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E igenetic Modifications of Nucleotide Excision Re air Genes in Oral S uamous Cell Carcinoma

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Abstract

Genotoxic exposure to tobacco carcinogens resulting in DNA damage is an important mechanism of oral squamous cell carcinoma (OSCC) etiology. Nucleotide Excision Repair (NER) pathway removes bulky DNA adducts, generated from tobacco exposure thereby playing a major role in initiation of OSCC. In addition to mutations, epigenetic modifcations have also been shown to target DNA repair genes thereby modulating oral tumor genesis. We therefore examined the role of epigenetic alterations modulating expression of three NER genes; XPC, XPB and XPD involved in removal of adducts caused by major classes of tobacco carcinogens and their contribution to OSCC

Methylation status of NER genes was assessed using methylation specifc PCR (MSP) in biopsies taken from 52 OSCC patients, their surrounding margins and 27 normal controls. The mRNA levels were determined using quantitative real time PCR (qRT-PCR) and Chromatin immuno precipitation (ChIP) analysis was performed to examine histone modifcations in selected NER genes.

We did not observe any signifcant difference in promoter methylation of NER genes between OSCC patients and controls. Increased XPB mRNA levels (p 0.04) and higher prevalence of H3 acetylation of XPB (p 0.04) gene were observed in OSCC patients as compared to controls.

Our fndings suggest the epigenetic modifcations regulating the expression of XPB gene may be involved in OSCC etiology.

Keywords: Oral squamous cell carcinoma; Nucleotide excision repair; DNA methylation; Histone modi cations

Abbreviations: OSCC: Oral squamous cell carcinoma; NER: Nucleotide excision repair; XPC: Xeroderma pigmentosum group C; MSP: Methylation-speci c PCR

Introduction

Whilst genetic alterations in oral squamous cell carcinoma (OSCC) have long been documented, the role of epigenetic changes in its etiopathogenesis is just being delineated. Epigenetic changes such as DNA methylation and histone modi cations have been shown to silence key genes involved in cell proliferation, di erentiation and genome integrity and thus may have a central role in oral tumorigenesis [1]. Environmental factors such as tobacco, alcohol and viruses have been shown to signi cantly contribute to cellular epigenetic patterns and thereby a ect changes in gene activation and cell phenotype [2].

Genotoxic exposure to tobacco carcinogens and consequent adduct formation resulting in DNA damage is an important mechanism of OSCC etiology [3]. erefore, repair of DNA damage and subsequent development of cancer will depend on e ciency of DNA repair mechanisms. Multiple cellular DNA repair pathways are operational to rectify/attenuate deleterious e ects of cellular DNA damage. Amongst major DNA repair pathways, Nucleotide excision repair [NER] is an important pathway which removes structurally diverse lesions such as bulky chemical adducts arising as a result of exposure to tobacco carcinogens [4]. Several critical genes participate in the NER process and have functions central to the ability of a cell to cope with di erent types of DNA damage and to maintain genomic integrity [5]. For example, Xeroderma pigmentosum group C (XPC) product in complex with HR23B forms a protein complex that recognizes and binds to damaged DNA sites thus, playing an important role in DNA damage–induced cellular responses, including cell cycle checkpoint regulation and apoptosis. e XPB and XPD helicases mediate strand separation at the site of the lesion a er damage recognition is done by the XPC-HR23B complex. XPD is a core component of transcription and repair factor; transcription factor IIH, while XPB is required for correct positioning of this transcription factor IIH on DNA prior to damage repair. Genetic and epigenetics defects in these genes resultin in reduced e cacy of NER repair pathways have been implicated in pathophysiology of several cancers such as lung cancer and bladde cancer [6-11]. However, the mechanisms leading to deregulated expression of these NER genes in OSCC are not clearly understood.

Since e ective DNA repair is at backbone of cancer free survival and a DNA repair system may be modulated in e ectiveness by epigenetic mechanisms, targeting these regulatory mechanisms a ecting DNA repair genes in OSCC may contribute to improved diagnosis, clinical management and outcome prediction of newly diagnosed OSCC cases. Moreover, information on role of epigenetic alterations in NER pathway genes that are speci cally involved in removal of adducts caused by tobacco carcinogens in pathophysiology of OSCC is lacking. With this background, we sought to examine the expression of three NER pathway genes (XPB, XPC and XPD genes) and epigenetic changes (DNA methylation and histone methylation and acetylation)

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which may modulate expression of these genes and thus their ability to eliminate adducts caused by tobacco carcinogens in OSCC.

Patients and Methods

Tumor samples

OSCC tumor tissues and adjacent margins were obtained from 52 patients with histopathologically confirmed OSCC patients, undergoing surgery prior to receiving any treatment at Post Graduate Institute Medical Education Research (PGIMER) Chandigarh, India. Noncancerous oral tissues (n=27) were obtained from trauma cases undergoing maxillofacial surgery served as controls. Absence of tumor cells in margins as well as control tissues was confirmed histopathologically. All the subjects were interviewed using a standard questionnaire regarding the demographic information, use of smoking and alcohol consumption, family history of cancer and medical history prior to diagnosis date of cancer. Smokers were defined as those subjects that reported that they had smoked a total of 100 cigarettes over their lifetime [12]. Patients with chewing habit were not included in the present study as this habit was found to be absent in control group. The study was approved by Institute Research Ethics Committee, PGIMER and all subjects enrolled in this study provided informed consent.

Nucleic acid extraction

Genomic DNA was extracted from the OSCC, margins and control samples using standard proteinase K digestion method and purified by phenol–chloroform extraction. Total RNA was isolated using the RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Real-Time reverse transcription PCR

For mRNA expression studies, total RNA (1 µg) was reverse transcribed with oligodT primer using the MMLV Reverse Transcriptase Kit (Fermentas Inc., Maryland, USA) in a total volume of 20 µl. Real-time polymerase chain reaction [PCR] was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems). Real time quantification of XPC, XPB and XPD genes was performed by SYBR-green chemistry using the primers and PCR conditions listed in Table 1. Beta actin was used as an internal control. All PCR reactions were performed in triplicate and the specificity of the amplifications was verified by melting curve analysis for all the samples. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Statistical analysis was done by the CT method (CT=CT test gene CT endogenous control).

Promoter methylation analysis

The methylation status of promoters of XPC, XPB and XPD genes was determined using methylation-specific PCR (MSP) [13]. Bisulphite modification of genomic DNA was performed using EZ DNA GOLD kit (Zymo research, USA) following manufacturer's instructions. Primers for MSP were designed by using the online program Methprimer (http://www.urogene.org/methprimer). All the primer sequences were designed to contain multiple CpG sites at 3' end to facilitate maximal discrimination between methylated and unmethylated DNA. Primer sequences and PCR conditions used for MSP are described in Table 1. Genomic DNA treated with SssI methylase served as positive control and blood DNA samples were used as negative control for MSP. All the samples analyzed in this study were amplified in duplicates to ensure the specificity of MSP.

Chromatin immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assays were performed using EZ ChIP kit (Upstate Biotechnology Inc., Massachusetts, USA) following the manufacturer's protocols with some modifications in 14 tumor tissues, 14 margins and 5 control samples. Briefly, tissue lysates were treated with micrococcal nuclease for 10 minutes at 37ºC and centrifuged at 15,000 rpm for 10 min at 4ºC. Supernatants were loaded on 1% agarose gels and determined to have reduced DNA lengths between 200 and 1,000 bp. The sonicated samples were precleaned with salmon sperm DNA/protein A agarose beads (Upstate Biotechnology). The soluble chromatin fraction was collected, and 3 µl of antibody for acetyl-H3 and dimethyl-H3-K9 (H3K9me2), or no antibody, was added and incubated overnight with rotation. All antibodies were purchased from Upstate Biotechnology. After rotation, chromatin-antibody complexes were collected using salmon sperm DNA/ protein an agarose beads and washed according to the manufacturer's protocol Immuno-precipitated DNA DNA says4determssholli1mageaned with d

Fischer's exact test or chi square test. Wilcoxon rank-sum test or independent t-test was used to test the di erences of gene expression between the cancerous and control tissues wherever each test was applicable. Logistic regression analysis was performed to determine association between methylation status and environmental factors a er adjusting for potential confounders. e level for a statistically signi cant di erence was set at $p<0.05$ for all the tests.

Results

Demographic characteristics of the OSCC patients and controls are given in Table 2. No signi cant di erence in mean age, gender, smoking and alcohol consumption was observed between OSCC patients and controls in our study cohort.

DNA methylation status of XPB, XPC and XPD genes in OSCC and association with tobacco and alcohol consumption

XPC promoter methylation was observed in 17.6% tumor tissues (9/51); however, none of the margin or control samples showed XPC promoter methylation. Although, 5.8% tumor (3/51) and 3.9% margin (2/51) samples showed promoter methylation of XPD gene, we did not nd XPD promoter methylation in any of control samples. Methylatedw level for a.14on rank-sl for a.oof Xha.oof Xha.oof X01g0.6(in) orwe -1
