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Epigenetic Modifications of Nucleotide Excision Repair Genes in Oral Squamous 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 modifications 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 specific 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 modifications in selected NER genes.

We did not observe any significant 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 findings suggest the epigenetic modifications regulating the expression of XPB gene may be involved in OSCC etiology.

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

Abbreviations: OSCC: Oral squamous cell carcinoma; NER: Nucleotide excision repair; XPC: Xeroderma pigmentosum group C; MSP: Methylation-specific 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 modifications have been shown to silence key genes involved in cell proliferation, differentiation 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 significantly contribute to cellular epigenetic patterns and thereby affect 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]. Therefore, repair of DNA damage and subsequent development of cancer will depend on efficiency of DNA repair mechanisms. Multiple cellular DNA repair pathways are operational to rectify/attenuate deleterious effects 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 different 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. The XPB and XPD helicases mediate strand separation at the site of the lesion after 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 resulting in reduced efficacy of NER repair pathways have been implicated in pathophysiology of several cancers such as lung cancer and bladder cancer [6-11]. However, the mechanisms leading to deregulated expression of these NER genes in OSCC are not clearly understood.

Since effective DNA repair is at backbone of cancer free survival and a DNA repair system may be modulated in effectiveness by epigenetic mechanisms, targeting these regulatory mechanisms affecting 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 specifically 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 ΔCT method (ΔCT=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 37°C and centrifuged at 15,000 rpm for 10 min at 4°C. 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 was recovered and analyzed by real time PCR. The primer pairs used for ChIP assays are shown in Table 1. Beta actin was used as an internal control. The ΔC_T values were determined by subtracting the average C_T value of the normalized input from the average C_T value of the corresponding immunoprecipitated samples. The fold change of target genes was calculated by dividing the $2^{-\Delta CT}$ of the controls from the $2^{-\Delta CT}$ of tumor tissue or noncancerous margin tissue.

Statistical methods

Statistical analyses were performed using SPSS software version 17.0. Differences in promoter hypermethylation were analyzed between the cancerous tissues and margins; cancerous and control tissues using

Gene	Real time PCR Primer sequences	Ta (°C)	Amplicon (bp)
XPC	F:5'-CCAGAGCAGGCGAAGACAAGA-3' R:5'-AAGCGGGCTGGGATGATGGAC-3'	60	215
XPB	F:5'-TTGGCGTCCCCTACGTCTAC-3' R:5'-CTGGTCCCGCAGGTATTCC-3'	60	68
XPD	F:5'-GCGGCAGAGATTCTTGGTAGA-3' R:5'-TGTCGAAAACGCCAAGTCTTC-3'	60	91
	Methylation Specific PCR Primer sequences		
XPC	M F:5'-AACGAAGGGGCGTGGTTAAGCG-3' M R:5'-TAAACAATTCCGCTTCCCGAATA-3' U F:5'-AATGAAGGGGTGTGGTTAAGTG-3' U R:5'-TAAACAAATTCCACTTCCCAAATA-3'	67.2 62.5-59.5 (-0.1C/ cycle)	175 110
XPB	EXT F:5'-GGTTTTTGAGGATTTGAGGGT-3' EXT R:5'-CCTACAATCTATCTTAAACCCAAAC-3' M F:5'-GGGAATTGATTTAGTAGTTTTTGTC-3' M R:5'-AACGTACGTTTAAACTCCCG-3' U F:5'-GGGAATTGATTTAGTAGTTTTTGTTGT-3' U R:5'-TCCAACATACATTTAAACTCCCAC-3'	60 60 60	306 120 123
XPD	EXT F:5'-TTTATGGATTTAGGTTATATTTTTTT-3' EXT R:5'-TCTTTTACCCATAACAACTACAACAAC-3' M F:5'-AATGAATAGTGGAGGATTTTCGTC-3' M R:5'-AAACTTCCGACTCAATCCGA-3' U F:5'-ATGAATAGTGGAGGATTTTTGTTGT-3' U R:5'-AAAAACTTCCAACTCAATCCAAA-3'	56.9 59 59	388 153 154
	ChIP Primer sequences		
XPC	F: 5'-GATGCAGGAGGTGGACTCTCT-3' R- 5'-GTAGTGGGGCAGCAGCAACT-3'	61.5	244
XPB	F:5'-TCTGCAGGAGGATCAGCT-3' R: 5'-GCAAGACTCAGGAGTCAC-3'	64.2	149
XPD	F:5' -TTGGGCCTAAGCCTACGAA-3' R:5'-GCAGGTGAGCCTAAGTCCTG-3'	52.7	189

Table 1: Primer sequences used for expression, methylation specific PCR and ChIP analysis of NER genes.

Fischer's exact test or chi square test. Wilcoxon rank-sum test or independent t-test was used to test the differences 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 after adjusting for potential confounders. The level for a statistically significant difference 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 significant difference 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 find XPD promoter methylation in any of control samples. Methylated alleles for XPB gene were found in 21.6% tumor (11/51), 17.7% margin (9/51) and 12.5% (3/24) control samples (Table 3).

We did not observe any significant association between tobacco consumption and alcohol consumption with promoter methylation of XPB, XPC and XPD genes (Table 4).

Expression of NER pathway genes in OSCC

mRNA expression of NER genes, XPB, XPC and XPD in tumors, corresponding margins and control tissues is shown in Table 5. We observed increased XPB mRNA levels in both margin (p 0.02) and tumor samples (p 0.04) as compared to control samples. mRNA levels of XPC and XPD genes were not found to be significantly different between margin and tumors as compared to control samples.

Association of DNA methylation status of NER genes with their expression in OSCC

To evaluate whether aberrant methylation observed in NER genes was associated with altered gene expression, we compared mRNA expression of these genes in methylated and non-methylated samples. Since, no promoter methylation of XPC and XPD genes was observed in control tissue samples, the association of DNA methylation with mRNA expression profiles was analyzed only in tumor tissues. Methylated tumor samples showed significantly decreased XPB (p 0.046) mRNA levels as compared to unmethylated samples. No significant difference was observed in mRNA levels of XPB and XPD genes in methylated as compared to unmethylated tumor samples (Table 6).

Histone modifications of XPB, XPC and XPD genes in OSCC

On examining the status of Histone 3 acetylation (H3Ac) and Histone 3 lysine 9 dimethylation (H3K9me2) in NER genes in OSCC, we observed significantly increased H3Ac of XPB gene in the tumor samples as compared to the control samples (p 0.04); however, no significant difference in H3K9me2 of XPB gene was observed between OSCC patients and controls (Table 7). No significant difference in either H3Ac or H3K9me2 of XPC and XPD genes was seen between either tumor and control samples or margin and control samples.

Discussion

Tobacco consumption is an established risk factor for OSCC and NER pathway has been reported to be a major pathway responsible for removal of tobacco associated carcinogens from DNA adducts in OSCC cells [9,14]. In the present study, we investigated the gene expression, promoter methylation and histone modifications of NER genes that may inactivate and/or decrease the effectiveness of DNA repair system in OSCC.

DNA methylation status of XPB, XPC and XPD genes in OSCC

Promoter methylation has been implicated as one of the important regulatory mechanisms for gene expression in carcinogenesis [15]. We examined the promoter methylation status of three key NER genes; XPB, XPC and XPD genes in tumors, their corresponding noncancerous margins and control tissues to determine the contribution of this epigenetic modification to oral carcinogenesis. To the best of our knowledge, this is the first study analyzing the promoter methylation of XPB, XPC and XPD genes, the key genes of nucleotide excision repair pathway that are involved in removal of structurally diverse lesions arising as a result of exposure to tobacco carcinogens. We did not find any significant difference in promoter methylation of NER genes among OSCC tissues, margins and controls. Our results are supported by a recent study in which the authors did not find any methylation in XPC gene in NSCLC [16]. Contrary to these findings, hypermethylation of XPC gene has been observed in patients with lung and bladder cancer [11-17]. These discrepant results may be attributed to differences in ethnicity or clinic-pathological features of samples tested in each study. Till date, no report is available on the role of XPB and XPD promoter methylation in cancer and ours is the first study to reveal the status of XPB and XPD promoter methylation in oral carcinogenesis. Although statistical analysis showed no significant difference in promoter methylation of XPB, XPC and XPD genes, however, we observed promoter methylation of XPC gene in only tumors and of XPD gene in only tumors and histopathologically confirmed normal margins. These findings suggest the pathogenic nature of methylation events in oral cancer as none of healthy controls were found to harbor XPC and XPD hypermethylation. Further, presence of methylated alleles for XPD gene in both tumors and histopathologically confirmed normal margins indicates that XPD promoter methylation may be an early event in carcinogenesis which appears before the onset of histopathological changes and may be further explored as an early biomarker of OSCC.

Characteristics	Patients (n=52)	Controls (n=27)	p value
Mean Age (yrs.)	48.32 ± 11.73	45.3 ± 8.87	0.469
Gender Males Females	 43 (82.7) 9 (17.7)	 26 (96.3) 1 (3.7)	 0.151
Tobacco consumption	33 (63.5)	18 (72.0)	0.611
Alcohol consumption	19 (36.5)	11 (40.7)	0.8

Table 2: Demographic characteristics of study population.

Genes	Subjects	Unmethylated n (%)	Methylated n (%)	p value
XPB	Controls Margins Tumors	21 (87.5) 42 (82.3) 40 (78.4)	3 (12.5) 9 (17.7) 11 (21.6)	 0.7 0.5
XPC	Controls Margins Tumors	24 (100) 51 (100) 42 (82.4)	0 (0.0) 0 (0.0) 9 (17.6)	 - 0.07
XPD	Controls Margins Tumors	24 (100.0) 49 (96.1) 48 (94.2)	0 (0.0) 2 (3.9) 3 (5.9)	 0.83 -

Methylation studies were performed in only 24 controls and 51 OSCC patients.

Table 3: Methylation frequencies of XPB, XPC and XPD genes in tumors, margins and control tissues.

Gene	Smokers (n=33)	OR(95% CI); p	Alcohol consumers (n=19)	OR(95% CI); p
XPB				
Methylated Unmethylated	23 (69.7) 10 (30.3)	7.39 (0.86-63.39); 0.07	5 (26.3) 14 (73.4)	1.54 (0.42-5.88); 0.72
XPC				
Methylated Unmethylated	6 (18.2) 27 (81.8)	1.04 (0.22-4.78); 1.0	3 (15.8) 16 (84.3)	0.78 (0.17-3.58); 0.99
XPD				
Methylated Unmethylated	1 (3.03) 32 (96.7)	0.25 (0.02-3.01); 0.29	1 (5.3) 18 (94.7)	0.88 (0.07-10.46); 0.98

Table 4: Association of Promoter methylation of XPB, XPC and XPD genes with tobacco and alcohol consumption.

Gene	Mean ± S.D. $2^{-\Delta Ct}$	p value
XPB		
Controls Margins Tumors	0.093 ± 0.23 0.18 ± 0.50 0.17 ± 0.51	 0.02 0.04
XPC		
Controls Margins Tumors	0.0036 ± 0.009 0.036 ± 0.21 0.195 ± 0.10	 0.25 0.49
XPD		
Controls Margins Tumors	0.016 ± 0.02 0.11 ± 0.50 0.15 ± 0.58	 0.29 0.28

Table 5: mRNA levels of XPB, XPC and XPD genes in tumor, margin and control tissues.

Gene	Mean ± S.D. $2^{-\Delta Ct}$	p value
XPB		
Methylated Unmethylated	0.031 ± 0.03 0.215 ± 0.57	0.046
XPC		
Methylated Unmethylated	0.47 ± 1.41 0.225 ± 1.15	0.638
XPD		
Methylated Unmethylated	0.002 ± 0.001 0.162 ± 0.61	0.08

Table 6: Expression of XPB, XPC and XPD genes in methylated and unmethylated OSCC samples.

However, these results should be interpreted with caution and future studies with larger sample size are warranted to confirm this finding. The present study is the first step to understand the methylation pattern of XPC and XPD genes in carcinogenesis.

Since tobacco and alcohol consumption are major risk factors for OSCC, we examined if these factors modulated promoter methylation of NER genes in OSCC. We did not find any significant association between alcohol or tobacco consumption and methylation status of XPB, XPC and XPD genes, suggesting that tobacco and alcohol consumption do not appear to affect methylation patterns in OSCC tissues. Although, reports on effect of tobacco consumption on methylation of XPB, XPC and XPD genes are lacking, an association of hyper-methylation with tobacco consumption has been reported for several genes in OSCC including p15, CYP1A1, CYP2A13, GSTM1 and E cad [18-20]. In a recent report, XPC protein attenuation and deficiency was shown to play an important role for tobacco smoke-caused DNA hypermethylation of important tumor suppressor genes [21]. To further delineate the association of promoter methylation status of XPB, XPC and XPD genes with tobacco consumption and the role of this association in OSCC initiation and progression, future studies with larger sample size are required.

Expression of NER pathway genes in OSCC

We determined the mRNA expression of XPB, XPC and XPD genes in tumor and control samples. Increased levels of XPB mRNA were observed in tumors and margins as compared to normal controls. XPB, a 3' to 5' helicase, is required for unwinding of DNA prior to damage repair [22] and for correct positioning of Transcription Factor IIH on the DNA before repair can occur. Thus, increased XPB expression in tumor tissues and margins may reflect an increased repair activity in these tissues. Our results are consistent with an earlier report in epithelial ovarian cancer in which increased levels of XPB mRNA were found in clear cell tumors [23]. These findings suggest that the high NER activity exerted by increased expression of XPB gene may reflect adaptive mechanisms exerted by cells against the swiftly accumulating DNA damage in tumorigenesis. Further, increased XPB expression has been reported to contribute to the development of drug resistance in platinum-chemotherapy [24]. These findings indicate that elevated DNA repair activity resulting from increased expression of XPB gene may lead to reduction in apoptosis which is a predominant mechanism of drug resistance in chemotherapy.

Further, on comparing expression levels of NER genes among methylated and unmethylated OSCC samples, mRNA expression of XPB gene was found to be significantly decreased in methylated samples as compared to unmethylated samples, confirming an inverse relation between methylation and gene expression and supporting the earlier reports showing methylation to repress gene expression [25,26]. Promoter methylation of XPC and XPD genes was not found to regulate the expression of these genes in our study thereby suggesting the involvement of alternative mechanisms [copy number variations, transcription factor production and recruitment, histone modifications and microRNA expression] in regulation of their gene expression.

Histone modifications of NER genes in OSCC

In addition to DNA methylation, histone modifications represent another important epigenetic mechanism that has a critical role in transcriptional regulation and carcinogenesis [2]. Since, the role of these epigenetic changes in oral carcinogenesis is not well known; we examined Histone 3 acetylation and Histone 3 lysine 9 dimethylation in chromatin associated with XPB, XPC and XPD promoter regions in tumors and control tissue samples. Interestingly, significant enrichment of H3 acetylation of XPB gene was found in OSCC samples as compared to controls and margins. These increased levels of H3 acetylation of XPB gene may account for the increased expression of this gene in OSCC patients as observed in our overall expression analysis. To the best of our knowledge, this is the first report studying the histone modification status of NER genes in OSCC. However, we did not find any significant difference in H3 acetylation and H3K9me2 between tumors and control samples for XPC and XPD genes. These findings are consistent with our gene expression data, where too we did not find any change in mRNA levels of these genes between tumors and

Histone Modification	Acetylation Fold Change Mean ± SD	p value	Methylation Fold Change Mean ± SD	p value
XPB				
Controls Margins Tumors	2.8 ± 0.9 4.0 ± 1.6 4.4 ± 2.3	 0.05 0.04	1.5 ± 2.2 3.1 ± 3.9 3.2 ± 4.2	 0.3 0.29
XPC				
Controls Margins Tumors	1.00 ± 0.6 1.2 ± 1.3 1.2 ± 0.7	 0.62 0.55	1.9 ± 2.9 3.4 ± 3.7 2.9 ± 3.7	 0.40 0.57
XPD				
Controls Margins Tumors	1. 5 ± 1.7 1.9 ± 1.9 2.8 ± 2.0	 0.69 0.22	1.5 ± 2.3 3.7 ± 3.9 3.3 ± 3.9	 0.26 0.32

Table 7: Histone Modifications of XPB, XPC and XPD genes in tumors, margins and control tissues.

control samples, suggesting that these histone modifications may not contribute significantly to regulation of XPC and XPD genes in OSCC. Our findings are supported by an earlier study in which the similar levels of global H3K9me2 were found in normal, dysplastic and in OSCC samples [27]. The association of histone modifications with gene expression and environmental factors was not studied as the number of samples included in ChIP analysis was very small. Further studies with adequate power and sample size are required to assess the association of histone modifications with gene expression and environmental factors in OSCC.

In summary, our data showed that XPB gene showed increased histone H3 acetylation correlating with its increased expression in tumor tissues. Further, promoter methylation of NER genes was not found to be associated with oral carcinogenesis; however, future studies with larger sample size are required for its validation. Thus, the present study indicates the role of histone modifications in regulating the expression of XPB gene in OSCC and may facilitate development of new approaches for treatment and diagnosis of OSCC.

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