MMP14 gene polymorphisms in chronic obstructive pulmonary disease

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Abstract. Proteinase/antiproteinase imbalance is a widely accepted theory for the pathogenesis of COPD. Among various proteinases, matrix metalloproteinases (MMPs) digest extracellular matrix of the lung and play significant roles in the development of COPD. Polymorphisms of an MMP that upregulate its activity may result in the degradation of the lung matrix. A case-control study was performed to investigate the association of polymorphisms of the MMP14 gene with COPD. Japanese subjects (96 COPD patients and 61 controls) and Egyptian subjects (106 COPD patients and 72 controls) were recruited. Each subject was genotyped for seven single nucleotide polymorphisms (SNPs) of the MMP14 gene; -165 G/T and -72 G/A in the promoter region, +221 C/T in exon 1, +6727 C/G and +6767 G/A in exon 5, +7096 T/C in exon 6, and +8153 G/A in exon 8. The distributions of the genotype frequencies of these SNPs were not significantly different between the COPD patients and the controls in either ethnic group after correction of multiple comparisons. In the haplotype analysis, however, the haplotype -165 T : +221 T : +6727 C : +7096 C had a significantly higher frequency in the Egyptian COPD group than the control group (p_{corr} =0.0063). The haplotype of the MMP14 gene, -165 T : +221 T : +6727 C : +7096 C, might be involved in the pathogenesis of COPD.

Introduction

COPD is characterized by a slowly progressive airflow limitation which is caused by peripheral airway inflammation and loss of lung elastic recoil resulting from parenchymal destruction. COPD is a complex disease influenced by both genetic and environmental factors. Cigarette smoking is the predominant environmental factor. Smoking is estimated to account for 80-90% of the risk of developing COPD (1). The oxidants or free radicals in cigarette smoke inactivate antiproteinases as well as directly damage components of the lung matrix (2). In addition, cigarette smoke recruits neutrophils and macrophages to the lung (3) and causes these cells to release reactive oxygen species and a variety of proteolytic enzymes (4,5). Consequently, these events lead to proteinase/antiproteinase imbalance resulting in degradation of the extracellular matrix (ECM) of the lung, which is the widely accepted theory for the pathogenesis of COPD. However, only a minority of smokers develops symptoms of the disease (6). Therefore, genetic susceptibility to smoking is thought to play an important role in the development of COPD.

Among various proteinases, α1-antitrypsin deficiency is the only proven genetic risk factor for COPD. However, there are increasing evidences that matrix metalloproteinases (MMPs) play a significant role in the pathogenesis of COPD. In animal studies, it has been reported that transgenic mice overexpressing human MMP1 in the lungs develop emphysema (7), and that MMP12-deficient mice do not develop emphysema after long-term exposure to cigarette smoke (8). More recently, Zheng *et al* (9) demonstrated that emphysema developed on the induction of MMP2, 9, 12, 13, and 14 along with cathepsins B, S, L, H, and K expression in the lungs. In humans, several studies (10-13) have demonstrated elevated levels of MMP1, 2, 9, 12, and 14 in bronchoalveolar lavage fluid, macrophages, or lung tissues from COPD patients, although there are some variations between the studies in the expression levels of specific MMPs. Since MMP14 activates proMMP2 on the cell surface in the presence of a low concentration of TIMP2 (14), it is suggested that the MMP2/MMP14/TIMP2 system plays a significant role in the MMP-mediated degradation of the ECM (11). Therefore, it is possible that polymorphisms of the MMP14 gene are responsible for upregulation of the protein activity leading to the activation of proMMP2, which results in degradation of the lung matrix.

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This study describes a case-control study designed to assess the association of MMP14 gene polymorphisms with COPD in Japanese and Egyptian populations.

Materials and methods

Subjects. This study included unrelated heavy smokers with and without COPD from Japanese and Egyptian populations. The Japanese subjects, 96 COPD patients and 61 controls, were recruited from Tsukuba University Hospital. The Egyptian subjects, 106 patients and 72 controls, were recruited from Cairo University Hospital and affiliated hospitals. Some of the Japanese and all of the Egyptian subjects were the same as those in a previous study (15). All the subjects were chronic heavy smokers with Brinkman's index (the number of cigarettes/day x the number of years) of >450. All the case subjects were diagnosed as having COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (16). The diagnosis was based on signs and symptoms, physical examination, chest radiography, and pulmonary function tests; FEV_1/FVC ratio of <70%. For the COPD group, we exclude those with FEV_1 >70% predicted in order to eliminate subjects with mild airflow limitation. The subjects with other significant respiratory diseases such as bronchial asthma, bronchiectasis and pulmonary tuberculosis were excluded. Controls were age- and smoking history-matched healthy volunteers. The study was approved by the ethics committees of the hospitals involved and informed consent was obtained from all the subjects.

Genotyping. The genomic organization and the nucleotide sequence of MMP14 have been shown in the Entrez Gene (Gene ID: 4323) and the Nucleotide (accession numbers: NC_000014 and AF158733) in the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/). The nucleotide positions in this study are numbered relative to the 3'-most transcription initiation site (17). Genomic DNA was extracted from whole blood using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Seven polymorphisms in the promoter region and exons were studied; -165 G/T (rs1003349 in the dbSNP database) and -72 G/A (rs4441188) in the promoter region, +221 C/T (rs1042703) in exon 1, +6727 C/G (rs2236302) and +6767 G/A (rs1042704) in exon 5, +7096 T/C (rs2236307) in exon 6, and +8153 G/A (rs3751489) in exon 8. Three of them, +221 C/T, +6767 G/A and +8153 G/A, were non-synonymous nucleotide substitutions.

Genotypes of +7096 T/C and +8153 G/A were determined by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. The forward and reverse primers used were 5'-GTAGTCTACACCCAC GCCTG-3' and 5'-GACAAACATCTCCCCTCGGA-3' for +7096 T/C, and 5'-GGAAGGATGGCAAATTCGTC-3' and 5'-CATTTCTAGGCAACAGCAGA-3' for +8153 G/A, respectively. The restriction enzymes for the RFLP analysis were *Hph I* (New England Biolabs, MA, USA) for +7096 T/C and *Nco I* (New England Biolabs) for +8153 G/A. *Hph I* produced 302- and 140-bp fragments for the wild-type allele +7096 T. *Nco I* produced 290- and 157-bp fragments for the mutant allele +8153 A. The fragments were then resolved on ethidium bromide-stained 1.5% agarose gel.

Table I. Primers and probes used for TaqMan allelic discrimination.

Target SNP	Primers and probes
-165 G/T	
Primer:	
Forward	5'-CACAAAAAGGCAACTTAGAGGTGTT-3'
Reverse	5'-GTGGTTGTTTTAGCCTGAATCCAAT-3'
Probe:	
	5'-[VIC]TTTCCTTCCATTTCTTG[MGB]-3'
	5'-[FAM]CCTTCCAGTTCTTG[MGB]-3'
-72 G/A	
Primer:	
Forward	5'-GTCCCCAACCAGGAAAGGA-3'
Reverse	5'-GGTTCCCTCTCTCGCTCTCT-3'
Probe:	
	5'-[VIC]CCCTCCCTTCTCCCAC[MGB]-3'
	5'-[FAM]CCCTCCCTTTTCCCAC[MGB]-3'
+221 C/T	
Primer:	
Forward	5'-ACCCGGTGGTCTCGGA-3'
Reverse	5'-TGCCGAGCGTGAGCAG-3'
Probe:	
	5'-[VIC]CCAAGACCCCCCCGTT[MGB]-3'
	5'-[FAM]CCCAAGACCCTCCCGTT[MGB]-3'
+6727 C/G	
Primer:	
Forward	5'-GGCTCGAGCATTCCAGTGA-3'
Reverse	5'-ACAAAATTCTCCGTGTCCATCCA-3'
Probe:	
	5'-[VIC]TCATGGCACCCTTTTA[MGB]-3'
	5'-[FAM]ATGGCACCGTTTTA[MGB]-3'
+6767 G/A	
Primer:	
Forward	5'-GGATGGACACGGAGAATTTTGTG-3'
Reverse	5'-CTACTCGCCATAAAGTTGCTGGAT-3'
Probe:	
	5'-[VIC]TGCCCGATGATGAC[MGB]-3'
	5'-[FAM]CTGCCCAATGATGAC[MGB]-3'

FAM, 6-carboxyfluorescein; MGB, minor groove binder.

Genotype analysis of -165 G/T, -72 G/A, +221 C/T, +6727 C/G and +6767 G/A was performed with allele-specific fluorogenic probes using the TaqMan technique. Primers and probes, designed and synthesized by Applied Biosystems (CA, USA), are shown in Table I. Probes were labeled with the fluorophores, 6-carboxyfluorescein (FAM) or VIC, and were minor groove binder (MGB) probes. PCR was carried out in a

Table II. Clinical features of study populations.

	Japanese			Egyptians		
	COPD	Control	p-value	COPD	Control	p-value
Subjects (n)	96	61		106	72	
Sex, M/F (n)	92/4	60/1		106/0	72/0	
Age (years)	68.0±1.0	67.8±1.2	NS	62.5±0.9	59.0±1.0	NS
Brinkman's index	1252.8±75.3	1066.6±61.4	NS	1050.0±60.3	990.9±69.6	NS
FVC % pred (%)	77.7±2.4	97.1±2.5	<0.001	56.3±1.0	92.3±0.9	<0.001
FEV_1 (liter)	1.0±0.1	2.5±0.1	<0.001	0.9±0.03	2.8±0.04	<0.001
FEV_1 % pred (%)	46.8±1.3	96.2±2.6	<0.001	30.3±1.1	85.9±0.4	<0.001
FEV_1/FVC (%)	43.4±1.3	81.4±1.3	<0.001	44.3±1.2	78.3±1.0	<0.001

NS, not significant. Brinkman's index is the number of cigarettes/day x the number of years. Data are presented as the mean ± SEM.

25 μl volume containing 20 ng of genomic DNA, 1x TaqMan Universal PCR Master mix (Applied Biosystems), 900 nM of each forward and reverse primer, and 200 nM of each FAM and VIC probe. Thermal cycling conditions were 50˚C for 2 min and 95˚C for 10 min, then 40 cycles of 95˚C for 15 sec and 60˚C for 1 min in the ABI PRISM-7000 (Applied Biosystems). The genotypes were determined by reading the fluorescent signals of FAM and VIC from the end products.

Statistical analysis. Clinical data were compared between the COPD and control subjects in each ethnic group using the two-sided Student's t-test. A value of $p<0.01$ was taken to be statistically significant. Both COPD and control groups were tested for Hardy-Weinberg equilibrium by comparison of the observed vs. the expected number of genotypes using χ^2 test with 1 degree of freedom. Fisher's exact test (2x3 table and 2x2 table) was used to analyze the distribution of genotype and allele frequencies. Lewontin's disequilibrium coefficient D' (18) was used to measure linkage disequilibrium between two loci. Maximum-likelihood haplotype frequencies were estimated using the expectation-maximization (EM) algorithm. Calculations were performed using the SNPAlyze program (Dynacom, Mobara, Japan). Fisher's exact test was used to test for the distribution of haplotype frequencies between the patient and control groups. The haplotype of interest was defined as one allele and the remaining haplotypes were pooled to form a second allele. p-values were corrected with Bonferroni's method. A p_{corr} value <0.05 was considered significant.

Results

The characteristics of subjects recruited in this study are given in Table II. All the subjects in both ethnic groups were heavy smokers. All the patients had moderate to very severe COPD according to GOLD classification of severity (16). No significant differences were detected in age and smoking history between the patients and the controls in either ethnic group.

Genotype frequencies of seven single nucleotide polymorphisms (SNPs) were analyzed (Table III). The distribution of each polymorphism for both ethnic groups conformed to expectations based on the Hardy-Weinberg analysis. For two SNPs, -72 G/A and +8153 G/A, all the subjects examined in the Egyptian population were wild-type homozygous. The genotype distribution of +6727 C/G showed a significant difference between the COPD and control groups in the Japanese population (p=0.047). However, after correction for multiple comparisons using the Bonferroni's method, the p_{corr} value was 0.392. There were no other significant differences in the genotype and allele frequencies between the patients and the controls in the two ethnic groups.

Pairwise linkage disequilibrium was analyzed between the SNPs having a minor allele frequency of >10%. Those SNPs were -165 G/T, +6727 C/G and +7096 T/C in the Japanese population, and -165 G/T, +221 C/T, +6727 C/G and +7096 T/C in the Egyptian population. In the Japanese population, significant linkage disequilibrium was detected in all the 3 pairs of SNPs (Table IV). However, among the 6 pairs of SNPs in the Egyptian population, +6727 C/G was not in significant linkage disequilibrium with +221 C/T and +7096 T/C (Table V).

Then haplotype analysis using all the SNPs having a minor allele frequency of >10% was performed for the patient and control groups. In the Japanese population, no significant differences were detected in the estimated haplotype frequencies between the patient and control groups. However, out of twelve haplotypes estimated in the Egyptian population, the haplotype -165 T : +221 T : +6727 C : +7096 C had a significantly higher frequency in the COPD than control group (p_{corr} =0.0063). The incidence of this haplotype was 12% in the Egyptian COPD patients, while it was 2% in the controls (Table VI).

Discussion

A case-control association study was performed for seven SNPs located in the promoter region or the exons of the MMP14 gene in order to analyze the association between these SNPs and the development of COPD. In the present study, the frequency of one haplotype in the Egyptian population was significantly higher in the patients than the controls after Bonferroni's correction. An analysis based on haplotypes is sometimes advantageous over an analysis based on individual

Table III. Genotype frequencies of MMP14 gene polymorphisms.

Genotype		Japanese			Egyptians		
		COPD	Control	p-value	COPD	Control	p-value
-165	G/G	32 (33)	19 (31)	NS	45 (42)	36 (50)	NS
	G/T	43 (45)	28 (46)		45 (42)	28 (39)	
	T/T	21 (22)	14 (23)		16 (16)	8 (11)	
-72	G/G	95 (99)	60 (98)	NS	106 (100)	72 (100)	NS
	G/A	1 (1)	1 (2)		0 (0)	0 (0)	
	A/A	0 (0)	0 (0)		0 (0)	0 (0)	
+221	C/C	0 (0)	0 (0)	NS	4 (4)	4 (6)	NS
	C/T	1 (1)	1 (2)		27 (25)	25 (35)	
	T/T	95 (99)	60 (98)		75 (71)	43 (59)	
+6727	C/C	70 (73)	53 (87)	0.047	81 (76)	53 (74)	NS
	C/G	26 (27)	8 (13)	(p_{corr} 0.329)	23 (22)	16(22)	
	G/G	0 (0)	0 (0)		2 (2)	3 (4)	
+6767	G/G	94 (98)	58 (95)	NS	85 (80)	62 (86)	NS
	G/A	2 (2)	3 (5)		18 (17)	10 (14)	
	A/A	0 (0)	0 (0)		3 (3)	0 (0)	
+7096	T/T	35 (36)	20 (33)	NS	46 (43)	39 (54)	NS
	T/C	48 (50)	33 (54)		42 (40)	27 (38)	
	C/C	13 (14)	8 (13)		18 (17)	6 (8)	
+8153	G/G	93 (98)	58 (98)	NS	106 (100)	72 (100)	NS
	G/A	2 (2)	1 (2)		0 (0)	0 (0)	
	A/A	0 (0)	0 (0)		0 (0)	0 (0)	

NS, not significant. Data presented as n (%).

Table IV. Pairwise linkage disequilibrium (D' above diagonal) and statistical significance (p-value below diagonal) in the Japanese population.

	-165 G/T	+6727 C/G	+7096 T/C
-165 G/T		0.461	0.846
+6727 C/G	0.008		1.000
+7096 T/C	<0.001	<0.001	

Table V. Pairwise linkage disequilibrium (D' above diagonal) and statistical significance (p-value below diagonal) in the Egyptian population.

	-165 G/T	+221 C/T	+6727 C/G	+7096 T/C
-165 G/T		1.000	0.416	0.227
+221 C/T	<0.001		0.026	0.924
+6727 C/G	0.031	0.802		0.156
+7096 T/C	0.037	<0.001	0.491	

SNPs for the detection of an association between the alleles and a disease phenotype (19). Haplotype analysis shows greater statistical power especially when each SNP constructing the haplotypes has a small but true contribution to disease susceptibility.

In the present study, special attention was paid to the following points in order to raise the statistical power. First, the selection criteria of the subjects were strict. The cases showing the intermediate phenotype with mild airflow limitation (FEV_1 >70% predicted) were excluded. We also removed the cases with recurrent episodes of reversible airflow limitation to exclude bronchial asthma. Furthermore, all the controls were exposed to enough cigarette smoke to develop COPD, and a strict match for smoking history was detected between the patient and control groups. Second, population stratification was minimized. The subjects recruited were all native Japanese and Egyptians. Since Japan is a single racial nation, the genetic background of the Japanese subjects is considered to be highly homogeneous. For the Egyptian population, subjects whose parents and grandparents were also born in Egypt were selected. All the genotypic frequencies in both ethnic groups were consistent with Hardy-Weinberg equilibrium. Therefore, it is unlikely that the present study had

Table VI. Haplotype frequencies of the MMP14 gene polymorphisms in Egyptians.

Haplotype	-165 G/T	+221 C/T	+6727 C/G	+7096 T/C	COPD	Control	p-value (p_{corr})
1	G	T	C	C	47 (22)	27 (19)	NS
2	T	T	C	T	45 (21)	39 (27)	NS
3	G	T	C	T	38 (18)	25 (17)	NS
4	G	C	C	T	30 (14)	27 (19)	NS
5	T	T	C	C	25 (12)	3 (2)	0.00052 (0.0063)
6	G	T	G	T	8 (4)	10 (7)	NS
7	T	T	G	T	7 (3)	0 (0)	NS
8	G	T	G	C	7 (3)	7 (5)	NS
9	G	C	G	T	5 (2)	3 (2)	NS
10	G	C	C	C	0 (0)	1 (1)	NS
11	T	C	G	T	0 (0)	1 (1)	NS
12	T	T	G	C	0 (0)	1 (1)	NS

NS, not significant. Numbers represent haplotype carriage frequencies with percentages in parentheses.

population stratification, genotyping errors, genetic drift or inbreeding.

MMPs play key roles in normal ECM remodeling such as embryonic development, cell migration and wound healing. Abnormalities of MMPs expression result in numerous pathophysiologic conditions including tumor metastasis (20), aortic aneurysm (21) and rheumatic diseases (22). Ohnishi *et al* (11), have reported that MMP2 and MMP14 are extensively expressed in emphysematous lung and that elastolytic activity is mainly derived from MMP2. They concluded that the MMP2/MMP14/TIMP2 system plays an important role in the pathogenesis of COPD. There are also several studies demonstrating the contribution of MMP2 to the pathogenesis of COPD (12,23). MMP14 can digest type I, II, and III collagens as well as other ECM molecules including gelatin, proteoglycan, fibronectin, vitronectin, and laminin-1 (24). In addition, MMP14 can activate proMMP2 on the cell surface (25). The catalytic domain of MMP14 binds to the N-terminal portion of TIMP2, which leaves the TIMP2 C-terminal region available for binding proMMP2. The cell surface-bound proMMP2 is then activated by a TIMP2-free MMP14. This process occurs only at low concentrations of TIMP2 (14). High levels of TIMP2 inhibit the proMMP2 activation by blocking all free MMP14. MMP2 digests type I, II, III, and IV collagens, gelatin, fibronectin, and elastin. Therefore, MMP14 degrades ECM molecules by activating proMMP2 as well as by itself. It is possible that polymorphisms of MMP14 lead to an upregulation of its activity and result in the development of COPD.

The functional effect of each polymorphism constructing the haplotype -165 T : +221 T : +6727 C : +7096C is not clear. The +221 C/T polymorphism is a non-synonymous SNP causing Pro8Ser, which might affect the protein function. The -165 G/T polymorphism in the promoter region may have an influence on the transcriptional activity. Even the synonymous +7096 T/C polymorphism might be able to affect the activity of MMP14 protein by changing the translational rate by modifying the mRNA stability or the ribosome binding. It is also possible that the haplotype -165 T : +221 T : +6727 C : +7096C is just in linkage disequilibrium with other functional polymorphisms that are responsible for the susceptibility to COPD.

In the Japanese population, the estimated haplotypes were not significantly different between the COPD and control groups. As the development of COPD is recognized to depend on multiple genetic factors and genotype-by-environment interactions (26), it is possible that different haplotypes in different ethnic groups are responsible for the susceptibility to COPD. Furthermore, since COPD is a heterogeneous entity including inflammation of the airways and destruction of lung parenchyma, different haplotypes may be responsible for each distinct entity of COPD.

Cigarette smoking is an important risk factor for abdominal aortic aneurysms as well as for COPD. It has been demonstrated that severe COPD patients have a significantly higher prevalence of abdominal aortic aneurysms than mild or moderate COPD patients (27). Aortic aneurysms are also characterized by destruction of the ECM in the arterial media by activated MMPs (28). It has been shown that MMP2, MMP9 and MMP14 may be the dominant proteases in the development of aneurysms (29). The MMP2/MMP14/TIMP2 enzyme system has also been detected in the arterial media

(30). While we have demonstrated in our previous studies (31,32) that +853 G/A polymorphism of the TIMP2 gene is associated with the development of COPD, the same polymorphism has been reported to be related to aneurysm (33). Therefore, it is worth examining the association of the haplotype -165 T : +221 T : +6727 C : +7096C in the MMP14 gene with the development of aneurysms.

In conclusion, the present study suggested that the MMP14 gene haplotype -165 T : +221 T : +6727 C : +7096C is associated with the development of COPD in the Egyptian population. It would be interesting to analyze the association of this haplotype with COPD in various ethnic groups. The haplotype -165 T : +221 T : +6727 C : +7096C of the MMP14 gene may be helpful for predicting the susceptibility to COPD.

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