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ORIGINAL ARTICLE

Matrix metalloproteinase 3 polymorphisms as a potential marker of enhanced susceptibility to lung cancer in chronic obstructive pulmonary disease subjects

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Abstract

Introduction and objective. Chronic obstructive pulmonary disease (COPD) is often accompanied by lung cancer. Among the genes that may play a role in the occurrence of COPD and lung cancer are those encoding the proteolytic enzymes, such as matrix metalloproteinases (MMPs) and their tissue inhibitors. The objective of this study was to find MMPs-associated markers useful in the identification of COPD subjects with increased susceptibility to developing lung cancer.

Materials and methods. We compared the frequency of single nucleotide polymorphisms in genes coding for matrix proteinases (*MMP1*, *MMP2*, *MMP3*, *MMP9*, *MMP12*) as well as tissue inhibitor of metalloproteinases (*TIMP1*) in two groups of subjects: COPD patients (54 subjects) and COPD patients diagnosed for lung cancer occurrence (53 subjects).The levels of the respective proteins in blood serum were also analyzed.

Results. The frequencies of 2 genotypes, *MMP3* rs3025058 and *MMP3* rs678815, were significantly different between the studied groups. In both cases, more heterozygotes and less homozygotes (both types) were observed in the COPD group than in the COPD + cancer group. A significantly higher TIMP1 level in blood serum was observed in the COPD + cancer group than in the COPD group. There were no statistically significant differences in MMPs blood levels between the studied groups. In addition, no genotype-associated differences in TIMP1 or MMPs blood levels were observed.

Conclusions. Homozygocity for *MMP3* rs3025058 and rs678815 polymorphisms is a potential marker of enhanced susceptibility to lung cancer development among COPD subjects.

Key words

COPD; matrix metalloproteinase; lung cancer, MMP, inhibitors of metalloproteinases

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by an airflow limitation that is not fully reversible, usually progressive and associated with an abnormal inflammatory response to noxious particles or gases. The main cause of COPD is cigarette smoking; other causes, such as outdoor air pollution, occupational exposure to dust and fumes, previous tuberculosis, childhood asthma and childhood respiratory infections, might increase the risk of COPD and lead to disease in nonsmokers [1]. Patients with COPD are at increased risk for both the development of primary lung cancer and poor outcome after lung cancer diagnosis and treatment. The association between COPD and lung cancer has been reported in numerous studies [reviewed in 2–6] and is independent of age or smoking habit [7].

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Smokers expose their bronchial epithelial cells to oxidants and reactive oxygen species (ROS), which induce oxidative stress. The cellular response that follows depends on the activation of the NF-κB and other transcription factors, and on the subsequent transactivation of inflammation-related genes. This initiates several signaling pathways depending on both genetic and epigenetic factors which, to some extent, overlap in the COPD and lung cancer. Chronic inflammation due to cytokine release and the resulting immune response contribute to both pathogenic processes. ROS and cigarette smoke components are able to induce DNA damage. The consequence is genomic instability, a factor sufficient for the development of cancer, reinforced by aberrant airway epithelial and matrix remodeling, epithelial-mesenchymal transition (EMT) and apoptosis [reviewed in 2, 5, 6]. At a certain stage, one tendency predominates: either the aggravating COPD conditions or the pre-cancerous pathway.

It is to be expected that the fate of individual subjects depends to some extent on their genetic predispositions. Indeed, twin studies showed that lung cancer susceptibility

is inherited in about 15–25% of cases, whereas COPD is inherited in 40–77% [6]. Hence, the recent studies seek to define the genetic susceptibility factors that contribute to the individual susceptibility to COPD and/or lung cancer. So far, the only gene that has been definitively proved to influence COPD susceptibility is *SERPINA1* that encodes α1-antitrypsin [8]. Most of the candidate gene studies reporting genetic associations with COPD have not been consistently replicated, making it difficult to draw reliable conclusions. In the study performed by Castaldi et al. [9], 27 genetic variants were found to have adequate data for quantitative meta-analysis, and 4 among them were significantly associated with COPD susceptibility: *GSTM1* null variant, *TGFB1* rs1800470, *TNF* rs1800629 and *SOD3* rs1799896. Genome-wide association studies found regions and genes which partly overlap in both COPD and lung cancer, and correspond to the pathogenic pathways mentioned above [reviewed in 2, 5, 6]. This allows for the seeking of an association between single nucleotide polymorphisms (SNPs) in the critical genes. A list of candidate SNPs examined in 12 genes and the respective source information can be found in the paper by Young et al. [10]. In that study, the control group of 'resistant smokers' was compared with COPD and lung cancer groups.

However, since not all COPD subjects develop lung cancer, it is important to identify the factors that determine the individual cancer susceptibility/resistance in this group. Therefore, a comparison of the frequency of several SNPs in 2 groups of subjects: patients with COPD and patients with COPD + lung cancer was carried out. The SNPs under study were located in genes engaged in tissue remodeling that code the following matrix metalloproteinases (MMPs) and their inhibitors: matrix metalloproteinase 1 (MMP1), matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 9 (MMP9), matrix metalloproteinase 12 (MMP12), tissue inhibitor of metalloproteinases 1 (TIMP1) [11–18]. In addition to SNPs, the blood serum levels of the respective proteins were also analyzed.

MATERIALS AND METHOD

DNA isolation and genotyping. Genomic DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. Certain polymorphisms were assessed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The PCR and RFLP conditions, together with the respective references, are presented in Table1. Other polymorphisms were assessed using TaqMan® SNP Genotyping Assays (Life Technologies) (Tab. 2). Thermal cycling was performed on the 7500 Real-Time PCR System (Life Technologies) using 100 ng DNA as a template. The initial denaturation cycle was at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The data were collected and analysed using SDS 2.4 software (Life Technologies).

Table 2. TaqMan SNP Genotyping Assays used in the study

Gene	Polymorphism	TaqMan® SNP Genotyping Assay ID
TIMP1	A/G rs6609533	C___2459015_10
MMP1	C/T rs1938901	C___3012119_30
MMP2	C/T rs1030868	C___3225949_1_
	A/C rs7201	C___3225976_10
MMP3	C/G rs678815	C___3047716_10
MMP9	A/T rs3918241	C__29689865_10

Estimation of MMPs and TIMP1 levels. The levels of TIMP1, MMP1, MMP2, MMP3, MMP9 proteins in the serum samples were determined by the following ELISA tests according to the manufacturer's protocols: TIMP1, active and pro-MMP2 (total MMP2) Quantikine ELISA Kit (R&D System Inc.); MMP1, MMP 3 and MMP9 (Merck Millipore – Calbiochem).

Statistical analysis. Differences in the protein levels in the serum between the subjects with COPD and those with COPD and lung cancer were analysed by Mann-Whitney U-test. The distributions of genotypes in the studied groups were compared using χ^2 test. In all tests significance was accepted at $p<0.05$. All statistical analyses were performed using Statistica 7 software (StatSoft).

RESULTS

The COPD + lung cancer (53 patients) and COPD-only (54 patients) groups were recruited among the clinic patients normalized according to gender, age, smoking habit and

Table 1. PCR primers and RFLP conditions for polymorphisms assessed by PCR-RFLP technique

Gene	Polymorphism	PCR primers and annealing temperature (Ta)	PCR product size	Restriction enzyme	Fragments identifying genotypes (base pairs)	Reference
MMP1	-1607 1G/2G rs1799750	F 5'-TGACTTTTAAAACATAGTCTATGTTCA-3' R 5'-TCTTGGATTGATTTGAGATAAGTCATAGC-3' Ta = 53°C	269 bp	AluI (Fermentas)	1G/1G = 241 + 28 1G/2G = 269 + 241 + 28 2G/2G = 269	[41]
MMP3	6A/5A rs3025058	F 5'-GGTTCTCCATTCCTTTGATGGGGGGAAAGA-3' R 5'-CTTCCTGGAATTCACATCACTGCCACCACT-3' Ta = 63°C	129 bp	PsyI (Fermentas)	6A/6A = 129 6A/5A = 129 + 97 + 32 5A/5A = 97 + 32	[42]
MMP9	-1562 C/T rs3918242	F 5'-GCCTGGCACATAGTAGGCCC-3' R 5'-CTTCCTAGCCAGCCGGCATC -3' Ta = 65°C	435 bp	PaeI (Fermentas)	C/C = 435 C/T = 435 + 247 + 188 T/T = 247 + 188	[43]
MMP12	-82 A/G rs2276109	F 5'-GAGATAGTCAAGGGATGATATCAGC-3' R 5'-AAGAGCTCCAGAAGCAGTGG-3' Ta = 60°C	199 bp	PvuII (Fermentas)	A/A = 199 A/G = 199 + 175 + 24 G/G = 175 + 24	[44]
	Asn357Ser rs652438	F 5'-GGGATAATTTGGCTCTGGTCTTCAA-3' R 5'-CCATGGGAACCATAGAAAAGA-3' Ta = 55°C	204 bp	MunI (Fermentas)	Asn/Asn = 180 + 24 Asn/Ser = 204 + 180 + 24 Ser/Ser = 204	[44]

diagnosed disease. The detailed characteristics of the subject groups were presented in a previous paper [19].

Genotype distribution. The *TIMP1* and *MMP*s genotypes distribution for COPD and COPD + cancer groups are shown in Table 3. The frequencies of both *MMP3* genotypes were significantly different between the studied groups. In both cases, more heterozygotes and less homozygotes (both types) were observed in the COPD group than in the COPD + cancer group. In the case of *MMP12* rs2276109 polymorphism, a higher frequency of G allele in the COPD + cancer group was observed, but this tendency was not statistically significant (p=0.054). There were no statistically significant differences between the groups for the other *MMP* or *TIMP1* polymorphisms.

Table 3. Distribution of genotypes in the studied groups of patients. The results for the polymorphisms marked with asterisks were published in the previous paper [19]

Genotype	COPD + cancer n=53 (%)	COPD n=54 (%)	χ^2 test
TIMP1 rs6609533			
G/G	28 (52.83)	27 (50.00)	p=0.770
A/G	25 (47.17)	27 (50.00)	
MMP1 rs1799750*			
1G/1G	12 (22.64)	17 (31.48)	
1G/2G	26 (49.06)	21 (38.89)	p=0.492
2G/2G	15 (28.30)	16 (29.63)	
MMP1 rs1938901			
C/C	27 (50.94)	28 (51.85)	
C/T	21 (39.62)	23 (42.59)	p=0.741
T/T	5 (9.43)	3 (5.56)	
MMP2 rs1030868			
C/C	23 (43.40)	24 (44.44)	
C/T	22 (41.51)	26 (48.15)	p=0.432
T/T	8 (15.09)	4 (7.41)	
MMP2 rs7201			
A/A	18 (33.96)	21 (38.89)	
A/C	25 (47.17)	23 (42.59)	p=0.859
C/C	10 (18.87)	10 (18.52)	
MMP3 rs3025058*			
5A/5A	16 (30.19)	9 (16.67)	
5A/6A	19 (35.85)	36 (66.67)	**p=0.006**
6A/6A	18 (33.96)	9 (16.67)	
MMP3 rs678815			
G/G	16 (30.19)	11 (20.37)	
C/G	19 (35.85)	34 (62.96)	**p=0.017**
C/C	18 (33.96)	9 (16.67)	
MMP9 rs3918242*			
C/C	42 (79.25)	40 (74.07)	
C/T	11 (20.75)	13 (24.07)	p=0.547
T/T	0 (0)	1 (1.85)	
MMP9 rs3918241			
T/T	42 (79.25)	36 (66.67)	
A/T	11 (20.75)	17 (31.48)	p=0.254
A/A	0 (0)	1 (1.85)	
MMP12 rs652438*			
Asn/Asn	51 (96.23)	49 (90.74)	p=0.251
Asn/Ser	2 (3.77)	5 (9.26)	
MMP12 rs2276109*			
A/A	36 (67.92)	46 (85.19)	
A/G	14 (26.42)	8 (14.81)	p=0.054
G/G	3 (5.66)	0 (0)	

TIMP1 and MMP's levels in blood serum. The blood serum levels of TIMP1 and MMPs were assessed using dedicated ELISA kits. A significantly higher TIMP1 level was observed in the COPD + cancer group in comparison with the COPD group (Fig. 1). There were no statistically significant differences in MMPs levels between the studied groups (Fig. 2). In addition, no genotype-associated differences in TIMP1 or MMPs levels were observed (data not shown).

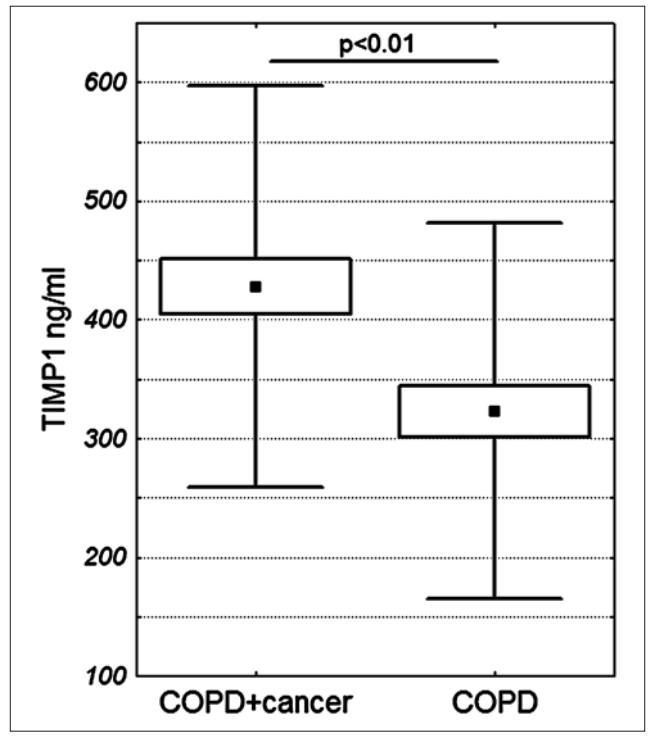

Figure 1. Box plots of mean TIMP1 levels in blood serum of the 2 groups of subjects: with COPD or COPD and lung cancer. In the box plots, the middle square represents the mean, the boxes denote standard error and the whiskers denote standard deviation. Significantly different in Mann-Whitney U-test, $p < 0.01$

DISCUSSION

A recent report indicates that previous lung disease (chronic bronchitis, emphysema, pneumonia, and tuberculosis) influences the lung cancer risk independently of smoking habit, and that these diseases are important for estimating individual risk of lung cancer [20]. On the other hand, Gierada et al. [21] reported a lack of association between emphysema and lung cancer. Nevertheless, the airway epithelial damage caused by exposure to cigarette smoke and the consequent inflammation, seem to be important factors in the development of tissue remodeling, dysfunction of apoptosis and promotion of angiogenesis.

Among a wide range of genes that may play a role in the occurrence of COPD and lung cancer are those encoding the proteolytic enzymes, such as matrix metalloproteinases, and their tissue inhibitors. MMPs are involved in a stromal connective tissue remodeling in inflammatory processes and necrosis, and facilitate angiogenesis and tumour growth

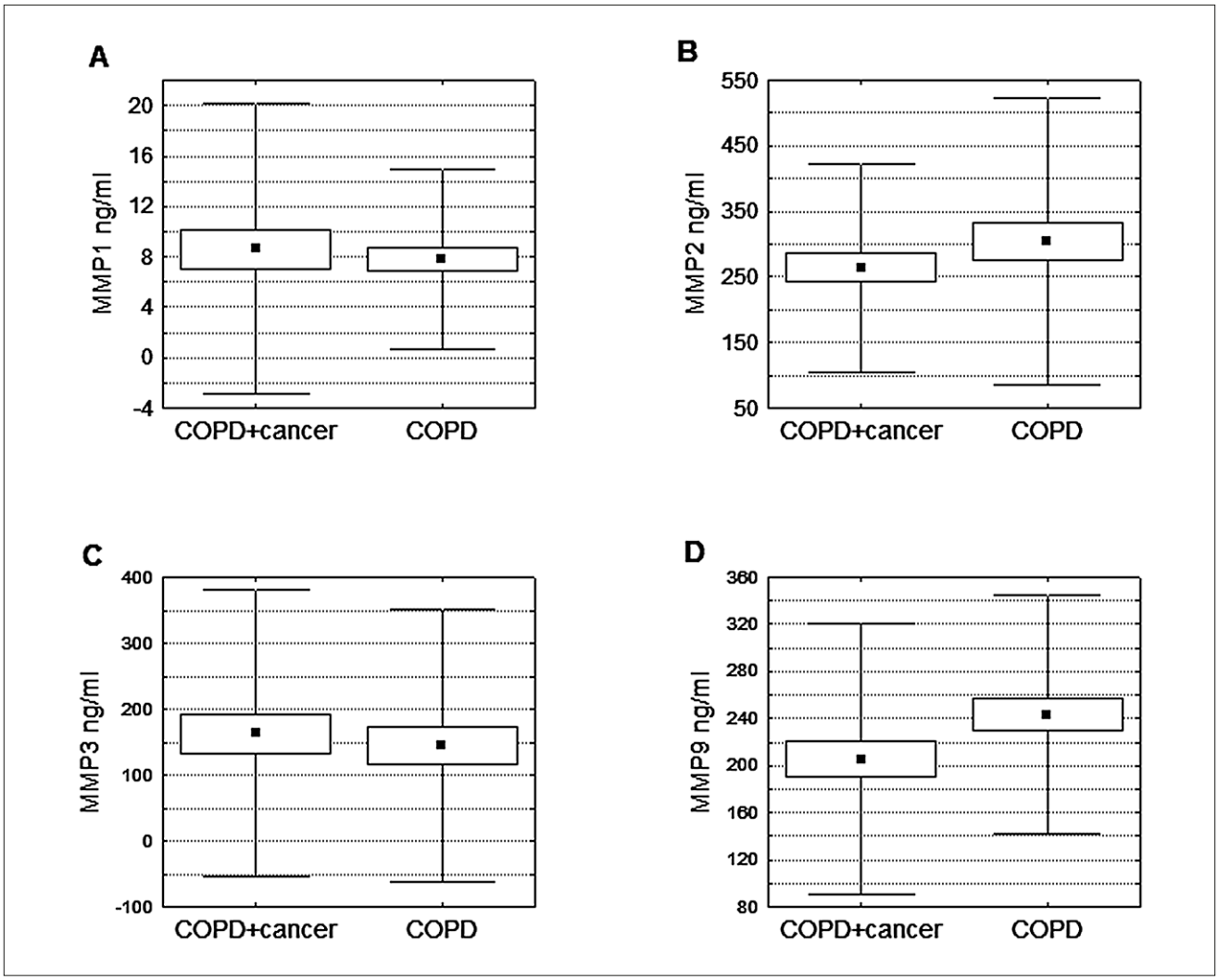

Figure 2. Box plots of mean MMPs levels in blood serum in the 2 groups of subjects: with COPD or COPD and lung cancer. (A) MMP1; (B) MMP2; (C) MMP3; (D) MMP9. In the box plots, the middle square represents the mean, the boxes denote standard error and the whiskers denote standard deviation. The differences are not statistically significant

[reviewed in 11, 13–15, 22, 23]; e.g. MMP9 and MMP12 belong to the markers of epithelial-mesenchymal transition [24, 25] that is involved in tumour growth [26], and COPD progression.

In the majority of studies, the significance of various *MMPs* polymorphisms for lung cancer risk or COPD was estimated by comparing healthy subjects with COPD or cancer patients. Overexpression of *MMP1* was associated with tumour invasion and metastasis formation [27]. Su et al. suggested that the *MMP1* polymorphism -1607 1G/2G rs1799750 and certain haplotypes of *MMP3* and *MMP12* may lead to an increased risk of lung cancer among non-smoking males [17, 18]. *MMP1* -1607 1G/2G rs1799750 polymorphism in relation to lung cancer risk was also examined by other authors and found to be significantly associated [28, 29]. Associations between *MMP2* C735T and C1306T polymorphisms and lung cancer risk were found in Asians but not in Caucasians [30]. In the study performed by Peng et al., *MMP2* rs2285053 T allele was associated with lower risk of lung cancer, whereas for all examined cancer types, *MMP2* rs243865, *MMP2* rs2285053 and *MMP7* rs11568818 were supposed to play allele-specific roles in cancer development [31]. According to this analysis, *MMP9* rs3918242 may not be a major risk factor for most cancer types [31]. However, in another study, the same *MMP9* rs3918242 polymorphism was associated with lower risk of lung cancer [32], similar to *MMP8* +17C/G rs2155052 polymorphism [33] and *MMP2* C1306T or C735T [34]. Also, *MMP9* P574R and R279Q polymorphisms were indicated as potential biomarkers for lung cancer and metastasis [35].

Whereas these studies are warranted by a small number of subjects and require confirmation in a larger population, they also suggest the important role of MMPs as initiators of carcinogenesis in lung cancer. They also point to the important role of the tissue inhibitors of metalloproteinases, alpha 1-antitrypsin, alpha2-macroglobulin and other protease inhibitors, which modulate the proteinases activity [8, 23].

In contrast to the above mentioned studies, in the presented study comparison was made between the occurrence of the *MMPs* and *TIMP1* polymorphisms in COPD patients and COPD patients developing lung cancer. Among the SNPs examined (Tab. 3), the only difference between the subject groups was found in the distribution of the two *MMP3* polymorphic alleles (*MMP3* rs3025058 and *MMP3* rs678815). In both cases, more heterozygotes and less homozygotes (both types) were observed in the

COPD group than in the COPD + cancer group. This is compatible with the notion that heterozygocity for *MMP3* polymorphisms reduces the risk of cancer, a phenomenon called 'heterozygote advantage'. According to Sellis et al., the heterozygote advantage is displayed by 'a substantial proportion of adaptive mutations'[36]. This effect stems from the susceptibility of heterozygotes to the invasion of new adaptive mutations.

Interestingly, MMP3 (also known as stromelysin2) belongs to proteases that are preferentially inhibited by TIMP1 [13]. A significantly higher TIMP1 level in blood serum was observed in the COPD + cancer group than in the COPD group (Fig. 1). As MMPs are involved in cancer growth and metastasis, their inhibitors seem to be suitable candidates for therapeutic factors [37, 38]. Hence, TIMP1 is typically associated with the inhibition of MMP induced cancer cell invasion. However, TIMP1 is overexpressed in many malignancies and alters the expression of approximately 600 genes *in vivo*, including *MMP1* and *MMP13* [39]. In parallel, the growth-stimulating function of TIMP1 has been described and explained in molecular terms [40]. Therefore, the high TIMP1 level found in subjects from the COPD + cancer group most probably reflects the progress of the malignancy.

CONCLUSIONS

The results of the presented study suggest that the homozygocity for *MMP3* rs3025058 and rs678815 polymorhisms is a potential marker of enhanced susceptibility to lung cancer development among COPD subjects. More extensive study including a larger cohort of patients is needed to confirm this relationship.

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Declaration of Interest
The authors declare that they have no conflict of interest.

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