



DNA Methylation Patterns in Saliva of Tobacco users with High-Risk Oral Potentially Malignant Disorders

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Authors' contributions

This work was carried out in collaboration among all authors. Author MP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MSC managed the analyses and supervision of the study. Author MNG managed the literature searches analyses of the study. Author AHH managed the validation and visualization of the study. All authors read and approved the final manuscript.

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ABSTRACT

Background: "Oral Potentially Malignant Disorder (OPMD)" is a well-known symptom that, if untreated, can be carcinogenic. It includes leukoplakia, erythroplakia or erythroleukoplakia. One of the typical premalignant lesions of the oral cavity is "oral leukoplakias (OLs)," which frequently precedes "OSCCs." OLs with dysplastic characteristics are considered to be at a higher risk of "malignant transformation." So, early diagnosis of "oral squamous cell carcinomas (OSCCs)" is desperately required to enhance patient prognosis and quality of life (QOL). As a result, we examined the distinctive promoter methylation presence in high-risk OLs.

Objectives: To detect, compare & correlate "DNA methylation" patterns in normal individuals, tobacco users without disease and tobacco users with the disease.

Methodology: With the participants' full consent, 48 saliva samples were obtained and prepared.

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DNA isolation, restriction digestion of genomic DNA, extraction of restriction enzyme digested genomic DNA, Polymerase Chain Reaction (PCR), and Agarose Gel Electrophoresis (AGE) were all carried out.

Expected results: This study will help us to assess the use of Saliva as an aid to identifying both high and low risk "Oral Potentially Malignant Disorders."

Conclusion: Peculiar promoter methylation of various genes was related to a high possibility of malignant transformation in OLS.

Keywords: Oral leucoplakia; oral squamous cell carcinoma; methylation; promoter methylation; gene silencing.

1. INTRODUCTION

Worldwide, "Oral cancer" is a foremost issue of public health [1–3] One third of the cases (37.5%) found in the Asian zone only [1]. A "pre-malignant lesion" term was introduced by Victor Babes (1875), is a condition, without treatment could progress to "cancer." Oral pre-malignant lesions (OPMLs) are affecting around 2.5% of people are a specific goal for the prevention of cancer.

Leucoplakia, erythroplakia or erythroleukoplakia are the important clinical signs, their significance stems from the huge percentage of cases where a biopsy shows dysplasia or alike "frank carcinoma" [4-5]. From the pre-cancerous phase to cancer, there is a sequential histopathological series that can be staged as normal, hyperplastic and carcinoma *in situ* [6-10]. Pre-cancerous lesions of the oral cavity can easily be detected by visual examination and have direct accessibility for further investigations such as cytology and biopsy [11]. Early detection of lesions significantly lower the morbidity and mortality rate, [12] delayed detections, specifically in countries of high incidence zone affects the survival rate even though there are advanced techniques for the treatment [3,4,13].

Pre-cancerous lesions of the oral cavity include miscellaneous lesions and conditions like leucoplakia, erythroplakia, lesions of the palate in submucous fibrosis, actinic keratosis, discoid lupus erythematosus, lichen planus, and reverse smokers collectively known as "potentially malignant disorders (PMDs)" [14]. It has been observed that within 0.5 to 16 years, 12.3% of these pre-cancerous lesions transformed into malignancies [10]. The existing 'wait and watch' strategy for tracking cancer advancement is dictated by the clinico-morphological problem about the identification, diagnosis, and early treatment of pre-cancerous lesions of the oral cavity [6,10]. Both over and undertreated

cases result in significant patient "morbidity" [7,9,10].

In this situation, where clinicopathologic analyses are inconsistent in identifying pre-cancer at progression threat and a sequential epigenetic and genetic changes advancement of the signal of disease, the detection of "molecular biomarkers" of progression of the disease may be extremely effective in the initial revealing of reversible lesions, letting for better diagnosis and treatment [7,10].

Time patterns of "abnormal methylation" increasing or decreasing could predict the rate and likelihood of "malignant transformation" as well as disease state reversal. Because of these factors, pathological "DNA methylation" is supposed to be an especially promising biomarker for testing for oral pre-cancer progression at early stages.

The transformed gene expression configurations allow for unique phenotypes are triggered by epigenetic modifications like "DNA methylation" and various histone modifications. While "DNA methylation" is essential for normal mammalian growth, "abnormal methylation" configurations have been associated with a number of differentiation-related diseases, including several types of human cancers. Considering the high frequency of this epigenetic transition in "oral epithelial dysplasia," research into promoter methylation of "tumor-suppressor genes" in the context of "OPMD" seems appropriate. Early epigenetic changes may potentially expose cells to further genetic abnormalities, allowing the "neoplastic process" to progress [1]. As a result, recognizing gene methylation as an effective marker could provide a sensitive method for detecting "OPMD." DNA methylation is a favorable target for "anticancer therapy" and aids in understanding the epigenetic pathway to cancer.

1.1 Rationale

The investigation of "epigenetic changes" in cancer, such as "oral cancer" and "pre-cancerous lesions," has sparked a lot of interest. Without modifying the structure or sequence of genes, these procedures impair or inactivate their functions. Methylation of the promoter site of certain genes participate in the regulation of the cell cycle is one example. Sympathetic gene expression in both natural and pathological conditions necessitates a thorough understanding of methylation patterns. Methylation shifts may be used as a possible and responsive molecular marker for identifying risk states, detecting cancer early, and monitoring prognosis. Better mastery of this epigenetic change may help with the diagnosis and prognosis of oral cancer, in addition, to the development of new therapeutic approaches.

1.2 Objectives

- To identify "DNA methylation" configurations in normal individuals.
- To identify "DNA methylation" configurations in tobacco users without the disease.
- To identify "DNA methylation" configurations in tobacco users with disease :
 - A) "Premalignant lesion" (Leukoplakia – high risk)
 - B) "Premalignant lesion" (Leukoplakia – Low risk)
- To compare & correlate the above three.

Hypothesis

- RESEARCH HYPOTHESIS:

"DNA methylation" in tobacco users without disease increases the risk of emerging "oral potentially malignant disorders".
- NULL HYPOTHESIS:

"DNA methylation" assessment may not be indicated in tobacco users without the disease to evaluate the risk of emerging "oral potentially malignant disorders".

2. METHODS

This cross-sectional research would be carried out at the OPD in the Department of Oral

Pathology & Microbiology, SPDC in collaboration with Human Genetics Unit, Sterling Accuris Diagnostic Human Genetic Unit, Ahmedabad.

2.1 Participants

Inclusion criteria

- Individuals with a habit of tobacco use in any form with/without the disease.
- Histopathologically diagnosed case of oral potentially malignant disorders.
- Both genders included.

Exclusion criteria

- Any other inflammatory condition like a sharp tooth, gingivitis, periodontitis in smokers users without the disease.
- Those who did not give consent.

2.2 Variables

- Outcome – "Oral potentially malignant disorders"
- Exposure – Cigarette Smoke.
- Dependent variable – "DNA methylation."
- Independent variable – Tobacco use.
- Confounding factors – Any other habit, genetic susceptibility, any other inflammatory condition.

2.3 Data Sources/ Measurements

- Sociodemographic details – Age, sex, socioeconomic status, education, occupation, etc.
- Tobacco use – Detailed history like duration, smoked/smokeless form, frequency/per day, placement, quadrant/overall, duration of chewing, associated with any other habit.
- "Oral Potentially Malignant Disorders" – Histopathologically diagnosed cases of "OMPDS".
- "DNA methylation"

While clinical assessment of a patient provisionally diagnosed with "oral potentially malignant disorders (OPMDS)" & habit of tobacco use in any form would be recruited for the study with their informed consent. They would be included in the study after confirmation of "oral potentially malignant disorders" from histopathological reports. Individuals who would approach the OPD for minor surgical procedures other than biopsy for precancerous or cancerous

lesions like “disimpaction”, “crown lengthening” procedures, etc. 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 the smoking habit but without the disease.

2.4 DNA Isolation

Saliva would then be subjected to DNA isolation using “HiPurA Mammalian Genomic DNA Purification Kit (HiMedia Labs.)”. The pureness and aggregation of DNA would be assessed by Nanodrop 2000 (ThermoScientific). About, 500 ng of genomic DNA from an individual specimen would be selected for sodium bisulfite translation by the EZ DNA methylation Gold Kit (Zymo Research, USA) as per the manufacturer’s directions. Genome-wide DNA methylation would be measured by the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc, USA) as per the manufacturer’s directions.

2.5 “Bisulfite sequencing PCR and quantitative real-time PCR for methylation analysis”

Bisulfite sequencing PCR (BSP) will be performed using MethPrimer-designed bisulfite conversion-specific primers. On a 1% agarose gel, the BSP substances will be measured. The quantitative real-time methylation-specific PCR will then be performed on a portion of the BSP products (qMSP). The qMSP reactions would be performed in a 7900HT Fast Real-Time PCR System Instrument (ABI, USA) with methylation-specific primers and FastStart Universal SYBR Green Master Mix (Rox) (Roche, Switzerland) [6].

2.6 BSP Cloning and Sequencing

After that, the BSP products will be chosen for cloning and sequencing. The MiniElute Gel

Extraction Kit will be used to purify another component of the BSP products (Qiagen Inc., USA). T4 DNA Ligase will be used to ligate about 165 ng of purified BSP product into the TA vector (PTZ57R/T) (Takara Bio Inc., Japan). Colony PCR using universal M13 forward and reverse primers and under normal conditions will be used to confirm the likely positive clones. The 3100 Genetic Analyzer will then sequence the positive clones (ABI, USA). The methylation status of each clone will be determined using the chromatogram obtained, and the percentage methylation measured [6].

2.7 Bias

Gender bias, Age bias (would be removed by regression analysis).

2.8 Sample Size

“Purposive sampling technique” is applied.

3 groups are as follows-

In each group, individuals would be enrolled randomly by using random table methods. So a total of 48 patients would be included in the given study. Therefore, 16 individual would be included in each group.

Group A: Normal healthy individuals.
Group B: Tobacco users with disease (OPMD).
Group C: Tobacco users without the disease.

2.9 Quantitative Variables

Tobacco use – A detailed case history.
“DNA methylation” – Quantitative real-time methylation-specific PCR (qMSP).

2.10 Statistical Methods

- The analysis would be performed using the “Predictive Analytics Software (SPSS 16.0 version)”.
- Repeated measures “ANOVA” (with post hoc as Bonferroni correction) would be used to link the continuous variables among the groups.
- Categorical variables would be evaluated using the “chi-square test or Fisher’s exact test”, as suitable.
- A p-value of < 0.05 would be measured statistically significant.

2.11 Analysis Plan

- “Tobacco”(Smoking) users with/without disease after informed consent would be included & detailed history of habit would be obtained.
- Histopathologically diagnosed cases of “OPMDS” would be included.
- The “DNA methylation” patterns of tobacco (smoking) users without disease would be compared & correlated with that of tobacco (smoking) users with disease (OPMDS) using statistically significant tests.

3. EXPECTED OUTCOMES/RESULTS

- This study will help us to assess the use of Saliva as a tool for identifying high and low risk “Oral Potentially Malignant Disorders”.

4. DISCUSSION

It is well-known fact that a progressively vital route for transcriptional inactivation of various “tumor suppressor and DNA repair genes” in “CpG island hypermethylation” in the gene promoter site has been observed in recent times [15]. Promoter hypermethylation is widely recognized as a key factor for the inhibition of tumor suppressor genes in advanced cancers. After observation of a large number of tumor specimens, serum, and saliva of head and neck cancer cases Sanchez-Cespedes et al. [3] and Rosas et al.,² discovered regular promoter hypermethylation of the tumor suppressor genes p16, DAP-K, and MGMT. Several other researchers have looked into the methylation profile of genes p16, DAPK, and MGMT in HNSCC, and observed that its a hopeful biomarker for after effects and early revealing of head and neck cancer reoccurrence [16-21]. Steinmann et al., found methylation of “tumor-related genes” was expressively higher (42%) in squamous cell carcinomas of the head and neck than in other samples (23%), but caution should be used when using these for diagnosis as few of them age-related. Even then, very few information about epigenetic changes in premalignant lesions, despite recent reports indicating a connection between premalignancy in endometrial, endobronchial, and cervical lesions and tumor-related genes like “CDKN2A/p16, DAP-K, and MGMT” [22-24]. In order to classify the time of these epigenetic changes in head and neck carcinogenesis, we explored the methylation status of the “p16, DAP-

K, MGMT, and GSTP1” genes in patients with leukoplakia.

Our findings indicate that several epigenetic mutations, similar to early genetic changes, have indeed occurred in “oral premalignant lesions” [25,26]. In premalignant oral lesions, we find promoter hypermethylation at p16 in 21 of 82 (25.6%), DAP-K in 28 of 87 (32.2%), and MGMT in 32 of 106 (30.2%).

There was no variance in the incidence of methylation of the “p16, DAP-K, and MGMT” genes in premalignant lesions relative to those previously recorded in “HNSCC” [2,3]. While DAP-K hypermethylation was observed in 18% of 111 HNSCC patients in the first series [3], 30% of the second series [2], that is so close to the occurrence found in oral premalignant lesions in the present research. Outcomes of the study suggest that methylation of genes took place initially while head and neck tumorigenesis, and subsequent genetic and epigenetic changes may have driven it.

“GSTP1” was not methylated in either of the premalignant lesions analyzed, meaning the gene is not a primary element for tumorigenesis in head and neck cancers relative to other cancers including breast, prostate, and renal cancers.¹⁴ In our finding, a single sample expressed “GSTP1 methylation.” While we are unable to clarify the initiation of the methylation, it seems that GSTP1 methylation is not a normal phenomenon in head and neck cancer. According to previous research, hypermethylation is not bounded to a solitary gene, but influences numerous genes at the same time. Various human neoplasms, including pancreatic adenocarcinoma, gastric cancers, and colorectal cancers, have been shown to have a hypermethylation phenotype. In the present study, it was also observed that “p16, DAP-K, and MGMT genes” was all methylated at the same time in some of the “precancer lesions” in this research. Twenty-three of the 68 (33.8%) methylation-positive “pre-malignant lesions” had epigenetic modifications on additional genes, and 8 of the 68 (11.8%) were methylated for all three genes studied at the same time, suggesting overall isolation of CpG island methylation in precancerous lesions, close to those seen in cancers. Deactivation of p16 gene was observed more often (56 percent vs. 11.1 percent) in oral premalignant lesions with DAP-K gene hypermethylation compared to those without, which was a significant variance. A significant

deactivation of MGMT gene was also observed in oral premalignant lesions with MGMT gene hypermethylation than those without (51.6% vs. 23.1%). Repeated methylation of numerous genes in oral premalignant lesions indicates that the system which generally defends CpG islands from methylation are dysfunctional in premalignant cells.

It's uncertain if a positive association among p16 and DAP-K methylation, as well as MGMT and DAP-K methylation, has any significance biologically, however it's probable such changes lead to tumorigenesis through various routes and mechanisms. DAP-K is a Ca²⁺/calmodulin-dependent serine/threonine kinase gene with ankyrin reappearances and death domain. This is just discovered to have significant tumour-suppressive properties, combining apoptosis regulation with metastasis control [27,28]. p16INK4a is an adverse regulator of the cell cycle that suppress the cyclin-dependent kinases CDK4 and CDK6. Methylation of p16INK4a [29,30] in cancer and premalignancy, and DAP-K lowest in cancers [3,9,12] is now well known as common epigenetic variations in a wide range of cancers.

A unique fascinating finding from the present research is "MGMT promoter methylation" is very common in nonsmokers' lesions. A DNA reconditioning protein, MGMT eradicates alkyl adducts out of the O6 site of guanine, shielding cells from the carcinogenic and cytotoxic responses of alkylating agents' lethal crosslinks [31]. The amount of MGMT in a tumor varies greatly depending on the type of tumor and also between tumors of the same type. p53 has previously been found to inhibit MGMT transcription at physiologic and supraphysiologic stages, despite the fact that changes in MGMT expression are unknown [3].

Transition mutations from the location G:A in K-ras as well as G:C to A:T in p53 are related to MGMT inactivation by promoter hypermethylation [32,33]. In lung carcinomas, a smoking-associated rise in MGMT expression has also been observed [34]. The detection of MGMT hypermethylation in nonsmokers may provide a different process of genetic instability by promoting p53 transitional mutations, instead of the normal smoking-related transversions [35-36], and by targeting another gene, such as K-ras. It's also conceivable such like "MGMT promoter methylation" causes an exceptional mutation phenotype marked via a slew of critical gene transition mutations. It is clear that further

research into MGMT hypermethylation in nonsmokers is required. Although "abnormal methylation" in oral premalignant lesions was commonly observed in current research, in case these epigenetic deviations are valuable adjuncts to the histopathologic evaluation of oral mucosal lesions for the assumption of the possibility of malignant transformation will need to be established by continuing follow-up of the study's group.

Few of the related studies on similar aspects were reported [37-40]. Studies on oral submucous fibrosis by Gadbaill et al. [41,42], and Hande et al. [43], were reviewed. Quazi et al., reported on the prevalence and pattern of tobacco use among tribal adolescent [44]. Studies by Yuwanati et al. [45], Borle et al. [46], and Khatib et al. [47,48], regarding treatment modalities were reported.

4.1 SCOPE

"DNA methylation" pattern could potentially be utilized as an early biomarker to indicate a malignant alteration in oral potentially malignant disorders. A better recognition of these epigenetic changes helps in greater assistance in the diagnosis and prognosis of oral cancer.

5. LIMITATIONS

As it is a cross-sectional study, there is a need for a longitudinal study to confirm the result.

IMPLICATIONS

"DNA methylation" pattern could potentially be utilized as an early biomarker to indicate a malignant alteration in oral potentially malignant disorders. Better recognition of these epigenetic changes helps in greater assistance in the diagnosis and prognosis of oral cancer.

6. CONCLUSION

By putting light in global and Indian scenario we come to the conclusion that :

1. The studies related to "DNA Methylation" in the saliva is a less explored field in India, as compared to tissue and blood. Therefore emphasis should be given to use saliva as a tool for detection of "DNA methylation" as a diagnostic marker as it is an easy and non-invasive and highly sensitive technique.

2. By screening different studies we came to the conclusion that “tumor suppressor gene” loci “p14, p15 and p16” are mostly affected, therefore we will be seeing changes in them.

CONSENT

As per international standard or university standard, patients’ written consent will be collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval will be collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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