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Association of matrix metalloproteinase -7 (-181*A/G*) promoter polymorphism in chronic pancreatitis

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Background & objectives: **Chronic pancreatitis is progressive and irreversible destruction of the pancreas. Matrix metalloproteinase-7 (MMP-7) is a secreted matrilysin, which contributes to angiogenesis and breakdown of basement membranes of pancreatic tissues. The present study was aimed to investigate the association of MMP-7 −181*A/G* (rs11568818) gene promoter polymorphism in patients with chronic pancreatitis.**

Methods: **A total of 100 chronic pancreatitis patients and 150 unrelated healthy individuals were included in this case control study. The genotyping of the *MMP-7* gene (− 181 *A/G*) (rs11568818) was carried out based on PCR-RFLP. The serum levels of MMP-7 were determined by ELISA. Association between genotypes and chronic pancreatitis was examined by odds ratio (OR) with 95% confidence interval (CI).**

Results: **The frequencies of the genotypes in promoter of MMP-7 were *AA* 49 per cent, *AG* 25 per cent and *GG* 26 per cent in chronic pancreatitis patients and *AA* 53 per cent, *AG* 38 per cent and *GG* 9 per cent in control subjects. Frequency of *MMP-7* −181*GG* genotype and − 181*G* allele was significantly associated with chronic pancreatitis compared to healthy subjects [OR = 1.58 (95% CI: 1.06 –2.36), *P* =0.019]. There was no significant difference in the serum MMP-7 levels in the patients compared to control subjects.**

Interpretation &conclusions: **The present study revealed a significant association of *MMP-7* -181*A/G* (rs11568818) *GG* genotype with chronic pancreatitis patients, indicating its possible association with the disease.**

Key words Chronic pancreatitis - genetic susceptibility - genotypes - inflammation - matrix metalloproteinase - myofibroblasts

Chronic pancreatitis (CP), an inflammatory disorder of the pancreas like acute pancreatitis, occurs when digestive enzymes attack the pancreas and nearby tissues, and is clinically characterized by recurrent attacks of abdominal or back pain, pancreatic stone formation, exocrine and endocrine pancreatic insufficiency in advanced stages[1]. Matrix metalloproteinases (MMPs) form an important family

of metal-dependent endopeptidases that represent the major class of enzymes responsible for degradation of extracellular matrix components[2]. MMPs are classified as five main classes: collagenases, gelatinases, stromelysins, membrane-type and others, on the basis of their putative substrate specificity and internal homologies[3]. Matrix metalloproteinase-7 (MMP-7) (matrilysin, pump-1 protease or PUMP-1), is among the smallest members of the MMP family. It is a protease with broad substrate specificity, being able to degrade elastin, proteoglycans, fibronectin and type IV collagen[2,4]. In addition, MMP-7 can cleave non-matrix substrates from the cell surface, including E-cadherin, pro-tumour necrosis factor α, and Fas ligand, which is called 'sheddase' function in apoptosis [5-7].

There are at least three regulatory mechanisms that influence activities of MMPs: regulation of transcription, activation of latent MMPs and inhibition of MMP function by tissue inhibitors of metalloproteinases. However, the most important step may be transcriptional regulation, since most MMP genes express only when active physiological or pathological tissue remodelling takes place. Growing evidence indicates that natural sequence variations in promoter regions of the MMP genes may result in variable expression of MMPs[8]. These polymorphisms have been associated with susceptibility to various diseases including acute myocardial infarction[9] rheumatoid arthritis[10] multiple sclerosis[11] and cancers[12-14]. A single nucleotide polymorphism (SNP) in the promoter region of the *MMP-7* gene, especially an A to G transition at the -181 base pair (-181 *A/G*) (rs11568818) has been proved to be functional *in vitro* and may influence coronary artery dimensions[15]. The present study was aimed at understanding the role of the biallelic polymorphism in the -181 promoter region of the *MMP-7* gene in the pathophysiology of chronic pancreatitis.

Material & Methods

Subjects: A total of 100 clinically evaluated consecutive chronic pancreatitis patients referred to the Gastroenterology unit of Gandhi Hospital and Osmania General Hospital, Hyderabad, India from January 2010 to February 2012 were included in the study, with the approval of protocol from the Institutional Ethics Committee. The patients were confirmed for chronic pancreatitis based on the clinical diagnosis, biochemical findings and imaging analysis. A total of 150 asymptomatic control subjects were randomly selected among the individuals visiting the Institute of Genetics and Hospital for Genetic Diseases, Hyderabad, for regular health check-up who had no complaints or evidence of pancreatitis or any other gastric problem. Informed written consent was obtained from all the subjects. The demographic characteristics such as sex, age, duration, addictions like smoking, and alcohol consumption were noted based on the standard proforma. Subjects who were consuming 80 g alcohol/day for a period of more than two years were considered as alcoholics[16,17].

DNA extraction: Venous blood (5 ml) was drawn from each individual in vacutainers containing EDTA and stored at 4°C. Genomic DNA was extracted from the peripheral blood samples by following the salting out procedure of Lahiri *et al*[18], and stored in TE buffer at -20°C until further use.

MMP-7 SNP genotyping: The MMP-7-181*A/G* (rs11568818) genotypes were determined as previously described by Zhang *et al*[19] by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assay[19]. The primers for amplifying the MMP-7 fragment were 5'-TGG TAC CAT AAT GTC CTG AAT G-3' (forward) and 5'-TCG TTA TTG GCA GGA AGC ACA CAA TGA ATT-3' (Reverse) [Bioserve, India]. The fourth nucleotide close to the 3' end of the reverse primer was mutated from T to A to create an EcoRI recognition site when the -181G allele exists. The PCR was performed in a 25 µl volume containing 100 ng DNA template, 2.0 ml of 10x PCR buffer, 1.5 mM $MgCl_2$, 1 U Taq-DNA polymerase (Bangalore Gene, India), 200 mM dNTPs and 200 nM each primer. The PCR cycling conditions were: 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 65°C and 30 sec at 72°C, and with a final step at 72°C for 5 min to allow for the complete extension of all PCR fragments. An 8-µl aliquot of PCR product was subjected to digestion at 37°C overnight in a 10 µl reaction containing 10 U EcoRI (Fermentas, USA) and 1x reaction buffer. After digestion, the products were separated on a 3 per cent agarose gel stained with ethidium bromide (Figure). As a result, the -181G alleles were represented by DNA fragments of size at 120 and 30 bp, the -181A alleles size of 150 bp, whereas the heterozygotes displayed a combination of both alleles of size (150, 120 and 30 bp). Ten per cent of the samples were randomly taken, and the assay

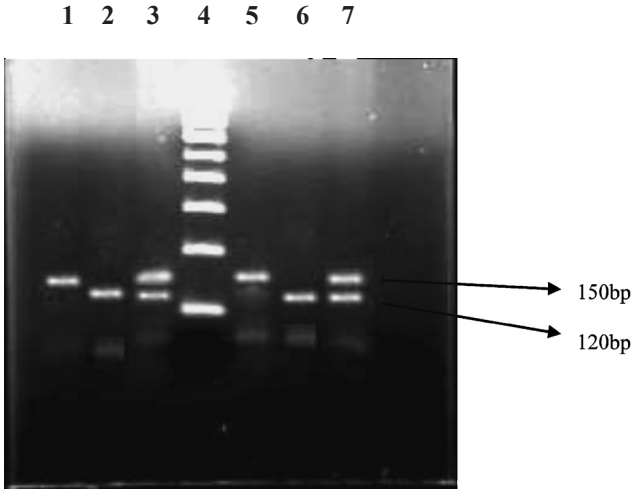

Fig. RFLP products of MMP-7 (-181 A/G) genotypes. Lanes 1&5-homozygous A/A (150 bp); lanes 2&6-homozygous G/G (120 bp); lanes 3&7-heterozygous A/G (150 bp/120 bp); lane 4- 100 bp ladder.

was repeated and found no bias in the genotyping. The findings were similar on replicative study with the results being 100 per cent concordant.

MMP-7 ELISA: Quantitative sandwich enzyme immunoassay for human MMP7 was performed as recommended by the manufacturer (Raybiotech®, Inc, Norcross, GA, USA). In brief, 100 µl of each standard and sample were added into the wells and incubated at room temperature for 2 h. After washing four times with washing buffer, the biotinylated antibody was added, and the plate was further incubated for 1 h; after two washings 100 µl of prepared streptavidin solution was added to each well and incubated for 45 min at room temperature with gentle shaking. The solution was discarded and washed properly using wash buffer, and 100 µl of tetra-methylbenzidine (TMB) one step substrate reagent was added to each well, and incubated for 30 min at room temperature in dark with gentle shaking. The absorbance was read at 450 nm immediately. The MMP-7 concentrations for each sample were calculated from the standard curve obtained.

Statistical analysis: Hardy-Weinberg analysis was performed to compare the observed and expected genotype frequencies using χ^2 test. Comparison of the *MMP-7* genotype distribution in the study groups was performed by means of two-sided contingency tables using χ^2 test. MMP-7 levels were compared using student t- test and ANOVA. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression.

Results

The demographic data of the samples under study are presented in Table I. The gender-wise distribution of patients and control subjects revealed no significant difference. Age (median)-wise comparison showed significant (P<0.001) difference in patients compared to control subjects. The proportion of smokers in CP patients (50%) was significantly higher than the healthy controls (30%) [OR = 2.333 (1.335-4.085), P=0.002]. Similarly, the frequency of alcoholics in chronic pancreatitis patients was significantly different from that in healthy controls [OR = 3.273 (2.839-5.849) P<0.001].

Genotype analysis: The frequencies of *A/A, A/G* and *G/G* genotypes in CP were 49, 25 and 26 per cent compared to 53, 38 and 9 per cent in the control subjects, respectively (Table II). The MMP-7 (-181 *A/G*) *GG* genotype frequency was higher in patients as compared to control group. When the MMP-7 (181A>G) *AA* genotype was used as the reference, it was found that CP patients with *MMP-7* (181*AG*) *GG* genotype were significantly associated with the risk for the disease (OR=2.99, 95% CI= (1.35-6.73), P=0.004). Moreover, in recessive model (OR=3.41, 95% CI=(1.60-7.37), P=0.001), the results showed that MMP-7 (-181*AG) GG* allele was conferring significant increased risk for CP. Further, the frequency of the -181*G* allele in chronic pancreatitis patients was significantly higher than that in healthy controls [OR=1.58 (95% CI: 1.06–2.36), P=0.019]. The Hapmap GIH frequency

Table I. Demographic and clinical data of chronic pancreatitis patients and control subjects

Characteristics	Patients (N=100)	Controls (N=150)
Gender		
Males	94 (94)	138 (92)
Females	6 (6)	12 (8)
Median age (yr)		
< 38	48 (48)***	91 (63)
≥ 38	52 (52)	59 (37)
Smoking		
Smokers	50 (50)**	45 (30)
Non-smokers	50 (50)	105 (70)
Alcoholism		
Alcoholics	72 (72)***	66 (44)
Non-alcoholics	28 (28)	84 (56)

Values in parentheses represent percentages.
P**<0.01, ***<0.001 compared to controls

of MMP7 (-181*A/G*) was found to be 37.5 per cent as per International HAP Map consortium[20] and the present study revealed 28.5 per cent, which could be due to the ethnic variation within different regions of Indian population. The *MMP-7* gene frequency in chronic pancreatitis patients was not found to be in Hardy Weinberg equilibrium at 5 per cent level of significance. The gene frequency of control subjects was in Hardy Weinberg equilibrium at 5 per cent level of significance.

MMP-7 serum levels: Table III gives the *MMP-7* levels with respect to *MMP-7* genotype in controls and chronic pancreatitis patients. In general MMP-7 levels were found to be increased in individuals with *G/G* genotype when compared to *A/G* and *A/A* genotypes though the difference was not significant. There was no significant difference between the mean levels of MMP-7 between chronic pancreatitis (0.29 ± 0.25 ng/ml) and controls (0.245 ± 0.17 ng/ml) [*P*=0.344].

Association of MMP-7 (181A>G) genotypes and serum levels with demographic and clinical characteristics of CP: Potential associations were explored between *MMP-7* genotypes and demographic and clinical characteristics such as median age, median duration of the disease, addictions like smoking and alcoholism, steatorrhoea, calcification, and atrophy (Table IV). No significant association of the demographic and clinical features was found in the present study.

Interaction of MMP-7 (181A>G) genotypes with environmental factors: On analyzing gene environment interaction, a significant modulation of chronic pancreatitis by MMP-7 (181A>G) *GG* genotype was found with alcoholism, but significance was lost after Bonferroni correction (Pc = 0.168) (Table V).

Discussion

Chronic pancreatitis is the progressive and permanent destruction of the pancreas resulting in exocrine and endocrine insufficiency and, often, with chronic disabling pain. Males are about three to four times more likely to be affected than females[21]. MMP-7 is the smallest known member of MMP

Table II. Frequency distribution of genotypes and of MMP-7 (-181 *A/G*) genotypes with risk of chronic pancreatitis

Genotype	Controls		Patients		OR (95% CI), *P* value
	N	%	N	%	
AA	79	53	49	49	1 (Ref)
AG	57	38	25	25	0.70 (0.38-1.33), 0.300
GG	14	9	26	26	2.99 (1.35-6.73), 0.004
Dominant model					
AA	79	53	49	49	1 (Ref)
AG+GG	71	47	51	51	1.15 (0.68-1.98), 0.607
Recessive model					
AA+AG	136	91	74	74	1 (Ref)
GG	14	9	26	26	3.41 (1.60-7.37), 0.001
A allele	215	71.6	123	61.5	
G allele	85	28.4	77	38.5	1.58 (1.06-2.36), 0.019

Table III. Mean serum levels (ng/ml) of MMP-7 in chronic pancreatitis patients and control subjects with respect to the genotypes

MMP-7	Patients (N)	MMP-7 level (ng/ml)	Controls (N)	MMP-7 level (ng/ml)
Genotype				
A/A	49	0.14 ± 0.04	79	0.135 ± 0.04
A/G	25	0.22 ± 0.65	57	0.349 ± 0.16
G/G	26	0.64 ± 0.25	14	0.573 ± 0.10
Total	100	0.29 ± 0.25	150	0.245 ± 0.17

Values are mean ± SD

Table IV. Intragroup comparison of MMP-7 genotypes and levels with respect to demographic and clinical parameters of chronic pancreatitis

Parameter	MMP-7 levels			
	A/A (Mean ± SD) (n)	*A/G* (Mean ± SD) (n)	*G/G* (Mean ± SD) (n)	*P* value
Median age (yr)				
< 38	0.13 ± 0.03 (29)	0.24 ± 0.08 (08)	0.67 ± 0.24 (11)	0.634
≥ 38	0.14 ± 0.05 (20)	0.21 ± 0.06 (17)	0.61 ± 0.29 (15)	
Sex				
Male	0.14 ± 0.04 (45)	0.22 ± 0.07 (24)	0.64 ± 0.26 (25)	0.715
Female	0.14 ± 0.06 (05)	0.29 (1)	-	
Median duration of disease(yr)				
< 1	0.13 ± 0.03 (24)	0.22 ± 0.08 (08)	0.67 ± 0.28 (08)	0.663
≥ 1	0.14 ± 0.05 (25)	0.22 ± 0.06 (17)	0.62 ± 0.27 (18)	
Smoking				
Non-smokers	0.13 ± 0.04 (26)	0.25 ± 0.07 (10)	0.70 ± 0.21 (15)	0.084
Smokers	0.14 ± 0.04 (23)	0.20 ± 0.05 (15)	0.55 ± 0.31 (11)	
Alcoholism				
Non-alcoholics	0.13 ± 0.04 (18)	0.26 ± 0.04 (02)	0.61 ± 0.32 (08)	0.829
Alcoholics	0.14 ± 0.04 (27)	0.22 ± 0.07 (23)	0.64 ± 0.26 (22)	
Steatorrhoea				
No	0.14 ± 0.04 (43)	0.23 ± 0.07 (21)	0.66 ± 0.26 (23)	0.431
Yes	0.12 ± 0.02 (06)	0.17 ± 0.03 (04)	0.50 ± 0.32 (03)	
Calcification				
No	0.14 ± 0.04 (34)	0.23 ± 0.06 (16)	0.61 ± 0.26 (19)	0.257
Yes	0.13 ± 0.04 (15)	0.21 ± 0.08 (09)	0.71 ± 0.27 (07)	
Atrophy				
No	0.14 ± 0.04 (43)	0.23 ± 0.07 (22)	0.63 ± 0.26 (25)	0.064
Yes	0.11 ± 0.02 (06)	0.19 ± 0.05 (03)	0. 95 (01)	
Diabetes				
No	0.14 ± 0.04 (46)	0.23 ± 0.07 (22)	0.64 ± 0.27 (25)	0.779
Yes	0.14 ± 0.07 (03)	0.17 ± 0.03 (03)	0.68 (01)	

Table V. Interaction of MMP-7 (-181*A*>*G*) genotypes with smoking and alcoholism

Genotypes	Controls		Chronic pancreatitis		
	Non-smokers (n)	Smokers (n)	Non-smokers (n)	Smokers (n)	OR (95 % CI) *P* value*
AA	56	23	26	23	1 (Ref)
AG	50	7	10	15	2.1 (0.66-7.17), 0.197
GG	11	3	15	11	3.67 (0.79-19.25). 0.072
	Non-alcoholics (n)	Alcoholics (n)	Non-alcoholics (n)	Alcoholics (n)	OR (95 % CI) *P* value
AA	48	31	18	27	1 (Ref)
AG	38	19	2	23	1.39 (0.58-3.34), 0.544
GG	5	9	8	22	2.81 (1.01-7.95), 0.043**

*P value was calculated only with smokers and alcoholics only; **corrected P value after Bonferroni correction = 0.168

family and possesses the highest extracellular matrix (ECM) degrading activity against a variety of ECM components, including elastin, gelatin, type IV collagen, fibronectin, vitronectin, laminin, entactin, aggrecan and proteoglycans[21,22]. It is also capable of triggering the activation of an MMP cascade[23].

Previous studies in chronic pancreatitis highlighted the role of MMP-7 based on expression data and immunohistochemical analyses[23]. MMP-7 is expressed exclusively in the metaplastic ductal epithelium of chronic pancreatitis patients and has been shown to regulate acinar cell apoptosis through proteolytic release of its pro-apoptotic molecule Fas ligand[24]. Sires *et al*[25] have reported that MMP-7 efficiently cleaves the basement membrane protein entactin, which bridges laminin and collagen type 4, and suggested a potentially important role for MMP-7 in the disruption of basement membranes by inflammatory cells.

The initial event in chronic pancreatitis is damage to one type or all tissues, compartments or cell types of the pancreas, leading to cell necrosis and/or apoptosis and subsequent release of cytokines/growth factors like tumour growth factor β1 either from migrating inflammatory cells, especially neutrophils, and/or nearby pre-existent epithelial or mesenchymal cells. In a second step, the damaged cells are phagocytosed by neutrophils and macrophages, and the released cytokines cause activation and proliferation of resident fibroblasts/pancreatic stellate cells in the immediate vicinity of the original site of damage and induce them to transform into myofibroblast cells. In the last phase, myofibroblasts produce and deposit ECM, which replaces the inflammatory infiltrate and affects the architecture and function of the surviving pancreatic tissues[26,27].

The present study revealed an increased frequency of *GG* genotype of *MMP 7* in chronic pancreatitis patients compared to control subjects, indicating its possible association with the disease. Earlier studies on transient transfection by Jormsjo *et al*[15] revealed that promoter activity of the -181*G* allele was 2- to 3-fold higher than that of the -181*A* allele. Similarly, higher risk in alcoholics was further enhanced due to interaction of MMP-7 -181*GG* genotype. The profibrotic role of MMP7 might be multiple considering its broad substrate specificity that includes basement membrane and extracellular matrix components. MMP-7 is required for efficient repair of damaged epithelium, and controls the transepithelial influx of neutrophils across the colonic mucosa in response to injury[28]. Re-epithelialization is a desired outcome of any form of injury in any tissue. In contrast, although neutrophils are an essential arm of innate immunity, an overabundance of these granulocytes can cause indiscriminate, severe, and potentially mortal damage. Reports on liver cirrhosis showed that MMP-7 was released from the bile ductular epithelial cells to the lumen during the tissue repair[29]. Thus, the presence of -181*G* allele in chronic pancreatitis may lead to an elevated expression of MMP-7, which results in pancreatic tissue damage by increased neutrophil influx as well as increased activation of other members of the MMP family such as MMP-2[30].

In conclusion, our results suggest a possible association of *MMP-7*(-181*A/G*) (rs11568818) gene polymorphism in the pathophysiology of chronic pancreatitis. However, this is a preliminary study and the results need to be confirmed in a large cohort.

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