

# MMP-1 polymorphisms and the risk of idiopathic pulmonary fibrosis

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**Abstract** Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrotic lung disorder of unknown etiology and unclear pathogenesis. Matrix metalloproteinase-1 (MMP-1) is strongly upregulated and may contribute to the abnormal remodeling that characterizes the disease. We conducted a case–control study of 130 IPF patients and 305 healthy controls to investigate associations between two polymorphisms of the MMP-1 gene promoter and IPF risk. First, using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis we studied the 2G polymorphism at  $-1,607$ , shown previously to generate the core of an AP-1 binding site and correlate with high transcriptional activity and risk for IPF. The frequency of the 2G/2G genotype was higher in IPF than in controls (63 vs. 49%;  $P < 0.008$ ; OR = 1.7; CI 1.15–2.79). Next, we studied a T/G SNP at position  $-755$ , which we identified by sequencing the MMP-1 promoter. Chromatin immunoprecipitation (ChIP) assay performed on IPF fibroblasts with either  $-755$  genotype revealed an AP-1 binding site for TT<sup>-755</sup> and GT<sup>-755</sup> genotypes. The frequency of this SNP revealed no signifi-

cant differences between IPF and healthy controls. However, when the study individuals were stratified by their smoking status, a significant increase in the T/T genotype frequency was observed in smoking cases compared with smoking controls (45 vs. 26%;  $P = 0.03$ ; OR = 2.3; CI 1.15–4.97). These findings indicate that polymorphisms of the MMP-1 promoter may confer increased risk for IPF and reveal a putative gene–environment interaction between the  $-755$  MMP-1 polymorphism and smoking in this disease.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal lung disease of unknown etiology. It is characterized by aberrant epithelial activation, fibroblastic foci formation, and progressive lung scarring that together lead to abnormal gas exchange (Selman et al. 2001, 2006; Hunninghake et al. 2007). Despite numerous studies, the pathogenic mechanisms implicated in the development of IPF are presently unknown. Recent findings have shown that, despite progressive scarring, a number of matrix metalloproteinases (MMPs) are upregulated at the transcriptional and protein levels indicating that MMPs may play a role in the abnormal remodeling that characterizes the disease (Pardo et al. 2006; Selman et al. 2000, 2006; Zuo et al. 2002; Pardo et al. 2008).

MMPs (also called matrixins) belong to a large family of peptidases consisting of 23 members. MMPs have long been associated with tissue remodeling and influence cell behavior, survival, and death. They are able to cleave the protein components of extracellular matrix and also proteolytically activate highly selective mediators, such as growth factors and cell surface receptors (Pardo et al. 2006; López-Otín et al. 2007). MMP-1 (also known as collagenase-1) is

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the prototype of some of the MMPs capable of degrading fibrillar collagens types I, II, and III. MMP-1 like many other MMPs is undetectable in normal resting tissues and is mainly expressed during physiological and pathological tissue remodeling *in vivo* suggesting a wide role in biology (Pardo et al. 2005).

The MMP-1 gene contains consensus sequences for DNA-binding proteins such as AP-1, AP-2, Ets/PEA-3, as well as responsive elements to glucocorticoids, retinoic acid, and cyclic AMP (Rutter et al. 1997). There is evidence to indicate that a functional polymorphism in the MMP-1 gene promoter that consists of a guanosine (G) insertion at position –1,607 generates a new 5'-GGA-3' core recognition sequence for members of the Ets family of transcription factors (Rutter et al. 1998). Importantly, the 2G genotype displays significantly higher transcriptional activity in normal and malignant cells compared to cells possessing the 1G allele (Rutter et al. 1998; Wyatt et al. 2002).

Given the prominent upregulation of this enzyme in IPF lungs, the purpose of our study was to examine this promoter polymorphism in a cohort of IPF patients. Our results showed that the frequency of the 2G/2G genotype is increased in this disease. In addition, by sequencing the MMP-1 promoter region we identified a new polymorphism at position –755 (G/T) that creates a potential binding site for transcription factors of the activation protein-1 (AP-1) family and thus may influence the transcriptional responsiveness of the MMP-1 promoter. We found that the TT genotype is increased in cigarette smoking IPF patients indicating a potential gene–environment interaction between the –755 MMP-1 polymorphism and smoking in IPF.

## Patients and methods

One-hundred and thirty unrelated IPF patients were included in this study. Diagnosis of IPF (67 males, 63 females,  $62.5 \pm 9.6$  years old) was supported by history, physical examination, pulmonary function studies, HRCT, and bronchoalveolar lavage findings (American Thoracic Society 2000). In 40% of the patients diagnosis was confirmed by morphology (open lung biopsy) based on typical microscopic findings of usual interstitial pneumonia (Katzstein et al. 1998). In the absence of biopsy, patients had to fulfill the criteria of the ATS/ERS international consensus (American Thoracic Society 2000). Patients with known causes of interstitial lung disease (i.e. collagen vascular disease, drug toxicity, environmental exposure), were excluded. Clinical data, including smoking status were extracted from case records. Smoking status was characterized as “never”, “former” (patients who stopped smoking at least 12 months before presentation), or “current” (patients

who were either still smoking or stopped smoking less than a year before presentation) (Selman et al. 2007). Smoking index (packs/year) was also documented.

The control group was comprised of 305 unrelated healthy subjects (189 males, 116 females,  $40.6 \pm 12.4$  years old). Forty-nine IPF patients and 107 controls were former or current smokers. Patients and controls were individuals with the same ethnic origin and with at least two generations born in Mexico. The protocol was approved by the Ethics Committee of the National Institute of Respiratory Diseases, México. All patients and control subjects were informed of the purpose of the study and their consent obtained.

## DNA extraction and MMP-1 genotyping assays

Genomic DNA was extracted from 10 ml of peripheral blood using the BD genomic DNA isolation kit (Maxim Biotech, San Francisco CA). The polymorphic site –1,607 of the MMP-1 promoter was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For each polymorphism, a DNA sequence containing the polymorphic site was amplified by PCR. The PCR primers used for amplifying a fragment of 269 bp for the polymorphism at –1,607 (G > GG) were:

Forward: 5'-TGACTTTTAAACATAGTCTATGTTCA-3'

Reverse: 5'-TCTTGGATTGATTTGAGATAAGTCATAGC-3'. The reverse primer was designed to introduce a recognition site for the restriction enzyme AluI (AGCT) by replacing a T with a G at the second position close to the 3' end of the primer. The 1G allele has this recognition site, but in the 2G allele this recognition site is destroyed by the insertion of a guanine (Zhu et al. 2001).

The PCR primers used for amplifying a fragment of 120 bp for the SNP at –755 (G > T) were: Forward: 5'-GATCCTCCACCTCAGCCTCTCCG-3' Reverse: 5'-CATGGTGAGACCCCATCTCT-3'. The forward primer was designed to introduce a recognition site for the restriction enzyme MspI (CCGG) by replacing an A with a C at the second position close to the 3' end of the primer. The G allele has this recognition site, whereas the T allele lacks this.

PCR was performed in a 25  $\mu$ l final volume containing 20 ng of genomic DNA, 1.5 mM MgCl<sub>2</sub>, 1  $\times$  Taq DNA polymerase buffer, 0.2 mM dNTPs, 1.5 mM of each primer and 2.5 U of recombinant Taq DNA polymerase (Invitrogen, CA). The PCR conditions were 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 64°C, and 30 s at 72°C, and with a final step at 72°C for 7 min. Cycling was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, CA).

The restriction endonucleases used were AluI (for –1,607 G > GG), and MspI (for –755 G > T). A 15- $\mu$ l aliquot of PCR product was digested overnight at 37°C in a 20- $\mu$ l

reaction containing 10 units of restriction endonuclease (New England BioLabs, Beverly, MA) and 2  $\mu$ l reaction buffer. After overnight digestion, the products were resolved on a 4% agarose gel stained with ethidium bromide. After electrophoresis, the homozygous 2G/2G alleles at  $-1,607$  were represented by a DNA band with a size of 269 bp, whereas the homozygous 1G/1G alleles were represented by DNA bands with sizes of 241 and 28 bp. The heterozygote (2G/1G) displayed a combination of both alleles (269, 241, and 28 bp).

For the SNP at  $-755$ , the homozygous T/T alleles were represented by a DNA band with a size of 120 bp, whereas the homozygous G/G alleles were represented by DNA bands with sizes of 97 and 23 bp. The heterozygote (T/G) displayed a combination of both alleles (120, 97 and 23 bp).

To validate this method, 20 representative gel-purified PCR products (Qiagen PCR purification kit) were directly sequenced by a sequencer (Prism 3130xl Genetic Analyzer, Applied Biosystems, CA), and the results were concordant.

#### DNA sequencing

In search for other possible functional SNPs, the complete promoter region of the MMP-1 gene was sequenced in pooled DNA obtained from ten Mexican subjects, by the cycle-sequencing method, using a BigDye Terminator, v. 3.1, sequence kit, and was analyzed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, CA). The gene sequence obtained was then analyzed using Chromas software.

We found a SNP at  $-755$  bp that is a substitution of G by T. For this SNP an AP-1 binding site was predicted to be present in the T allele when possible transcription factor binding sites were examined with the TRANSFAC (Transcription Factor Database, <http://www.cbrc.jp/research/db/TFSEARCH.html>) program.

#### Cell culture

Lung fibroblasts were derived from IPF lung tissues obtained by open lung biopsy. Lung fibroblasts were isolated by trypsin dispersion, and cells were cultured at 37°C in 5% CO<sub>2</sub>-95% air containing Ham's F-12 medium (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL) and 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin, and 2.5 mg/ml of amphotericin B. Eight cell lines were explored to identify the presence of  $-755$  TT,  $-755$ GT, and  $-755$  GG genotypes were used.

#### Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay was performed according to the manufacturer's protocol (ChIP assay kit by

Upstate Biotechnology, Lake Placid, NY). Briefly,  $1.5 \times 10^7$  fibroblasts were grown on 100 mm dishes. Formaldehyde was added to the medium to a final concentration of 1% for cross-linking at room temperature for 10 min. Cells were washed with cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride PMSF, 1  $\mu$ g/ml aprotinin and 1  $\mu$ g/ml pepstatin A) and pelleted by centrifugation. Cell pellets were then lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, with proteases inhibitors mentioned above). After 10 min incubation on ice, cells were sonicated to shear DNA to lengths between 200 and 1,000 base pairs. Samples were then immunoprecipitated with 10  $\mu$ l c-Jun monoclonal antibody (Cell Signaling Technology) according to the specifications provided by the vendor (Upstate Biotech).

ChIP PCR analysis was done by using 5  $\mu$ g of ChIP DNA and the appropriate primers to amplify the region  $-825$  to  $-631$  of the MMP-1 promoter. The expected PCR product was 195 bp. PCR was performed in a 25  $\mu$ l final volume containing 5 ng of genomic DNA, 1.5 mM MgCl<sub>2</sub>, 1 $\times$  *Taq* DNA polymerase buffer, 0.2 mM dNTPs, 1.5 mM of each primer and 2.5 U of recombinant *Taq* DNA polymerase (Invitrogen, CA).

The PCR conditions were 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, and with a final step at 72°C for 7 min. Cycling was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, CA). PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide.

#### Real time PCR

Total RNA was extracted from lung fibroblasts using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) and reversed transcribed into cDNA (Advantage RT-for-PCR Kit; Clontech, Palo Alto, CA) according to the manufacturer's instructions. Real-time PCR amplification was performed using i-Cycler iQ Detection System (BioRad, Hercules, CA), using TAQMAN probes (PE Applied Biosystems, Wellesley, CA) labeled with FAM (Hs00899660\_g1 for MMP-1 and eukaryotic 18S rRNA endogenous control). Standard curves were calculated referring the threshold cycle ( $C_t$ ) to the log of each cDNA dilution step as previously described (Pardo et al. 2005). Results are expressed as the number of copies of the target gene normalized to 18S rRNA.

#### Statistical analysis

Data are expressed as means  $\pm$  SD. Mantel-Haenszel chi square test was used to determine the significance of the association between MMP-1 promoter genotypes and IPF (Epi Info v6.04b; Stone Mountain, GA). Associations

between IPF and MMP-1 genotypes were estimated using odds ratio (ORs) and their 95% confidence intervals (CIs). Adjustment for multiple tests was made by multiplying the *P* value by the number of SNPs (Bonferroni method). *P* < 0.05 was considered statistically significant. Conformance of the allele frequencies with the Hardy–Weinberg equilibrium was tested using a Fisher's exact test.

## Results

### Genotype and allele frequency of the –1,607 MMP-1 promoter polymorphism

This case/control study included 130 patients with IPF and 305 normal controls. The distribution of the MMP-1 –1,607 allele and genotype frequencies for cases and controls are shown in Table 1. The percent of the 2G/2G genotype in IPF patients was significantly higher than that in controls (63 vs. 49%; *P* < 0.008). This finding indicates that the 2G/2G genotype carriers may display an elevated risk for developing IPF (OR = 1.7; CI 1.15–2.79). Allelic analysis also showed a significant increase in the frequency of the 2G allele in the patients group (*P* = 0.006; OR = 1.6, CI 1.13–2.29), while the frequency of the 1G allele was considerably lower (*P* = 0.006, OR = 0.62, CI 0.44–0.88).

### Characterization and analysis of the –755 (G/T) MMP-1 promoter polymorphism

Sequencing of the complete MMP-1 promoter revealed a T/G SNP at nucleotide position –755. When this SNP was evaluated for possible transcription binding sites using the Transcription Factor Database (TRANSFAC) program, an AP-1 binding site was predicted to be present in the T allele (Fig. 1).

To corroborate this potential binding activity at position –755, we performed a ChIP assay using lung fibroblasts from IPF patients with the TT<sup>–755</sup>, GT<sup>–755</sup>, and the GG<sup>–755</sup> genotypes. After formaldehyde cross-linking, chromatin immunoprecipitation was performed with an antibody against AP-1. The precipitated DNA was subjected to PCR

with the use of specific primers for the MMP-1 promoter region that were located between nucleotides –825 and –631. PCR characterization of the precipitated DNA revealed an association of the MMP-1 promoter at –755 bp with AP-1 in the lung fibroblasts that have the T allele (Fig. 2, cells with T/T and G/T genotypes), whereas no amplification was observed in the immunoprecipitated chromatin from cells with the G/G genotype (Fig. 2). In the negative control (PCR without antibody) no amplification was detected (IgG). Only the total input sample gave a clear PCR signal, confirming that AP-1 is specifically bound to the promoter.

The frequency of –755 G/T polymorphism is increased in smoker IPF patients

When we examined the frequency of the –755 G/T MMP-1 polymorphism using our entire study group of cases (*n* = 130) and controls (*n* = 305) we found no significant differences between IPF and healthy controls (Table 2). However, as illustrated in Table 3, when we stratified them by their smoking status, a significant increase in the genotype frequency of the T/T polymorphism was observed in the smoking cases compared with the smoking controls (45 vs. 26%; *P* = 0.03; OR = 2.3; CI 1.15–4.97). A significant increase in the frequency of T allele was also observed in the smoking cases (68 vs. 48%; *P* = 0.03; OR = 1.68, CI 1.01–2.74). When we compared the frequency of the –755 T/T genotype in the IPF cohort, a significant increase was observed in the smoker IPF patients [45 vs. 12% in non-smoker IPF (*P* = 0.00007)]. Consequently, the T allele was also significantly increased in smokers IPF (68 vs. 33%; *P* = 0.00002). In contrast, no differences were found in the control population (T/T genotype 26% in smokers versus 21% in non-smokers).

### Haplotype analysis of –1,607 and –755 polymorphic loci

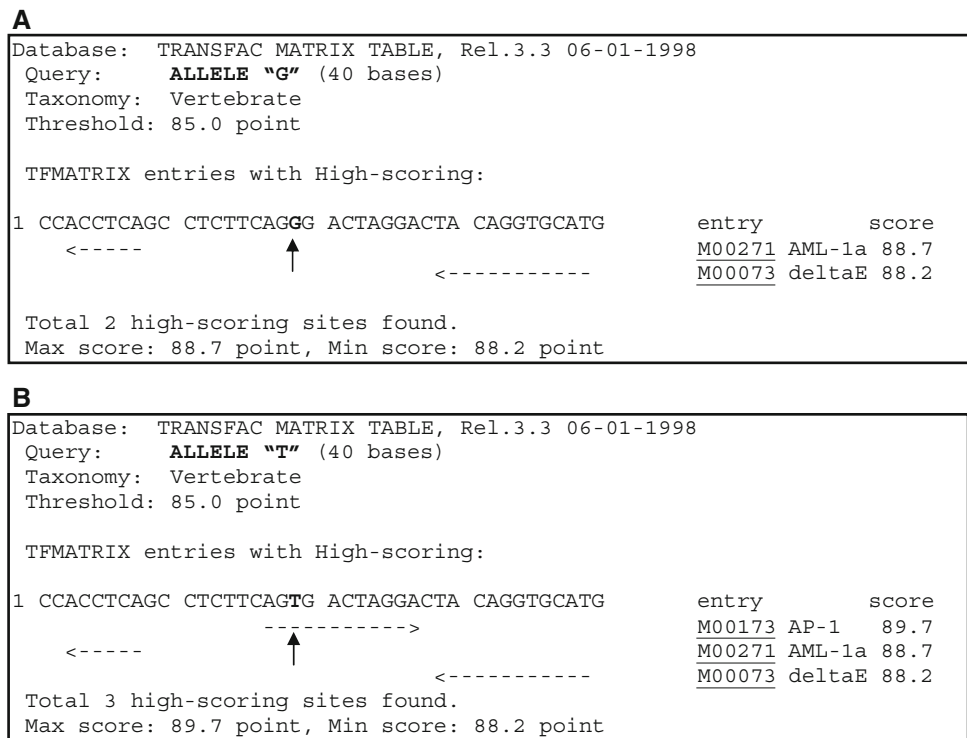
Analysis of haplotypes of the two polymorphic loci (–1,607 and –775) showed a marginal but significant increase of the 1G/1G<sup>–1,607</sup>–G/T<sup>–755</sup> haplotype in the con-

**Table 1** Genotype and allele frequencies of the MMP1 –1,607 polymorphism in cases and controls and their association with risk of IPF

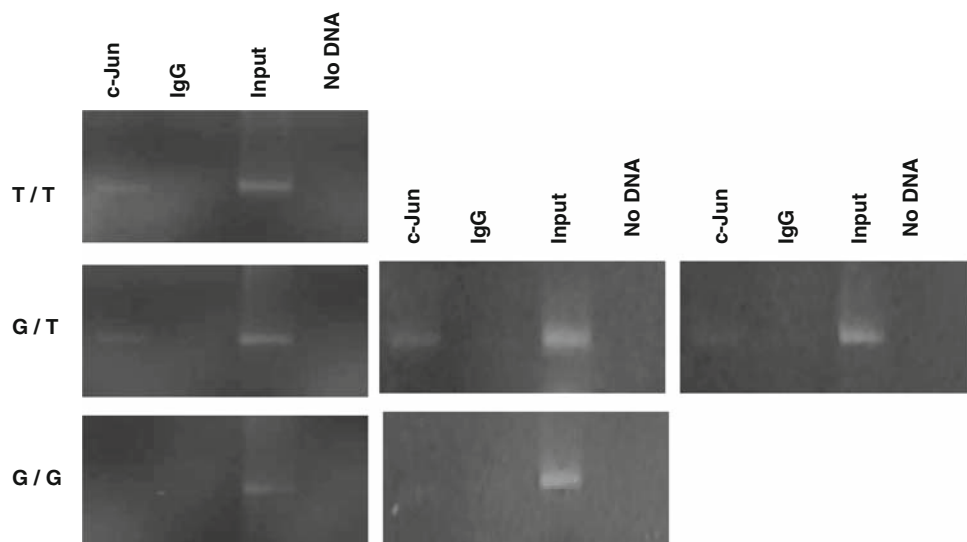
*pC* *P* value with Bonferroni correction, *OR* odds ratio, *CI* confidence interval  
Bold values indicate statistically significant results

MMP-1 genotype	IPF patients <i>n</i> = 130		Controls <i>n</i> = 305		<i>pC</i>	OR	CI (95%)
	No.	Freq.	No.	Freq.			
–1,607 1G/2G							
1G/1G	10	(0.08)	36	(0.12)	0.23		
1G/2G	38	(0.29)	119	(0.39)	0.10		
2G/2G	82	(0.63)	150	(0.49)	<b>0.008</b>	<b>1.79</b>	<b>1.15–2.79</b>
Alleles							
1G	58	(0.22)	191	(0.32)	<b>0.006</b>	<b>0.62</b>	<b>0.44–0.88</b>
2G	202	(0.78)	419	(0.68)	<b>0.006</b>	<b>1.61</b>	<b>1.13–2.29</b>

**Fig. 1** Analysis of binding sites in the  $-755$  promoter region using the TRANSFAC software. **a** Sequence of the G allele. **b** Sequence of the T allele; an AP-1 binding site is shown. *Black arrow* points at the single nucleotide polymorphism, G in **a** and T in **b**



**Fig. 2** Analysis of the AP-1 site-specific binding activity by chromatin immunoprecipitation and PCR. Chromatin from lung fibroblast cell cultures from IPF patients with the T/T, G/T (three different cell lines), and G/G (two different cell lines) genotypes were used. Anti c-Jun antibody precipitates DNA fragments containing the T allele, but does not precipitate DNA fragments with the G/G genotype. Positive control: no immunoprecipitated chromatin (*input*). Negative control: samples immunoprecipitated with IgG



trol group [ $P = 0.05$ , OR = 0.14; CI 0.01–0.96 (Table 4)]. After adjustment for smoking, the haplotype  $2G^{-1,607}-T^{-755}$  was significantly higher in smoker patients compared with smoker controls (33 vs. 24%  $P = 0.04$ ; OR = 1.6; CI 1.12–3.4) (Table 5). Consequently, in the non-smoking group the frequency of the same haplotype was lower in IPF cases (0.24) than control group (0.37) ( $P = 0.0009$ , OR 0.57, CI, 0.41–0.80). Also, a higher frequency of the  $2G^{-1,607}-G^{-755}$  haplotype was present in IPF non-smokers.

No significant deviations from the Hardy–Weinberg equilibrium in the distribution of MMP-1 SNP genotypes in IPF patients and healthy controls were detected ( $P < 0.05$ ).

#### In vitro baseline expression of MMP-1

Several IPF cell lines were explored searching for the different MMP-1 haplotypes. Only one 1G/1G at the  $-1,607$  position was found. We examined MMP-1 expression in this cell-line compared with three fibroblasts lines that carried the 2G/2G polymorphism (Fig. 3). We found that in the only 1G/1G fibroblast line MMP-1 expression was lower than in the other three cell-lines carrying the 2G/2G. Interestingly, the presence of the  $-755$  T allele was associated with a further increase of the MMP-1 expression (Fig. 3).

**Table 2** Genotype and allele frequencies of MMP-1 –755 in cases and controls and their association with risk of IPF

MMP-1 genotype	IPF patients <i>n</i> = 130		Controls <i>n</i> = 305		pC
	No.	Freq.	No.	Freq.	
–755 G/T					
G/G	50	(0.38)	98	(0.32)	0.31
G/T	48	(0.37)	140	(0.46)	0.10
T/T	32	(0.25)	67	(0.22)	0.51
Alleles					
C	58	(0.69)	191	(0.32)	0.82
T	202	(0.31)	419	(0.68)	0.82

pC P value with Bonferroni correction

**Table 3** Risk of IPF related to MMP-1 –755 genotype by smoking

Smokers	IPF patients <i>n</i> = 49		Controls <i>n</i> = 107		pC	OR	CI (95%)
	No.	(Freq.)	No.	(Freq.)			
–755 G/T							
G/G	12	(0.25)	32	(0.29)	0.61		
G/T	15	(0.30)	47	(0.44)	0.16		
T/T	22	(0.45)	28	(0.26)	<b>0.03</b>	<b>2.30</b>	<b>1.15–4.97</b>
Alleles							
G	39	(0.42)	111	(0.52)	<b>0.04</b>	<b>0.60</b>	<b>0.37–0.95</b>
T	59	(0.68)	103	(0.48)	<b>0.04</b>	<b>1.68</b>	<b>1.01–2.74</b>

pC P value with Bonferroni correction, OR odds ratio, CI confidence interval

Bold values indicate statistically significant results

## Discussion

IPF is a chronic, progressive, and often fatal form of interstitial lung disease, characterized by injury and activation of lung epithelial cells, fibroblastic foci formation and abnormal tissue remodeling. Although the primary contrib-

uting factors and the mechanisms involved in repair and remodeling have not been elucidated, recent work comparing IPF lungs with normal lungs or from other interstitial lung diseases have demonstrated that several MMPs, primarily MMP-1, are highly upregulated in IPF (Pardo et al. 2006; Selman et al. 2000, 2006; Zuo et al. 2002; Pardo et al. 2008). Moreover, it has been recently indicated that increased levels of circulating MMP-1 and MMP-7 may serve as molecular biomarkers for IPF, since both MMPs are able to distinguish patients with this disorder compared with normal subjects or patients with other chronic lung diseases (Rosas et al. 2008).

In the present study, we carried out a case/control association study to investigate the hypothesis that polymorphisms in the MMP-1 gene promoter associate with risk for IPF. The focus was on a –1,607 polymorphism and a newly discovered G/T polymorphism at –775. Our results showed that: (a) the 2G allele and the 2G/2G genotype at –1,607 associate with increased IPF risk; (b) the G/T SNP at –775 shows no association with IPF if smokers and non-smokers are grouped together but if separated then the TT genotype at –775 associates with increased risk for IPF in smokers; (c) in smokers the haplotype 2G/T associates with increased disease risk; and (d) the AP-1 factor may bind these or bind near these polymorphic sites. The observations may indicate that MMP-1 promoter polymorphisms associate with IPF via perhaps their interaction with AP-1. Moreover, one or both of –1,607 and –775 loci may be involved in gene–environment interactions that in turn modulate IPF susceptibility.

The frequency of the 2G/2G and 1G/1G genotypes were increased and decreased, respectively, in IPF cases indicating that this genotype may modulate susceptibility to IPF. Of interest, similar results have been found in chronic liver disorders where there were significantly more 2G homozygotes in patients with liver cirrhosis than in patients with chronic hepatitis C virus infection (Okamoto et al. 2005). It is well known that the 2G type of SNP at –1,607 in the

**Table 4** Individual haplotypes of MMP-1 promoter in cases and controls and their association with risk of IPF

MMP-1 –1,607/–755 haplotype	IPF patients <i>n</i> = 130		Controls <i>n</i> = 305		pC	OR	CI (95%)
	No.	Freq.	No.	Freq.			
1G 1G/G G	3	(0.025)	2	(0.005)			
1G 2G/G G	6	(0.05)	16	(0.052)			
2G 2G/G G	41	(0.316)	64	(0.210)			
1G 1G/G T	1	(0.008)	18	(0.058)	0.05	0.14	0.01–0.96
1G 2G/G T	20	(0.158)	55	(0.181)			
2G 2G/G T	27	(0.208)	76	(0.251)			
1G 1G/T T	8	(0.058)	17	(0.058)			
1G 2G/T T	14	(0.108)	39	(0.128)			
2G 2G/T T	10	(0.075)	18	(0.058)			

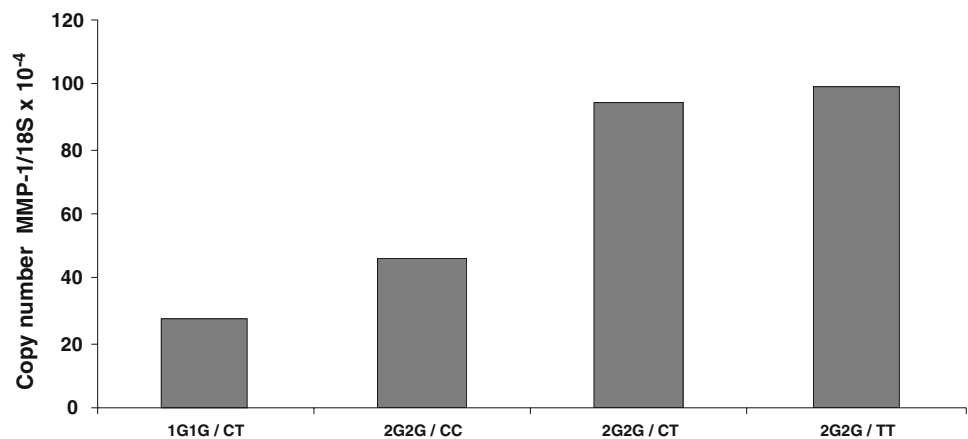
pC P value with Bonferroni correction, OR odds ratio, CI confidence interval

**Table 5** Risk of extended haplotypes of MMP-1 by smoking

MMP-1 -1,607/-755 haplotype	IPF patients <i>n</i> = 130 (Freq)	Controls <i>n</i> = 305 (Freq)	<i>p</i> C	OR	CI (95%)
Smoking					
1G/G	(0.10)	(0.14)	0.09		
2G/G	(0.32)	(0.35)	0.48		
1G/T	(0.25)	(0.27)	0.64		
2G/T	(0.33)	(0.24)	<b>0.04</b>	<b>1.60</b>	<b>1.12–3.40</b>
Non-smoking					
1G/G	(0.11)	(0.09)	0.60		
2G/G	(0.54)	(0.39)	0.004	<b>1.87</b>	<b>1.38–2.54</b>
1G/T	(0.11)	(0.15)	0.055		
2G/T	(0.24)	(0.37)	<b>0.0009</b>	<b>0.57</b>	<b>0.41–0.80</b>

*p*C *P* value with Bonferroni correction, *OR* odds ratio, *CI* confidence interval

Bold values indicate statistically significant results

**Fig. 3** MMP-1 mRNA expression was analyzed by real-time RT-PCR and the 18S rRNA gene was used for normalization

promoter of MMP-1 creates a sequence, 5'-GGA-3', that is the core recognition sequence of the binding site for Ets family transcription factors. The promoter containing the 2G allele displays significantly higher transcriptional activity than the 1G promoter (Rutter et al. 1998). Our findings in few lung fibroblast cell-lines suggest that the 2G allele increases MMP-1 expression. More recent studies have shown that this polymorphism, together with an adjacent AP-1 binding site, significantly affects the induction of MMP-1 by hydrogen peroxide (Ranganathan et al. 2001), and that MMP-1 production is higher in human foreskin fibroblasts from 2G homozygotes than in those from 1G homozygotes when stimulated with epidermal growth factor or interleukin1 (Wyatt et al. 2002). Although the details as to how the 2G polymorphism may increase disease risk are not known, the available literature supports the notion that the 2G allele contributes to increased transcriptional activity of the MMP-1 promoter (perhaps by enabling AP-1 binding). The increased activity most likely results in increased protein levels that may have a negative impact on lung tissue remodeling and then this abnormality may enhance the risk for developing IPF.

A new single nucleotide genetic variant (G/T) at -755 of the MMP-1 promoter identified in the present study was shown to associate with the presence of IPF in smokers. The TT genotype appeared to be a risk factor for IPF in smokers. Cigarette smoking has been associated with IPF in a number of case-control studies with patients of different ethnic backgrounds as well as with patients with familial pulmonary fibrosis indicating that a history of ever smoking may confer increased risk for IPF (Taskar et al. 2006; Steele et al. 2005). In this context, our findings indicate that the -755 polymorphic site may modulate an interaction with smoking resulting in an increase in the susceptibility to develop the disease.

Similar results have been reported for this and other genes in other human diseases (Zhu et al. 2001). For example, it has been demonstrated that the MMP-1 -1,607 2G/2G genotype enhances lung cancer susceptibility especially in current smokers (Zhu et al. 2001). Likewise, Vitamin D receptor *Taq-I* TT polymorphism is associated with both the presence and the progression of periodontitis in smokers, while no association was detected in non-smoking individuals (Nibali et al. 2008). When the region containing the -775 polymor-

phism was subjected to analysis by the Transcription Factor Database Program, an AP-1 binding site was predicted in the T allele, which was corroborated by chromatin immunoprecipitation assay. Of interest, an AP-1 core recognition sequence was generated by the 2G allele at  $-1,607$ . Together these findings indicate that AP-1 may be an important factor in IPF pathogenesis especially in the context of the polymorphisms investigated in the present study.

Although the putative role of MMP-1 in IPF is unclear, it has been demonstrated that this enzyme is primarily expressed by the alveolar epithelial cells, but also by macrophages in smoker patients (Selman et al. 2000). MMP-1 specifically degrades fibrillar collagens, which are the major component of the lung extracellular matrix. Moreover, the identification of a large variety of non matrix substrates of MMPs, including of MMP-1 indicates that many molecular pathways may be involved. However, based on the present findings where alleles and haplotypes of the two MMP-1 polymorphic sites were shown to associate with susceptibility to IPF and along with the available literature, we postulate that both MMP-1 polymorphic sites ( $-1,607$ ,  $-775$ ) play an important role in IPF pathogenesis, probably via their ability to modulate transcriptional regulation where the AP-1 factor may be involved.

It is likely that gene–environment (smoking) interactions occur and that both of these polymorphisms modulate transcription via AP-1 involvement. A larger confirmatory study involving other populations is required to assess the population-specificity and the relative contribution of these polymorphisms to disease.

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