Association of Promoter Polymorphism of MMP-1 rs1799750 and MMP-3 rs3025058 with circulating levels of MMP-1 and MMP-3 in patients with Rheumatoid Arthritis

Sagare Aparna1,2, Dandekar Sucheta3 and Rajadhyaksha Anjali4
1. Department of Biochemistry, Seth G.S. Medical College and KEM hospital, Mumbai, INDIA
2. Department of Biochemistry, Dr. D.Y. Patil Medical College, Navi Mumbai, INDIA
3. Department of Biochemistry, Eras Medical College, Lucknow, INDIA
4. Department of Medicine, Seth G.S. Medical College and KEM hospital, Mumbai, INDIA
*aparnaa4s@yahoo.com

Abstract
The genetic background of Rheumatoid arthritis (RA) is only partly understood and several genes seem to be involved. Matrix metalloproteinases (MMPs) are involved in joint destruction in rheumatoid arthritis (RA). MMP1 (interstitial collagenase) and MMP3 (stromelysin 1) as most studied enzymes in RA. In the present study, we have investigated the genotypic and haplotypic relationships of the MMP-1 and MMP-3 genes with circulating levels of these MMPs. Functional relevance in single-nucleotide polymorphisms (SNPs) in rs1799750 (1G/2G, MMP-1 promoter) and rs3025058 (5A/6A, MMP-3 promoter) genotyped in 60 RA patients and 60 healthy control is used to determine association of polymorphisms with the severity of the disease.

The MMP-3 SNPs were associated with serum MMP-3 level (P <0.05) and were associated with disease activity score (DAS28). We observed that MMP-1 SNPs were lacking in association with disease activity. Our findings conclude that several closely linked polymorphisms in the MMP-1—MMP-3 loci have an important role in determining the circulating levels of these MMPs in RA and that MMP-3 polymorphism is associated with the disease severity.

Keywords: Rheumatoid Arthritis, Matrix metalloproteinases, Promoter polymorphism, Extracellular Matrix.

Introduction
Rheumatoid arthritis (RA) is an autoimmune inflammatory disease with unknown aetiology. Synovial inflammation and hyperplasia, autoantibody production, cartilage and bone destruction are the main characteristics of the disease. Various environmental factors and genetic mechanism are involved in pathogenesis of RA. Currently, many studies are focusing to find out the relationship of new genes and its association with disease susceptibility in RA. Progressive destruction of cartilage and bone manifests as RA outcome ranges from mild to severe polyarthritis. RA is inflammatory disease in the release of abnormal amount of ECM proteases. These ECM proteases involve a large family of proteins grouped in several subfamilies, named the matrix metalloproteinases (MMPs) as a group of zinc-dependent endopeptidases which are involved in the degradation of every component of the extracellular matrix. Much of the destruction in RA is mediated by abnormal release of matrix metalloproteinase (MMPs) in synovium stimulated by persistent inflammation. Many MMPs regulated their transcriptional level by various growth factors, hormones and cytokines.

Stromelysin -1 (MMP-3) is mainly secreted by synovial cells, fibroblasts and cartilage cells and osteoclasts involved in cartilage degradation. MMP-3 is directly involved in degradation of proteoglycan in the extracellular matrix. It also activates other proteins like fiber adhesive proteins, laminin and other MMPs like MMP-1 (collagenase). The undigested or degraded products of both further release into joints causing joint inflammation. The production of MMPs is highly monitored at the level of gene expression. The gene of MMP-1 and MMP-3 is located on long arm of chromosome 11 which covers length of 8 kb and expressed in variety of the cells such as fibroblasts, chondrocytes, endothelial and epithelial cells and in various tumor cells. MMP-1 expression gets affected by various single nucleotide polymorphism at promoter region. MMP-1 at position 1607 defines promoter region of human gene creating two different alleles 1G and 2 G as an effect of insertion/ deletion of guanine position. Also, MMP-3 expression is controlled at the transcriptional level by a promoter gene. At the position 1171, two alleles 5A and 6A are found at the transcription start site in human MMP-3 gene. This promoter polymorphism 5A/6A results into variable transcription activity of MMP-3.

A relationship between the MMP-3 5A/6A (rs3025058) polymorphism and circulating levels of MMP-3 in RA has been found in two studies. Although another study found no relationship among serum levels and polymorph status. The latter study also found no relationship between the MMP-1 1G/2G (rs1799750) polymorphism and circulating MMP-1 levels, but these studies failed to explain their relationship as both MMP are situated on same chromosome. Also, there is lack of evidence on a research of Indian ethnicity origin.
We believed it would be of interest to further evaluate the relationship between polymorphisms in the MMP-1 and -3 region and the circulating levels of MMP-1 and -3. This study aimed to investigate the correlation between serum levels of MMP-1 and MMP-3 and their gene polymorphism in RA, to provide an objective basis for prognosis evaluation and to their relation with disease activity of RA.

Material and Methods

Between April 2018 to December 2019, 60 RA patients were enrolled from KEM hospital, Mumbai. Study population comprises of age 38-72 yrs. including both sexes with age matching 60 healthy controls. All RA patients were diagnosed in accordance with the RA classification criteria by standards of American Rheumatism Association (ACR) and the European League Against Rheumatism (EULAR) modified in the year 2009 by Aletha et al. ²

The RA disease activity determination criteria were: medium rest pain, morning stiffness more than 1 h, joint swelling ≥3, joint tenderness points >5 and ESR >28 mm/h; CRP.

We calculated DAS 28 from standard health assessment questionnaire.¹¹ Patients with diabetes and metabolic abnormality diseases, liver and kidney dysfunction, severe cardiac dysfunction, other joint diseases, malignant tumours, pregnancy and lactation were excluded from the study. DAS28 was calculated by formula containing values of SJC, TJC, VAS and ESR for defining RA severity

DAS28=0.56*√(TJC)+√0.28*(SJC)+0.70*Ln(ESR)+0.014 *VAS

• DAS 2.6- 3.1 Low disease activity (n= 3)
• DAS 3.2 to 5.1 Moderate disease activity (n= 44)
• DAS > 5.1 High disease activity (n=13)

10 ml of venous blood samples were collected in plain and EDTA bulb after taking their written consent and serum MMP-1 and MMP-3 levels were estimated by ELISA using Elabsciences commercial kit. For extraction of genomic DNA, we used Epigentek mini kit for isolation of DNA from blood PCR and restriction fragment length polymorphism analysis was applied to determine genotypes of MMP- 3 (rs3025058) and for MMP-1 (rs1799750):

PCR and RFLP for MMP-1 and MMP-3 polymorphism

MMP3 5A6A (rs3025058) Primers: Forward: 5’-GTTCTCTCATTCCTTTGATGGGGGGAAGA-3’,
Reverse: 5’-CTTCCTGGAATTCAACTACGCTGCCACCA CT-3’¹⁰

PCR master mix contained 2 μl diluted genomic DNA approximately (~ 20 ng), 2 μl of 10 × reaction buffer, 0.6 μl of 50 mM MgCl₂, 2 μl of 2 mM dNTPs, 1.6 μl of each 10 μM primer, 0.16 μl of 5 U/μl Taq polymerase and 10.04 μl of ddH₂O. Cycling setting is: 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C, with final extension at 72°C for 5 minutes.

MMP1 1G2G (rs1799750) Primers:
Forward: 5’-TGACTTTAAAACATAGTCTATGTTCA-3’,
Reverse: 5’-TCTTGAGATTTGAGATAAGTCATAg C-3’.

PCR Master mix contains 2 μl (~ 20 ng) of diluted genomic DNA, 2 μl of 10 × reaction buffer, 1 μl of 50 mM MgCl₂, 4.8 μl of 2 × polymerate additive, 2 μl of 2 mM dNTPs mix, 1 μl of each 10 μM primer, 0.16 μl of 5 U/μl Taq polymerase and 6.04 μl of ddH₂O. Cycling setting is: initial denaturation 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 30 seconds at 72°C, with final extension at 72°C for 5 minutes. Generally, each digestion reaction was performed in a 20 μl volume made up of 10 μl of PCR product, 1 × appropriate reaction buffer, 1 × bovine serum albumin (BSA), ddH₂O and appropriate restriction enzyme Tth111I (for MMP3 5A6A polymorphism). Incubation: at 65°C for 3 hours and Alul (for MMP1 1G2G polymorphism). Incubation: at 37°C for 16 hours.

The digested products were resolved on 2% agarose gels on electrophoresis. Gels containing ethidium bromide were loaded with 10 μl of each digest sample and 5 μl of DNA marker (100 bp DNA ladder thermoscientific) for 120 volts for 45 min. Bands were observed through UV transilluminator suggestive of alleles exhibit 97 bp bands for MMP

Statistical Analysis: Numerical data were reported as means and standard deviation (± SD). Differences between means were analyzed using one-way analysis of variance and the chi-square test respectively. All statistical analyses were performed using the SPSS 26.0 software (Chicago, IL, USA); p values<0.05 remains significant. HW equilibrium for polymorphism of genotype distribution of each was tested with goodness of fit. LD index was used to estimate the strength of LD between each pair of marker. LD block was defined by four gamete rule. The haplotype frequencies were estimated using an expectation maximization likelihood frequency of haplotype. SNPSTAT and Haplovie 4.2 were used to generate haplotype frequency and linkage disequilibrium.

Results

The study included 120 patients 60 RA (18 males and 42 females, mean age 50.0±8.5 years) and 60 control subjects (39 males and 21 females, mean age 41.7±11.6 years). The percentage of females was higher among RA cases (70%) compared to controls (35%).

Common characteristics of the disease and the control group are shown in table 1. It has been found that progressive rise in serum levels of MMP-3 in disease severity of RA and it
remained significantly high as compared with control. Our results showed that plasma MMP-1 level remained significantly high in RA when compared with control. It was observed that no significant change in their levels occurs as disease progresses. Progressive increased levels in inflammatory marker like ESR and CRP in RA are observed and remained significant when RA cases are compared with controls. DAS activity distributes cases as mild, moderate and severe.

Table 2 shows frequency distribution of alleles of MMP-1 and MMP-3 genes. The genotypic distribution of the MMP-1 gene polymorphism (rs1799750) is suggestive of no deviations from Hardy–Weinberg equilibrium found for the MMP-1 or MMP-3 alleles for both control and patient group.

![Fig. 1A: Genotyping of MMP-1 promoter polymorphism lane 1- 6 RA, control 7-9 and lane10 DNA ladder (product size 29 bp for 1G and 269 bp for 2G)](image1)

![Fig. 1B: PCR product of MMP-3 gene separated on 2 % agarose gel lane 1-9 shows RA, 10-100 bp DNA ladder, 11-20 control size of PCR product 97 bp for 5A and 129 bp for 6A)](image2)

Table 1
Disease characteristics in study (*p < 0.05)

<table>
<thead>
<tr>
<th></th>
<th>Mild RA (n=3)</th>
<th>Moderate RA (n=44)</th>
<th>Severe RA (n=13)</th>
<th>All RA patients (n=60)</th>
<th>Control (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age yrs</td>
<td>59±3</td>
<td>49.3±7.4</td>
<td>46.8±10.7</td>
<td>50.0±8.5</td>
<td>41.7±11.6</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>3/0</td>
<td>9/35</td>
<td>6/7</td>
<td>18/42</td>
<td>39/21</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>41±7</td>
<td>41.1±16.2</td>
<td>52.9±17.3</td>
<td>44.3±16.8*</td>
<td>13.2±3.9</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.9±1.5</td>
<td>9.1±11.0</td>
<td>22.4±27.1</td>
<td>12.11±17.2*</td>
<td>1.35±0.6</td>
</tr>
<tr>
<td>DAS</td>
<td>3.23±0.11</td>
<td>4.14±0.49</td>
<td>5.38±0.22</td>
<td>4.3±0.7</td>
<td>----</td>
</tr>
<tr>
<td>MMP 1 (ng/mL)</td>
<td>18.4±3.07</td>
<td>19.1±4.6</td>
<td>16.6±4.1</td>
<td>18.8±4.8*</td>
<td>4.3±3.0</td>
</tr>
<tr>
<td>MMP 3(ng/mL)</td>
<td>50.9±1.2</td>
<td>62.1±15</td>
<td>74.9±20.9</td>
<td>67.5±23.8*</td>
<td>22.0±4.7</td>
</tr>
</tbody>
</table>
The frequency of the 5A/6A allele genotype was higher in RA patients (71.6%) than controls (26.7%) and the frequency of the 1G/2G allele was higher in RA (65%) than controls (5%) and this difference remains statistically significant by chi square analysis (p <0.0001).

Haplotype association response in RA and control is suggestive of haplotypic association among MMP1 and MMP 3. Specifically, combination of 2G- 5A, 1G -6A, 2G- 6A remain statistically significant with non-significant allelic combination 2G- 6A (Table 3).

The linkage disequilibrium index D’ value was found to be 0.96 and pairwise correlation between rs3025058 and rs1799750 was very high. LD value suggests that the two SNPs coinherited roughly 96 % of the time and r² = 0.8 is suggestive of alleles that tend to occur on the same haplotype having almost same allele frequencies. It was noted that the majority of 2G alleles of rs179975 (64 %) was linked with the most frequent haplotype 5A and 1G associated with 6A. Significant Haplotype association suggested that MMP-1 and MMP-3 allele are in equilibrium as alleles located on the same chromosome.

**Discussion**

Large-scale genome wide association studies (GWAS) show interrelation of SNP markers with RA disease susceptibility. Many common risk alleles may affect genetic component of disease susceptibility; however, it remains unknown to date owing to the limited power of current GWAS. The association between MMP-3 and MMP -1 gene polymorphism in RA was poorly understood; MMP-3 is believed to play an important role in destruction of joint in RA. and there are few common polymorphisms in the promoter sequence of these gene which may be correlated with RA susceptibility.

Results of our study suggest levels of serum MMP-3 correlated well with disease severity in RA but MMP -1 failed to have such a pattern. We have also found that serum levels of MMP-3 are correlated with markers of inflammation ESR and CRP levels. Studies reveal that MMP1 expression occurs in cells of the invading front of the activated RA synovial tissue and is correlated with erosive arthritis.

Also, it is seen that Integrated MMP-1 levels are correlated with the number of new joint erosions. Overexpression of MMP-1 provides a molecular mechanism for extensive degradation of ECM, which results in tissue remodelling and repair during development and inflammation. It is observed that MMP-3 activates latent MMP-1, enhancing MMP-1 activity in vitro up to 12-fold. 

Study of Kobayashi et al suggests the elevated serum levels of MMP-3 were found in patients with early RA and it might predict that bone damage will remain useful marker for predicting early stages of RA. Few studies have described earlier increased levels of both MMPs in the serum and synovial fluid of patients with RA and other inflammatory joint diseases and it correlated well with markers of inflammation and with the radiographic damage in early RA.

Deletion–insertion polymorphism of MMP-1 rs1799750 creates additional guanine (G) which in turn produces E26

---

**Table 2**

<table>
<thead>
<tr>
<th>MMP-3</th>
<th>Healthy control N= 60 (%)</th>
<th>Rheumatoid Arthritis N= 60 (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A</td>
<td>42 (70.0%)</td>
<td>9 (15 %)</td>
<td>-</td>
</tr>
<tr>
<td>5A/6A</td>
<td>16 (26.7%)</td>
<td>43 (71.6%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>6A</td>
<td>2 (3.3%)</td>
<td>8 (13.3%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Case, control ratio</th>
<th>Chi square</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2G-5A</td>
<td>0.577</td>
<td>0.350,0.812</td>
<td>52.258</td>
<td>4.8 X 10^-13</td>
</tr>
<tr>
<td>1G-6A</td>
<td>0.177</td>
<td>0.293,0.057</td>
<td>22.873</td>
<td>1.7X10^-6</td>
</tr>
<tr>
<td>2G-6A</td>
<td>0.156</td>
<td>0.199,0.122</td>
<td>3.41</td>
<td>0.0648</td>
</tr>
<tr>
<td>1G-5A</td>
<td>0.090</td>
<td>0.158,0.019</td>
<td>14.14</td>
<td>2X10^-4</td>
</tr>
</tbody>
</table>
with increasing level of serum MMP-3, but remains insignificant on the disease activity or severity of RA.

Various MMPs involved in disease pathology of RA are regulated by epigenetic mechanism by producing conformational changes in chromatin fibre which in turn controls gene expression which might be the result of involvement of DNA binding proteins like transcription factors. Inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL) increase involvement of transcription factors NFκappa B (NF-KB) and AP-1 which bind to promoter region of MMP thus regulates MMP gene.

Study by Nagasawa et al. suggested that inflammatory cytokine IL-1 and TNF-α may upregulate MMP-3 mRNA expression while IL-4 and TNF-β can downregulate it.

It was observed by Vanessa et al. that NF-KB, a transcriptional factor affects 5A and 6A allele in macrophages with high 5A allele transcription supplemented by this transcription factor. Stimulation to inflammation to macrophages may also alter expression of MMP-1 by modifying MMP-1 1G/2G polymorphism. This mechanism results in high MMP-1 activity irrespective of variation in genotype of MMP-1 1G or 2G.

Study by Ye et al. found another transcription factor named ZBP89 which may affect promoter polymorphism of MMP-3 which in turn increases in the release of these enzymes. Our findings support this possible mechanism of inflammation and release of these inflammatory markers in regulating cascade of transcription of MMP genes resulting changes in their circulating levels.

Both genes of MMP-1 and MMP-3 have been mapped on chromosome 11 the long arm in the region 11q22.3, distance between the two near about of 37.64 kilobases. The phenotypic and genotypic appearance of both gene still remains unknown.

The biological function of this phenomenon is not much understood. Our observation suggests that MMP-3, SNPs rs3025058 and MMP-1 rs1799750 are located in the same LD block and the association remains significantly strong. Study by Chen et al. supports findings of our study suggestive of association of both SNPs with high serum MMP-1 and serum MMP-3 level as they are in complete equilibrium.

Our study shows a significant linkage disequilibrium among the 1G/2G MMP1 and the 5A/6A MMP-3 polymorphism. However, our observation tells that this phenomenon can be attributed to the proximity of the MMP-1 and MMP-3 genes which is suggestive of alteration of transcriptional activity of these MMPs. Expressions of MMP-1 and MMP-3 are often synchronised and their promoters may contain some regulatory elements.
Fig. 2A and 2B: Box and whisker plot shows serum MMP-1 and Serum MMP-3 level stratified by polymorph status. This graph suggest that highest serum MMP-1 level is associated with 1G allele and suggestive of highest Serum MMP-3 associated with 6A allele.

**Conclusion**

In summary, our study provides further insight into the interrelationship among MMP-1–MMP-3 loci and serum MMP-1–MMP-3 levels. It stipulates that several closely linked polymorphisms in the MMP-1–MMP-3 loci may define important role in assessing the circulating levels of these MMPs in RA. MMP-3 gene polymorphism may affect disease activity in RA. Further research needs to be done to determine the link between genetic variants of MMP-1 and -3 and their expressions and how they are concerned to circulating protein levels and disease severity.

In addition, further evaluation of functional characterization of MMP-1 and -3 variants correlation with clinical features will be needed to determine the role of genetic variation at these loci in the pathogenesis of RA.

**Acknowledgement**

We gratefully acknowledge Sakal India Foundation’s - Oka Research Fellowship for the year 2018-2019 for providing financial support to our study.

**References**


16. Lepetsos Panagiotis, Pampanos Andreas, Kanavakis Emmanouil, Maria Tzetis, Dimitrios Korres, Papavassiliou Athanasios G. and Nicolos Efthathopoulos, Association of MMP-1/1607 1G/2G (rs1799750) polymorphism with primary knee osteoarthritis in the Greek population, *Journal of orthopedic Research, 32(9),1150-1160 (2014)*


(Received 30th June 2021, accepted 10th August 2021)