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Role of matrix metalloproteinase-9 polymorphisms in basement membrane degradation and pathogenesis of oral submucous fibrosis

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ABSTRACT

Oral submucous fibrosis (OSMF) is regarded as a collagen and collagenase metabolic disorder. Aberrant expression of matrix metalloproteinases (especially MMP-9) plays important role in remodeling of extracellular matrix (ECM) during development of OSMF. Single nucleotide polymorphisms (SNPs) in *MMP-9* promoter and coding region have been demonstrated to be associated with several diseases. In this case-control study, 196 controls and 189 OSMF patients were genotyped at four *MMP-9* polymorphic sites on −1562C > T, R279Q, P574R and R688Q loci by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) method to determine the susceptibility to OSMF. The functional effect of SNPs to the development of OSMF was analyzed by studying the expression of MMP-9, collagen type-I and IV in oral biopsy tissues. R279Q SNP was found to be associated with OSMF [OR 1.5, CI (1.04–2.43), $p = 0.03$]. The 574R and 668Q alleles were significantly associated with early age group at 2.08 ($p = 0.007$) and 2.12 ($p = 0.011$) fold risk to OSMF respectively. MDR analysis showed that -1562C > T and R688Q SNPs were in strong synergy (IG = 0.95%, $p = 0.0032$) with increased risk of OSMF. Genotypic and functional study revealed definitive role of *MMP-9* coding SNPs R279Q, P574R and R668Q in the pathogenesis of OSMF with strong predictive and prognostic value to determine OSMF at early stages in the areca chewers. Pathologically, over-expression of MMP-9 leads to decrease in collagen type-IV and epithelial thinning which contribute to basement membrane degradation along with continuous accumulation of collagen type-I enhanced by *MMP-9* −1562C > T, R279Q, P574R and R688Q SNPs, resulting into early onset of OSMF.

1. Introduction

Oral submucous fibrosis (OSMF) is a chronic inflammatory condition of oral cavity characterized by progressive fibrosis of lamina propria and underlying connective tissue. It is considered as a potential pre-malignant disorder with a malignant transformation rate of 2.3–7.6%. Histopathologically, the disease is characterized by imbalance between synthesis and degradation of collagen in extracellular matrix (ECM) of oral mucosa. The habit of chewing areca nut is found to be the principal etiological factor behind the fibroblastic proliferation and pathogenesis of OSMF. The pro-fibrotic cytokines such as TGF-α, TGF-β, IL-1, IL-6, platelet-derived growth factors (PDGF) and basic fibroblast growth factors (FGF) were upregulated in OSMF (Haque et al., 1998). TGF-α alters collagen synthesis by activation of procollagen genes and upregulation of lysyl oxidase (LOX) activity, reducing its degradation by activation of tissue inhibitor of matrix proteinase (TIMPs) (Rajalalitha and Vali, 2005). TGF-β activates expression of inflammatory mediator COX-2 and PI3K/Akt and ERK/JNK/p38 MAPK pathways in OSMF (Khan et al., 2012; Lu et al., 2008). Tissue remodeling and fibrosis are dynamic processes that involve ECM production and degradation, leading to tissue repair or pathological condition (Bousquet et al., 1992). Several studies have shown the role of Matrix metalloproteinase-9 (MMP-9) in the tissue remodeling and pathogenesis of OSMF (Rajendran et al., 2006; Mukherjee et al., 2012).

Abbreviations: OSMF, oral submucous fibrosis; ECM, extracellular matrix; FGF, fibroblast growth factors; LOX, lysyl oxidase; TIMP, tissue inhibitor of matrix proteinase; MMP-9, matrix metalloproteinase-9; EMT, epithelial to mesenchymal transition; SNP, single nucleotide polymorphisms; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; MDR, multifactor dimensionality reduction; TBA, testing balanced accuracy; CVC, cross-validation consistency; BCIP/NBT, 5-bromo,4-chloro,3-indolylphosphate/nitro-bluetetrazolium; H&E, hematoxylin/eosin; OR, odds ratio; CI, confidence interval; LD, linkage disequilibrium

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Upregulated expression of MMP-9 in microarray analysis of OSMF also suggests being an indicative marker for the epithelial to mesenchymal transitional (EMT) mechanism in OSMF (Das et al., 2013). Further depletion of S100A4 protein has been found to reverse arecoline-induced TIMP1 and MMP-9 expression of arecoline-stimulated fibroblasts (Yu et al., 2013). Again, several types of cancer affecting different organs (like Oral cavity, Prostate, Lung, Breast, Ovary) have been found to involve higher expression of MMP-9 (Aalinkeel et al., 2011; Cox et al., 2000; Kim et al., 2014; Mehner et al., 2014; Sun et al., 2008; Yu et al., 2014). Thus, upregulation of MMP-9 would predominantly result from regulation at genetic, epigenetic and physiological levels (Fanjul-Fernández et al., 2010; Cock-Rada et al., 2012).

Several single nucleotide polymorphisms (SNPs) in *MMP-9* gene at regulatory and coding regions were identified; of these, −1562C > T promoter polymorphism (rs3918242) and three coding SNPs R279Q (rs17576, G/A, Exon-6), P574R (rs2250889, C/G, Exon-10), and R668Q (rs17577, G/A, Exon-12) have been widely studied in connection with several diseases and has rare allele frequency > 0.05. SNP −1562C > T is located at the transcriptional start site and affects promoter activity and the binding of transcriptional repressor protein. The C allele shows low activity and T allele shows high-activity of promoter and influences differential expression of MMP-9 (Park et al., 2000). R279Q is located in the gelatinase specific fibronectin type II domain which enhances substrate binding (O'Farrell and Pourmotabbed, 2000). P574R and R668Q are located in the hemopexin domain, which is involved in homodimerization, and binding to substrates and TIMP1 inhibitor (Murphy and Knäuper, 1997). It has been believed that these polymorphisms may modify or alter the interaction between MMP-9 and its substrate or alter MMP-9 activity.

The aim of the present study was to investigate whether SNPs in promoter and coding region of *MMP-9* gene may constitute the risk for OSMF, and to elucidate the mechanism by which MMP-9 and its genetic variants may influence the pathogenesis of OSMF. This is the first attempt focused on these four polymorphisms (−1562C > T, R279Q, P574R, R688Q) at the level of genotype and their functional counterpart as risk factor for susceptibility to OSMF.

2. Materials and methods

2.1. Selection of subjects

Peripheral blood sample (3–4 ml) was collected from 189 clinically and histopathologically confirmed OSMF patients (having the habit of chewing areca nut, Khaini, Betal quid, cigarette in any form) as cases, and 196 healthy normal volunteers with normal oral epithelium and without the above oral habit for less than six months as controls, from the Outpatient Department of Dr. R. Ahmed Dental College & Hospital, Kolkata, India. Written consent was obtained from each of the study participants; the study design was duly approved by the Human Ethics review committee of the institute.

2.2. MMP-9 genotyping

Genomic DNA was extracted from peripheral white blood cells of cases and controls using phenol-chloroform method (Sambrook and Russel, 2000).The genotypes for the *MMP-9* polymorphisms were detected using the Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) method. PCR was performed with 25 µl PCR reaction mixture containing 100 ng genomic DNA, 2.5 mM dNTPs, 1 pg of each primer (Hu et al., 2005; Ke et al., 2001), (Supplementary Table 1.) 1U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 1× Taq Buffer under the following condition: an initial denaturation (95 °C, 5 min), followed by 35 cycles of denaturation (94 °C, 30 s), annealing at different temperatures (30 s), (Supplementary Table 1) followed by elongation (72 °C, 30 s), with a final elongation step (72 °C, 5 min) in GeneAmp® PCR system 9700 (PE Applied Biosystem, Foster City, CA). Negative controls lacking DNA templates were included in all batches of PCR. The amplified PCR products were digested with specific restriction enzymes (NEB, Ipswich, MA, USA) as per manufacturer's guidelines (Supplementary Table 1.) All the restriction digestion products were separated by electrophoresis on a 6% polyacrylamide gel and visualized under Gel Doc™ XR + Imaging System (BioRad, USA) after staining with ethidium bromide (EtdBr). The representative images of RFLP-genotyping are shown in the Supplementary Fig. 1.

2.3. SNP-SNP interaction analysis

SNP–SNP interactions between the four SNP-s were studied by a non-parametric and genetic model-free approach called multifactor dimensionality reduction (MDR) that uses a data reduction strategy. The input file was in text format with 0 for wild type, 1 for heterozygous, and 2 for homozygous mutant in each case. Evaluation of SNP-SNP interactions with case–control data set was performed by the MDR program (Moore et al., 2006). A naive bayes classifier in the context of a 10-fold cross-validation was used to estimate the testing balanced accuracy (TBA) of each one dimensional attribute of the 2-factor to 10-factor models. The cross-validation consistency (CVC) was also calculated, which measures the number of times out of 10 divisions of the data that the same best model was found. All these analyses were implemented in the open-source MDR software package version 2.0 beta 8.1 (http://sourceforge.net/projects/mdr/) (Hahn et al., 2003). 1000 permutations were performed using MDRPT beta version 0.4.9 program (https://sourceforge.net/projects/mdr/files/mdrpt/).

2.4. RNA extraction and reverse transcriptase (RT)-PCR

Oral biopsy tissues obtained from OSMF and healthy control subjects were washed thrice with sterile 1× PBS and then total RNA was isolated using Trizol reagent following manufacturer's instructions (Invitrogen, USA). MMP-9 and GAPDH (internal control) mRNA from tissues were amplified using the OneStep RT-PCR kit (QIAGEN, USA) in a 25 µl reaction volume containing 1 µg RNA, 1× of 5× QIAGEN OneStep RT-PCR Buffer, 400 µM of each dNTP, and 0.6 µM of each primer, (Supplementary Table 2) under the following conditions: reverse transcription at 50 °C for 30 min, followed by PCR activation step (95 °C,15 min); 35 cycles of denaturation (95 °C, 30 s), annealing at different temperatures (30 s), (Supplementary Table 2) extension (72 °C, 1 min); and final extension (72 °C, 10 min). The amplified RT-PCR products were subjected to electrophoresis on 2% agarose gels and stained with EtdBr.

2.5. Western blotting analysis

Tissue extracts were prepared by homogenization in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1%SDS, 100 µg/ml PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1%NP-40 on ice for 30 min, then centrifuged at 13000 rpm for 5 min; total protein content in the supernatant was estimated by Bradford reagent (BioRad Inc., USA). 50 µg of protein samples were subjected to western blot analysis. Primary Anti-human MMP-9 Polyclonal (Goat, AF911, R&D Systems, USA), Anti-Collagen Type-I (Rabbit, Santacruz) and Anti-Collagen Type-IV (Rabbit, Santacruz) and Anti-β-actin (Mouse, Sigma- Aldrich) at 1:100 dilution were used, followed by ALP-conjugated anti-goat IgG, anti-rabbit IgG and anti-mouse IgG (GENEI, Banglore, India) at 1:1000 dilution. The protein bands were visualized with 5-bromo,4-chloro,3-indolylphosphate/nitrobluetetrazolium (BCIP/NBT) solution (GENEI, Banglore, India).

2.6. Immunohistochemistry and epithelial thickness determination

Paraffin embedded tissue sections of normal subjects and OSMF