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. Post- translational analysis using Western Blot has shown relatively high intensity of active MMP-9 in comparison to the normal (p-value 0.001) by using student t-test. The study is concluded on the basis of significant up regulation of active MMP-9 (Q279R-polymorfized; 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:

a) Clonally selection of fibroblasts with a high amount of collagen production during the long-term exposure to areca quid ingredients [09].
b) Stimulation of fibroblast proliferation and collagen synthesis by areca nut alkaloids [10].
c) Fibrogenic cytokines secreted by activated macrophages and T lymphocytes in the OSF tissue [11].
d) Decreased secretion of collagenase [12].
e) Deficiency in collagen phagocytosis by OSF fibroblasts [13].
f) Production of collagen with a more stable structure (collagen type I trimer) by OSF fibroblasts [14]
g) Stabilization of collagen structure by (+) catechin and tannins from the areca nut [15]
h) An increase in collagen crosses linkage as caused by up regulation of lysyl oxidase by OSF fibroblasts [16].

Genetic susceptibility is also associated with OSF, as raised frequencies of HLA-A10, -B7 and DR3 are found in OSF patients compared to normal subjects. Further HLA typing done by use of' the polymerase chain reaction (PCR) also demonstrates significantly increased frequencies of HLA A24, DDRB I-I I and DRB3 0202/3 antigens in 21 OSF patients when compared with the healthy controls [17].

The malignant transformation of OSF is often related to genetic polymorphism in MMP genes [18]. Matrix metalloproteinase (MMPs) are thought to participate in the pathogenesis of OSF and MMPs have also been implicated in connective tissue remodeling, ECM homeostasis maintenance and Angiogenesis. Activity of MMPs is regulated at multiple levels such as gene transcription and synthesis of inactive zymogens, posttranslational activation of zymogens and interactions of secreted MMPs with tissue inhibitors of metalloproteinase (TIMPs) [34]. MMP9 (matrix metallopeptidase-9, gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase). Location 20q11.2-q13.1, MMP-9 highly expressed in a wide variety of human cancers [19-24]. The DNA sequence contains 13 exons and the transcript length: 2,335 bps translated to a 707 residues protein. MMP-9 is a Zn^{+2} dependent endopeptidase. The nascent form of the protein shows an N-terminal signal sequence ("pre" domain) that directs the protein to the endoplasmic reticulum. The “pre” domain is followed by a propeptide-"pro" domain that maintains enzyme-latency until cleaved or disrupted, and a catalytic domain that contains the conserved zinc-binding region. A hemopexin/vitronectin-like domain is also seen, that is connected to the catalytic domain by a hinge or linker region. MMP-9 is first synthesized as inactive proenzyme or zymogens. Activation of proMMP-9 is mediated by plasminogen activator/plasmin (PA/plasmin) system. The regulation of MMP-9 activity is also controlled through TIMP-3[32].

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 no 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 1ʎ of genomic DNA template, 4ʎ of 10X PCR buffer, 0.2ʎ of MgCl2, 0.3 ʎ of Taq DNA polymerase , 2.5ʎ of dNTPs and 1ʎ of forward and reverse primer. The PCR cycling conditions were 5 min at 95^0C followed by 30 cycles of 30sec at 95^0C, 30sec at 53^0C and 30sec 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 10ʎ of PCR product was digested at 30^0C for 5hours reaction, containing 2unit/reaction of Sma-I and 1ʎ NE buffer.14ʎ of PCR.

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 96bp, the wild type were represented by a DNA band of 227bp, whereas the heterozygote displayed a combination of both alleles (227, 181 and 96bp).

2.2 RT-PCR

Total RNA was prepared using TRIzol reagent. Single stranded DNA was synthesized from mRNA in a 15µl reaction mixture containing 100mg random hexamer and 200U of 'Moloney murine leukemia virus reverse transcriptase'. The reaction mixture was diluted with 20µl of water and 3µl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). The PCR reaction mixture 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 95^0C, 1 min of annealing at 60^0C, and 1 min of extension at 72^0. The sequences of primers used were as follows: GAPDH forward: 5′ATGGGGAAGGTGAAGG

TCGG-3′reverse: 5′ GGATGCTAAGCAGTTGGT-3′MMP-9 forward: 5′-GCGGAGATTGGGAACCAGCTGTA-3′ reverse: 5′-GACGCGCCTGTGTACACCCACA-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 lyates were collected as described previously. Briefly, cells were solubilized with sodium dodecyl sulfate-solubilization buffer (5 mM EDTA, 1mM MgCl2, 50 mM TriseHCl, pH 7.5, and 0.5% Trition X-100,2 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethyl-maleimide) for 30 minutes on ice. After that the cell lysates were centrifuged at 12,000g at 4ºC 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 in OSF patients. 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 had shown the significant up-regulation compared to normal having a p-value of 0.024, by using student t-test. The post translational measurement of MMP-9 was done by western blot analysis, which has shown the relative intensity of active MMP-9. It was found to be significantly higher than the normal (p-value 0.001), by using student t-test.

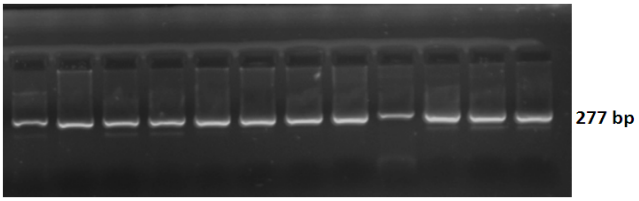

Figure 1: PCR Conformation by Agarose gel electrophoresis

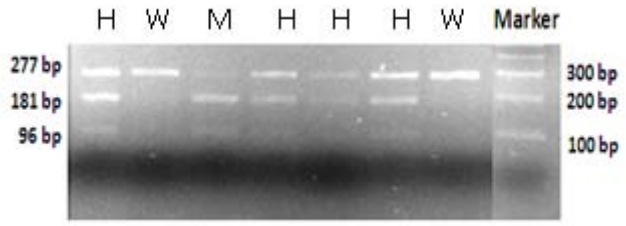

Figure 2: Image of restriction fragment length polymorphism run in agarose gel.

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

	Control (n=40)	*Patient (n=40)*
Age	35.87±12.20	35.01±12.45
Gender		
Male	25(62.5%)	24(60.0%)
Female	15(37.5%)	16 (40.0%)

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

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

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:

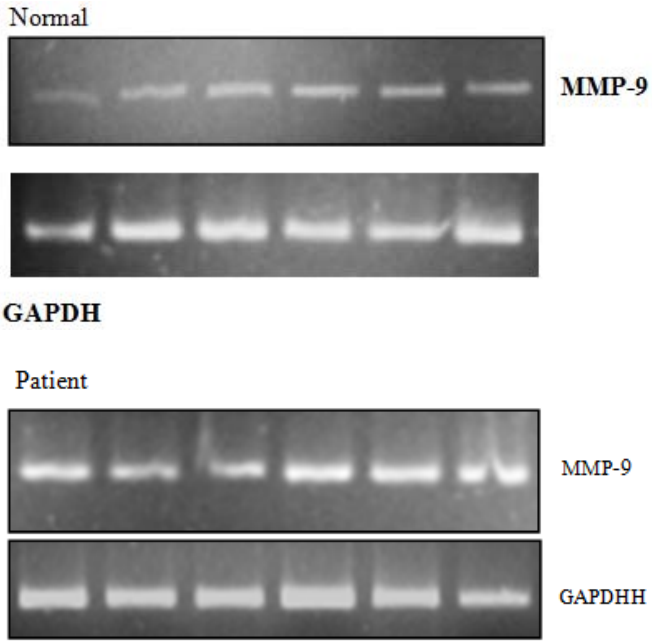

Figure 3: Showing 2% Agarose Gel picture confirming presence of c-DNA products of MMP-9(227 bp) after semi quantitative RT-PCR

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

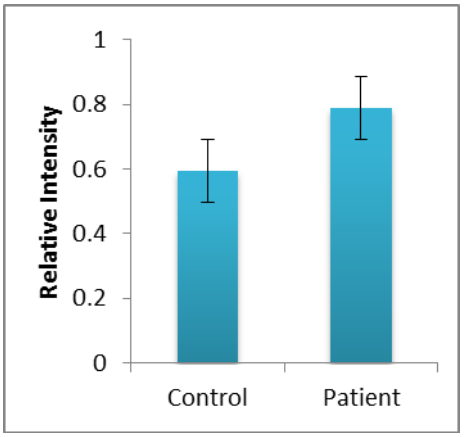

Figure 4: Semi-quantitative RT-PCR showing the up regulation MMP-9 m-RNA expression in OSF patients(PT) compare to control (N)

Western Blot Analysis:

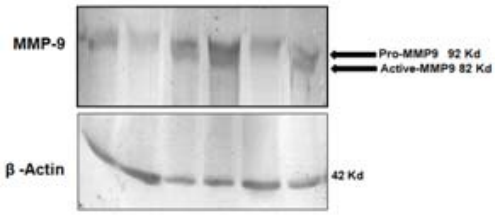

Figure 5: Western blot showing the up regulation MMP-9 protein expression in OSF patients (PT) compare to control (N)

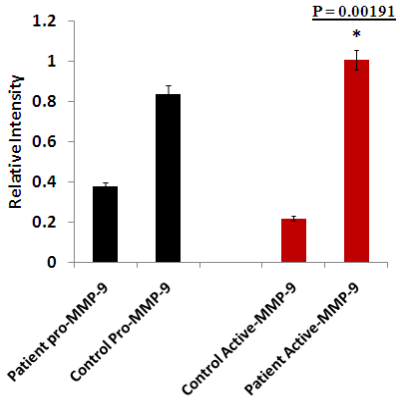

Figure 6: Differential protein expression of MMP-9 in patient and control

4. Conclusion

Our study was performed for the early detection/diagnosis of oral submucous fibrosis (OSF), by using the phenomenon of 'MMP9 Q279R polymorphism' as a potential biomarker. In the present pilot study, 40 healthy individuals were considered as controls and 40 patients suffering from OSF were selected as experimental group. A detailed history of both patients and controls were recorded. First of all DNA was isolated from blood samples and then PCR of the isolated DNA was performed, followed by RFLP technique to obtain the genotypic sequence of samples. In control, the frequencies of wild type (Q/Q), heterozygous (Q/R) & mutant (R/R) were 22.5%, 50% & 27.5% where as in patients these were 17.5%, 45.5% & 27.5%.

The analysis of odds in overall genotype has shown the ratio of R/R compared to Q/Q, which was 1.75 (0.41 -7.48) and p-value was 0.575. The analysis of odds ratio in age group <35years, the genotype analysis has shown the ratio in R/R compared to Q/Q = 1.80 (0.18-19.10) and p-value = 0.920, and in age group>35years, has shown an odd ratio of 0.66(0.08-4.97) and p-value = 0.969. The analysis of odd ratio in male genotype analysis shows the ratio in R/R upon Q/Q = 2.50(0.36-18.58) and p-value = 0.276, and in female the odd ratio was 1.25(0.10-16.31) and p value was 0.833. The RT-PCR analysis of MMP-9 has shown a significant up-regulation compared to normal having p value 0.024, using student t-test. The post translational analysis of MMP-9 was done by Western Blot analysis show the relative intensity of active MMP-9 significantly higher than the normal group (p value 0.001), using student t-test.

The experimental population was showing a presence of MMP-9 Q279R polymorphism in patient group, which was statistically cross validated by presenting a significant up regulation of MMP-9 polymorphism in both transcriptional levels as well as by polymorphised MMP-9. We hereby conclude that the polymorphised MMP-9 shows the role in the pathogenesis of oral submucous fibrosis. Thus on the basis of such significant up regulation, we can use the polymorphised MMP-9 as a potential biomarker for an early, low-cost clinical diagnosis for OSF, which will be helpful to prevent the possibility of any advanced malignant manifestation.

5. Acknowledgment

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