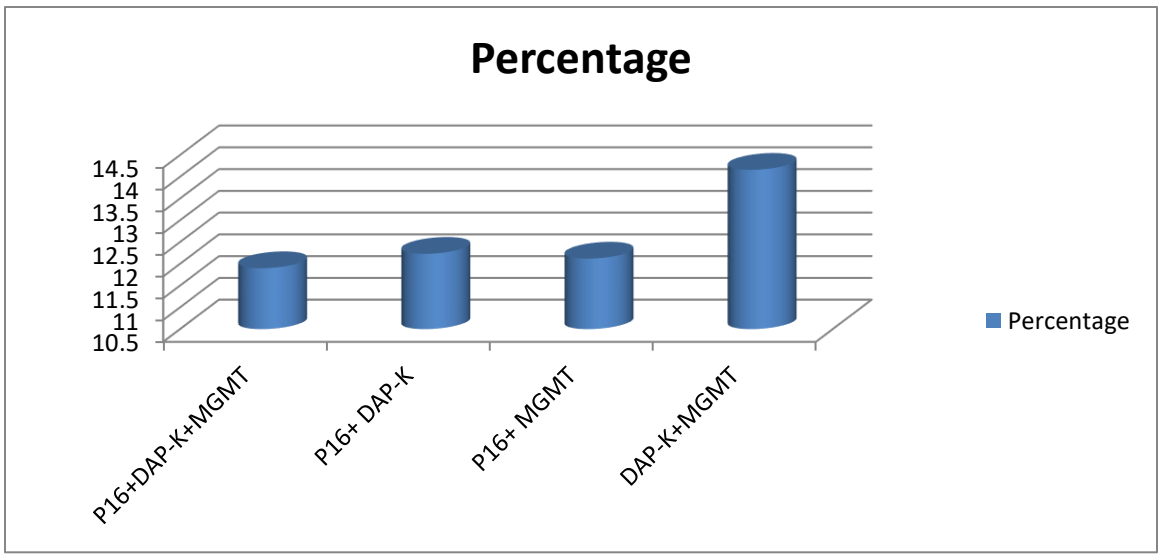
Habit other than tobacco	**Percentage**
Alcohal	33.33
No other habit	66.66



Graph 9 : Presence of habit other than tobacco

Table 10: Combination of DNA methylation of gene p16, gene DAP-K and gene MGMT in tobacco users with oral premalignant lesions.

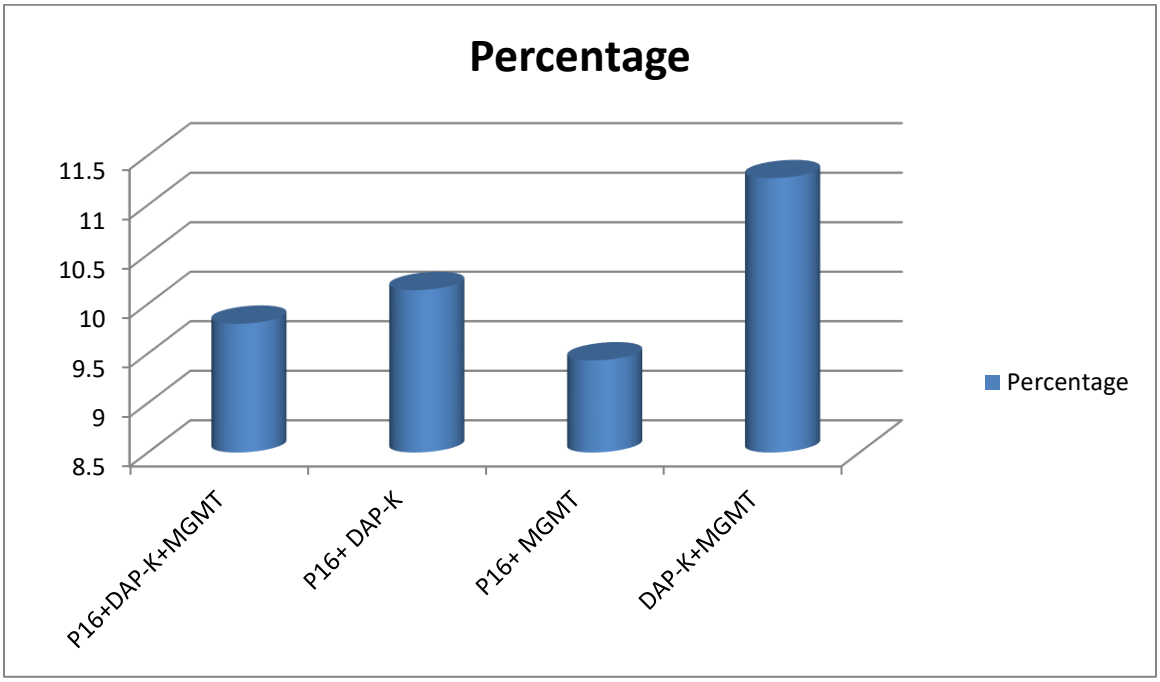
Combination of Genes	DNA methylation %	P value
P16+DAP-K+MGMT	11.9	
P16+ DAP-K	12.23	0.07
P16+ MGMT	12.12	
DAP-K+MGMT	14.16	



Graph 10: Combination of DNA methylation of gene p16, gene DAP-K and gene MGMT in tobacco users with oral premalignant lesions.

Table 11: Combination of DNA methylation of gene p16, gene DAP-K and gene MGMT in tobacco users with no oral premalignant lesions.

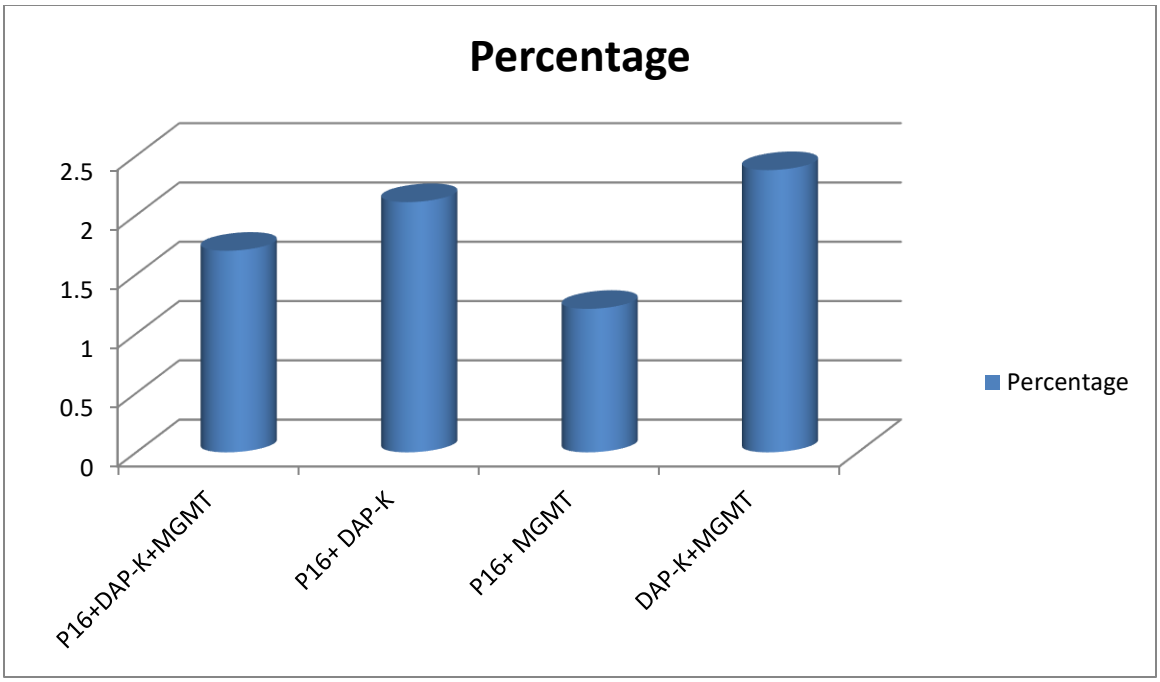
Combination of Genes	DNA methylation %	P value
P16+DAP-K+MGMT	9.8	
P16+ DAP-K	10.14	0.07
P16+ MGMT	09.43	
DAP-K+MGMT	11.27	



Graph 11: Combination of DNA methylation of gene p16, gene DAP-K and gene MGMT in tobacco users with no oral premalignant lesions.

Table 12: Combination of DNA methylation of gene p16, gene DAP-K and gene MGMT in normal healthy subjects

Combination of Genes	DNA methylation %	P value
P16+DAP-K+MGMT	1.7	
P16+ DAP-K	2.11	0.07
P16+ MGMT	1.21	
DAP-K+MGMT	2.38	



Graph 12: Combination of DNA methylation of gene p16, gene DAP-K and gene MGMT in normal healthy subjects

Discussion

Tobacco consumption is considered as most important risk factor for development of OPMDs. Tobacco either in the smokeless form, smoked form may contribute to development of OPMDs.[11,12] It is not clear whether DNA methylation is associated with tobacco use in patients with OPMDs.[13,14] Therefore this study was conducted to identify, correlate and compare "DNA methylation" among healthy people, tobacco users having premalignant disease, and tobacco users donot having premalignant disease.

It was inferred that methylation of p16, MGMT and DAP-K was quite significant in tobacco users having diseases. Further it was also observed that methylation of p16, MGMT and DAP-K was also quite significant in tobacco users having no diseases. Therefore it can concluded that DNA methylation" in tobacco abusers without any existing oral disease elevates the probability of development of "oral potentially malignant disorders". Hence the null hypothesis that "DNA methylation" evaluation might not be used in tobacco abusers without any existing oral disease to analyse the probability of development of "oral potentially malignant disorders" was rejected.

In our research there were some very important useful findings regarding the possibility of use of deoxyribonucleic acid methylation as potential biomarker for progression of premalignancy and malignancy of oral cavity which need to be further followed up. In this study methylation of DNA at p16 was observed in 25.6% of the oral premalignant lesion in tobacco users, methylation of DAP-K was observed in 32.3% lesions while methylation of MGMT was observed in 30.3 oral premalignant lesions. The finding was statisrically significant. ($p \leq 0.01$).

In a systematic review it was found that most of the studies utilised busulfide conversion method and Ms-Pcr technique for measurement of the methylation of deoxyribonucleic acid with proper procedures for quality control.[15,16] These methods are standard methods with well proven validity worldwide. Another important feature was that most of the studies used those tissue samples for carrying out analysis of methylation which were confirmed with the biopsy procedure.[17,18] It was also observed that 2 studies demonstrated that saliva can be another medium for analysis of methylation of the deoxyribonucleic acid in subjects with premalignancy and malignancy of oral cavity. There is added advantage with use of saliva as medium for analysis because it is non invasive in nature. But a research demonstrated reporting of lower production of DAPK induced methylation as compared with blood and tissue samples.[11,12] It has been found that patterns of methylation are specific for specific tissues. Besides profile of methylation for a tissue is different on analysing saliva or blood. It is being argued that methylation is the reason for different expression of different genes. Therefore specific tissue samples may be helpful in revealaing correct epigenetic addition of methyl groups to the dinucleotide of deoxyribonucleic acid. It will be helpful in proper analysis of the pathway of the disease.[19,20]

Alongwith tissue sample there can be use of saliva and whole blood for the analysis of methylation. But there is some disadvantage with these samples. In case of whole blood there are different cells which have different patterns for methylation process.[21,22] As a result whole blood become non specific sample for analysis. In case of saliva there are chances of contamination of saliva due to debris of food, microorganisms and residual cells. However there are few studies which have demonstrated better results by using saliva and whole blood samples for detecting the biomarkers specifically found in saliva like KIF1A and EDNRB And there has been evidence of significant correlation between the blood samples and the tissue samples. ($p < 0.001$).[20-24]

Methylation of all the genes i.e p16, DAP-K and MGMT was observed in 11.9% of oral premalignant lesions. 38.51% of oral premalignant lesions were found to have methylation of two genes. 12.23% cases were found to associated with methylation of p16 and DAP-K, 12.12% cases were found to have methylation in the p16 and MGMT and 14.16% cases of oral premalignant lesions showed methylation of DAP-K and MGMT. The difference was non significant statistically. ($p \geq 0.05$)

Methylation of all the genes i.e p16, DAP-K and MGMT among tobacco users with no oral premalignant lesions was observed in 9.8% cases. 31.2 cases were found to have methylation of two genes. 10.14% cases were found to associated with methylation of p16 and DAP-K, 09.43% cases were found to have methylation in the p16 and MGMT and 11.27% cases showed methylation of DAP-K and MGMT. The difference was significant statistically. ($p \leq 0.05$). Methylation of all the genes i.e p16, DAP-K and MGMT among normal healthy individuals was observed in 1.7% cases. 2.11 % cases were found to associated with methylation of p16 and DAP-K, 1.21% cases were found to have methylation in the p16 and MGMT and 2.38 % cases showed methylation of DAP-K and MGMT. The difference was significant statistically. ($p \leq 0.05$).

Several contemporary DNA libraries lack good smoking description, and the emergence of vaporizers has turned conventional cotinine analysis based methods of detecting status of smoking unusable in several currently undergoing investigations.[15-21] They previously demonstrated that modification level of cg05575921 for whole plasma DNA may forecast cigarette usage with high accuracy. However, it is yet to be determined if epigenomics activity in saliva should be utilised in the same way.[17-25]

To evaluate and examine the efficacy of cg05575921 in diagnosing and measuring smoking tobacco, authors utilised DNA of four hundred biochemically verified people who smoke or people who dont smoke. A prototype including gender, age, and epigenetic modification level with a regressive epigenetic modifications sensitivity to smoking seemed to have ROC analysis alongwith AUC analysis for estimating smoking level of 0.995 through using blood deoxyribonucleic acid.[14-19] The ROC AUC in identifying cigarette consumption employing salivary Deoxyribonucleic acid remained 0.971, with a graph illustrating the connection between DNA epigenetic methylation and each day cigarette intake that looked virtually identical to just that observed from blood deoxyribonucleic acid.[20-25] The AUC with salivary deoxyribonucleic acid was increased to 0.981 by providing comments from some other epigenetic indicator meant to adjust for cellular diversity.

Ultimately, modification of cg05575921 was not substantially different than counterparts in thirty one participants who had stop cigarettes ten years or more ago.[13-16] The modification status of cg05575921 in deoxyribonucleic acid from blood or saliva indicates cigarettes habit and tobacco cigarette use, according to the researchers. These methylation tests for scientifically determining level of smoking, authors believe, will be useful in scientific, medical, and social purposes.[15-19]

cg05575921 alteration reduces in consequence to cigarettes, according to multiple studies. However, the amplitude and dose specificity of such reaction are yet unknown due to technical difficulties. This uncertainty is a roadblock to using deoxyribonucleic acid alteration in clinical settings to detect and track status of smoking.[23-25]

By examining the global and Indian contexts, we may conclude that studies of "DNA Methylation" in saliva are understudied in India compared to tissue and blood. As a result, saliva should be used to identify "DNA methylation" as a diagnostic marker because it is a simple, non-invasive, and highly sensitive approach. After reviewing several research, we determined that the "tumour suppressor gene" loci "p14, p15, and p16" are the most affected, and so we will detect alterations in them

Conclusion

Methylation of p16, MGMT and DAP-K was quite significant in tobacco users having diseases. Further it was also observed that methylation of p16, MGMT and DAP-K was also quite significant in tobacco users having no diseases. Therefore it can concluded that DNA methylation” in tobacco abusers without any existing oral disease elevates the probability of development of “oral potentially malignant disorders.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Perklin DM. GLOBOCAN 2012 v2.0 cancer incidence and mortality worldwide: IARC CancerBase No. 1.0. Lyon (France): International Agency for Research on Cancer; 2012. Available from: http://globocan.iarc.fr [accessed on 30th April 2015].
2. Rosas SL, Koch W, da Costa Cavalho MG, et al. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res. 2001;61(3):939–942.
3. Sanchez-Cespedes M, Esteller M, Wu L, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res. 2000;60(4):892–895.
4. Huber MA (2006) A review of premalignant oral conditions. Tex Dent J 123:502–509.
5. Gupta B, Ariyawardana A, Johnson NW. Oral cancer in India continues in epidemic proportions: evidence base and policy initiatives. Int Dent J 2013;63 (1):12–25.
6. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009;45(4–5):309–16.
7. Bessell A, Glenny AM, Furness S, Clarkson JE, Oliver R, Conway DI, et al. Interventions for the treatment of oral and oropharyngeal cancers: surgical treatment. Cochrane Database Syst Rev 2011;9:CD006205.
8. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J ClinOncol 2013;31(36):4550–9.
9. Napier SS, Speight PM. Natural history of potentially malignant oral lesions and conditions: an overview of the literature. J Oral Pathol Med 2008;37(1):1–10.
10. Towle R, Garnis C. Methylation-mediated molecular dysregulation in clinical oral malignancy. J Oncol2012;2012:170–2.
11. Mishra R. Biomarkers of oral premalignant epithelial lesions for clinical application. Oral Oncol 2012;48(7):578–84.
12. Pitiyage G, Tilakaratne WM, Tavassoli M, Warnakulasuriya S. Molecular markers in oral epithelial dysplasia: review. J Oral Pathol Med 2009;38 (10):737–52.
13. Mehanna HM, Rattay T, Smith J, McConkey CC. Treatment and follow-up of oral dysplasia – a systematic review and meta-analysis. Head Neck 2009;31 (12):1600–9.
14. Rajaraman P, Anderson BO, Basu P, Belinson JL, D'Cruz A, Dhillon PK, et al. Recommendations for screening and early detection of common cancers in India. Lancet Oncol 2015;16(7):e352–61.
15. Sankaranarayanan R, Ramadas K, Thomas G, Muwonge R, Thara S, Mathew B, et al. Effect of screening on oral cancer mortality in Kerala, India: a cluster randomized controlled trial. Lancet 2005;365(9475):1927–33.

16. Kulkarni V, Saranath D. Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues. Oral Oncol 2004;40(2):145–53.
17. Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007;36(10):575–80.
18. Bird A. Perceptions of epigenetics. Nature 2007;447(7143):396–8.
19. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev 2011;25(10):1010–22.
20. Baumgartel K, Zelazny J, Timcheck T, Snyder C, Bell M, Conley YP. Molecular genomic research designs. Annu Rev Nurs Res 2011;29:1–26.
21. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358(11):1148–59.
22. Al-Kaabi A, van Bockel LW, Pothen AJ, Willems SM. p16(INK4A) and p14(ARF) gene promoter hypermethylation as prognostic biomarker in oral and oropharyngeal squamous cell carcinoma: a review. Disease Markers 2014.
23. Zeilinger S, Kuhnel B, Klopp N, Baurecht H, Kleinschmidt A, Gieger C, et al. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PLoS ONE 2013;8(5):e63812.
24. Guida M, Sanguedolce F, Bufo P, et al. Aberrant DNA hypermethylation of hMLH-1 and CDKN2A/p16 genes in benign, premalignant and malignant endometrial lesions. Eur J GynaecolOncol. 2009;30(3):267–270.
25. Iliopoulos D, Oikonomou P, Messinis I, Tsezou A. Correlation of promoter hypermethylation in hTERT, DAPK and MGMT genes with cervical oncogenesis progression. Oncol Rep. 2009;22(1):199–204.