Clinical Development of Biomarker to Detect Oral Carcinoma in Relation to Genetic Polymorphism at MMP-9

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Abstract: Premalignant/potentially malignant oral lesion and condition such as oral submucous fibrosis are known to be transformed into oral cancer. The malignant transformation is often associated with genetic polymorphism which is reflected by the altered expression of proteins related to ECM degradation, fibroblast proliferation and angiogenesis. Protein such as metallo matrixproteinase-9 that loose substrate binding activity due to SNP at Q279R polymorphism at active site results in the degradation of type-4 collagen in basement membrane. The prime objective of this study is to develop biomarker for early detection of oral carcinoma, in relation to MMP-9 polymorphism at Q279R. As a method, Genomic DNA were extracted from blood samples (5ml, from OSF and control subjects) for genotypic analysis using restriction fragment length polymorphism (RFLP) subsequently after amplification through polymerase chain reaction (PCR). The expressions of MMP-9 Polymorphism in transcriptional and post-translational levels were analyzed by RT-PCR and Western blot respectively. Statistical validation was performed by estimating P-values using student-t test and odds ratio analysis. It was found that the odd ratio of R/R compared to Q/Q was 1.75 (0.41 - 7.48) and p-value was 0.575. The RT-PCR analysis of MMP-9 has shown the significant up-regulation of transcription compared to that of normal p-value 0.024, observed in student t-test. The study is concluded on the basis of significant up regulation of active MMP-9 (Q279R-polymorized; 82kDa) in patients than that of healthy control, we can conclude by establishing this polymerized protein as a biomarker for the early detection of oral submucous fibrosis to prevent the oral carcinoma.

Keywords: mmp-9, oral submucous fibrosis, pcr, rflp, snp.

1. Introduction

Oral submucous fibrosis is a chronic debilitating disease of the oral cavity characterized by inflammation and progressive fibrosis of the sub mucosal tissues (lamina propria and deeper connective tissues). Oral submucous fibrosis results in marked rigidity and an eventual inability to open the mouth [3, 4]. The buccal mucosa is the most commonly involved site, but any part of the oral cavity can be involved, even the pharynx [5]. This condition is well recognized for its malignant potential and is particularly associated with areca nut chewing, in 1952; Schwartz coined the term atrophica idiopathica mucosa oris to describe an oral fibrosis, which he discovered in 5 Indian women from Kenya [1]. Joshi subsequently coined the term ‘oral submucous fibrosis (OSF)’ for the same condition in 1953[2]. There are compelling evidences to implicate the habitual chewing of areca nut with the development of OSF. It occurs predominantly in the Indian subcontinent where the habit is more prevalent. The frequency of this habit in population affected by OSF ranged from 35% to 100% (Bhonsle RB et al 1987). This has been reported to be higher among OSF patients than in the general population. In a study of 100,000 villagers in Maharashtra (India), 4.2% of females who chewed areca nut and did not use tobacco, suffered from OSF. Betel quid chewing is a habit practiced predominately in Southeast Asia and India that dates back for thousands of years. It is similar to tobacco chewing in westernized societies. The mixture of this quid, or chew, is a combination of the areca nut (fruit of the Areca catechu palm tree, erroneously termed betel nut) and betel leaf (from the Piper betel, a pepper shrub), tobacco, slaked lime (calcium hydroxide), and catechu (extract of the Acacia catechu tree)[3]. Lime acts to keep the active ingredient in its freebase or alkaline form, enabling it to enter the bloodstream via sublingual absorption. Arecoline, an alkaloid found in the areca nut, promotes salivation, stains saliva red, and is a stimulant.

In most patients with oral submucous fibrosis, areca nut was chewed alone more frequently than it was chewed in combination with pan (i.e., betel leaf + lime + betel catechu, with or without tobacco) or had higher areca nut content [4, 8]. Thus chewing areca nut may be an important factor in the aetiology of OSF. Previous studies on the pathogenesis of OSF have suggested that the occurrence may be associated with the following:
MMP-9 expression is regulated by several cytokines and growth factors, including interleukins [32]. MMP-9 is usually seen in invasive and highly tumorigenic cancers [32]. The main substrates of the gelatinase are type IV collagen and gelatin, and these enzymes are distinguished by the presence of an additional domain inserted into the catalytic domain. This gelatin-binding region is positioned immediately before the zinc-binding motif, and forms a separate folding unit that does not disrupt the structure of the catalytic domain. The gelatinase are MMP2 and MMP9 [33]. MMP9 cleaves collagen IV at a Gly|-Leu bond. It cleaves type IV and type V collagen into large C-terminal three quarter fragments and shorter N-terminal one quarter fragments [31]. Physiologically, MMP-9 in coordination with other MMPs, play a role in normal tissue remodeling events. MMP9 may act essentially in local proteolysis of the extracellular matrix, in leukocyte migration as well as promote tumor growth, metastasis and angiogenesis [29]. Single-nucleotide polymorphisms (SNP) in the coding region (especially non-synonymous SNPs) may influence the protein activity and therefore may be associated with cancer development and metastasis [29]. We used the public SNP database and literatures to select no synonymous coding SNPs in the MMP-9 gene and we also identified three non synonymous SNPs (Q279R, P574R, and R668Q) with a rare allele frequency of >0.05.SNPs, which are useful markers for diseases in haplotype-based association studies and in linkage disequilibrium analysis [26, 27, 28]. A common A/G polymorphism of the MMP-9 exon-6 gene leading to an amino acid substitution in the enzymatic site (active) has also been described as rs17576 (known as Gln279Arg or Q279R) that is a SNP in Exon 6 of the MMP9 gene. The rs17576 (A>G), G-allele encodes the Arg (R) and A-allele encodes the Gln (Q). The clinical relevance of these polymorphisms to OSF is unclear. The Q279R (exon 6, base A to G) is located in the gelatinase specific fibronectin type II domains, which presumably enhance substrate binding [29]. The Q279R mutation leads towards the substitution of a positively charged amino-acid (arginine) by an uncharged amino acid (glutamine) at position 279 within the active site of MMP9. Although the functional impact of this polymorphism on the protein is unknown, this variant is noteworthy; it is located in the coding sequence of a highly conserved fibronectin type II-like repeats which confers MMP-9 with high affinity binding to type IV collagen, type I gelatin and elastin. The digestion of type IV collagen in the epithelial basement membrane has been suggested to be a key regulatory event in the initiation of fibrosis. Q279R mutation represents a partial loss-of-function within the proteinase whose presence reduces the development of fibrosis; these findings implicate MMP-9 as a key molecule in the pathogenesis of OSF [30].

No data are available regarding the relation of Gln279Arg polymorphism with the occurrence of OSF. As MMP activity in vivo could depend on either functional genetic polymorphisms or biochemical factors, hence a pilot study on MMP-9 (A/G) polymorphism in between patients and controls were perform to confirm the SNP at MMP-9 in OSF cases. To establish MMP9 polymorphism as a potential biomarker, initially a semi-quantitative RT-PCR was performed in transcriptional level (in mRNA) and expression of polymorphised MMP-9 at translational level was finally analyzed by Western blot method.

2. Materials and Methods

Blood samples of patients diagnosed with oral submucous fibrosis were collected from different hospitals in Kolkata. Blood samples were also collected from healthy individuals with no oral lesions or any history of the same (considered as controls). Information about tobacco chewing habit and
smoking (cigarettes/bidis) for each patient as well as healthy individual were noted. Around 5 ml of blood was collected from each individual in sterile tubes using disposable syringe. To each tube a proportionate amount of EDTA as an anticoagulant.

2.1 PCR-RFLP Assay

The extracted Genomic DNA (from blood through DNA isolation) was quantified and checked Spectrophotometrically. Ethidium bromide (EtBr) stained 0.8% agarose gel electrophoresis was used to confirm presence of genomic DNA in samples from both patients and controls. The MMP9 genotype was determined by the PCR–RFLP assay. The PCR primers were used to amplify the MMP9 SNP Q279R. The forward primer was 5′-ATG GGT CAA AGA ACA GGA-3′ and the reverse primer was 5′-GGT AGA CAG GGT GGA GGG-3′. The A-G mutation at the second nucleotide was close to the 3′ end. PCR was performed with a 25 µl volume containing 1 μ of genomic DNA template, 4× of 10X PCR buffer, 0.2 μ of MgCl2, 0.3 μ of Taq DNA polymerase, 2.5× of dNTPs and 1 μ of forward and reverse primer. The PCR cycling conditions were 5 min at 950C, 1 min of annealing at 60 0C, and 1 min of extension at 72 0C and with a final step at 72 0C for 10 min to allow for the complete extension of all PCR fragments. For a negative control, each PCR reaction was done by distilled water instead of DNA in the reaction mixture.

Restriction enzyme (Sma-I) digestion of MMP-9 gene: A 10μ of PCR product was digested at 300C for 5 hours reaction, containing 2 unit/reaction of Sma-I and 1.4 μl of NE buffer. After digestion, the products were separated on 2.0% agarose gel stained with EtBr. On electrophoresis, the polymorphism were represented by DNA bands of 181 and 96 bp, the wild type were represented by a DNA band of 227 bp, whereas the heterozygote displayed a combination of 227, 181 and 96 bp.

2.2 RT-PCR

Total RNA was prepared using TRIzol reagent. Single stranded DNA was synthesized from mRNA in a 15μl reaction mixture containing 100ng random hexamer and 200U of ‘Moloney murine leukemia virus reverse transcriptase’. The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). The PCR reaction contains 10μmol of forward and reverse primers and 2U of Tag-DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for MMP-9 in a thermal cycler. Each cycle consisted of 1 min of denaturation at 950C, 1 min of annealing at 600C, and 1 min of extension at 720C. The sequences of primers used were as follows: GAPDH forward: 5′ATGGGGAAGGTGAAGG and reverse: 5′-GGATGCTAAGCAGTTGGT-3′; MMP-9 forward: 5′-GGGAGATTGGGAACCAGCTGTA-3′ and reverse: 5′-GACGGCCCTTGTTACACCCCACA-3′. The PCR products were analyzed by agarose gel electrophoresis and a 227 bp band for MMP-9 was observed. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, a relative proportion of mRNA synthesis could easily be determined in each experiment. The intensity of each band (after normalization with GAPDH mRNA) was quantified by the photographed gels with a densitometer.

2.3 Western Blot

For Western Blot analysis, cell lysates were collected as described previously. Briefly, cells were solubilized with sodium dodecyl sulfate-solubilization buffer (5 mM EDTA, 1mM MgCl2, 50 mM TrisHCl, pH 7.5, and 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethyl-maleimide) for 30 minutes on ice. After that the cells lysates were centrifuged at 12,000g at 4°C and the protein concentrations are determined with Bradford reagent using bovine serum albumin (standard). Equivalent amounts of total protein per sample of cell extracts were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immediately transferred to PVDF membranes. The membranes were blocked with phosphate buffered saline containing 5% bovine serum albumin for 2 hours, rinsed, and then incubated with primary antibodies anti-b catenin (1:500) in phosphate buffered saline containing 0.05% Tween 20 for 2 hours. After three washes with Tween 20 for 10 minutes, the membranes were incubated for 1 hour with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above, and treated with 1:1000 BCIP/NBT solution for 30 minutes. After a series of washing steps, protein expression was detected by chemiluminescence using an ECL detection kit, and relative photographic density was quantified by scanning the photographic-negatives on a gel documentation and analysis system. Each densitometric value was expressed as the mean ±SD.

3. Result

The population was showing presence of MMP-9 Q279R polymorphism in OSF patient. The up regulation of MMP-9 polymorphism in transcription level as well as the polymorphised MMP-9 protein was distinctly observed OSF patients. The odd ratio of R/R compared to Q/Q was significantly higher than the normal (p-value 0.001), by using student t-test. The post translational measurement of MMP-9 compared to normal having a p-value of 0.024, by using student t-test. The analysis of MMP-9 had shown the significant up-regulation in OSF patient. The up regulation of MMP-9 polymorphism in transcription level as well as the polymorphised MMP-9 protein was distinctly observed OSF patients. The odd ratio of R/R compared to Q/Q was significantly higher than the normal (p-value 0.001), by using student t-test.

![Figure 1: PCR Conformation by Agarose gel electrophoresis](image-url)
After Restriction Digestion: 2 fragments of size 181 bp & 96 bp
In case of wild type: Single band of 277bp (AA)
In case of mutants: Two bands of 181bp & 96bp (GG)
In case of heterozygous: Three bands of 277bp, 181bp, & 96bp (GA)

Table 1: Demographic Table

<table>
<thead>
<tr>
<th></th>
<th>Control (n=40)</th>
<th>Patient (n=40)</th>
<th>OR (CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35.87±12.20</td>
<td>35.01±12.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25(62.5%)</td>
<td>24(60.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15(37.5%)</td>
<td>16(40.0%)</td>
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</table>

Table 2: Distribution Of Genotype Of MMP-9 Among The Patients And Controls

<table>
<thead>
<tr>
<th></th>
<th>Control (n=40)</th>
<th>Patient (n=40)</th>
<th>OR (CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w</td>
<td>9(22.5%)</td>
<td>7(17.5%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>20(50.0%)</td>
<td>18(45.0%)</td>
<td>1.15 (0.30-4.39)</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>11(27.5%)</td>
<td>15(37.5%)</td>
<td>1.75 (0.41-7.48)</td>
<td>0.575</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35</td>
<td>n=18</td>
<td>n=21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3(13.3%)</td>
<td>3(14.2%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>10(55.5%)</td>
<td>9(42.8%)</td>
<td>0.90 (0.10-7.87)</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>5(27.7%)</td>
<td>9(42.8%)</td>
<td>1.80 (0.18-19.10)</td>
<td>0.921</td>
</tr>
<tr>
<td>&gt;35</td>
<td>n=22</td>
<td>n=19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>6(27.2%)</td>
<td>4(21.0%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>10(45.4%)</td>
<td>9(47.3%)</td>
<td>0.60 (0.09-3.63)</td>
<td>0.798</td>
</tr>
<tr>
<td>M</td>
<td>6(27.2%)</td>
<td>6(31.5%)</td>
<td>0.66 (0.08-4.97)</td>
<td>0.969</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>n=25</td>
<td>n=24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>6(24.0%)</td>
<td>3(12.5%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>11(44.0%)</td>
<td>11(45.8%)</td>
<td>2.00(0.31-13.73)</td>
<td>0.654</td>
</tr>
<tr>
<td>M</td>
<td>8(32.0%)</td>
<td>10(41.6%)</td>
<td>2.50(0.36-18.58)</td>
<td>0.276</td>
</tr>
<tr>
<td>Female</td>
<td>n=15</td>
<td>n=16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3(2.0%)</td>
<td>4(25.0%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>9(6.0%)</td>
<td>7(43.7%)</td>
<td>0.58(0.06-4.79)</td>
<td>0.554</td>
</tr>
<tr>
<td>M</td>
<td>3(2.0%)</td>
<td>5(31.2%)</td>
<td>1.25(0.10-16.31)</td>
<td>0.833</td>
</tr>
</tbody>
</table>

The observed MMP-9 heterozygous mutant genotype distribution was found to be high in patient’s age group less than 35.

3.1 Semi-Quantitative RT-PCR Analysis:
Normal

Differential m-RNA expression of MMP-9 in OSF Patient compare to normal.


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