MMP2 -1306C>T POLYMORPHISM IN PATIENTS WITH COPD

Tanya Tacheva[1], Dimo Dimov[2], Pavlina Chelenkova[3], Rumena Petkova[3], Stoyan Chakarov[4], Tatyana Vlaykova[1]

[1]Department of Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora
[2]Department Internal Medicine, Medical Faculty, Trakia University, Stara Zagora
[3]STS Ltd., Sofia Bulgaria
[4]Department Biochemistry, Faculty of Biology, Sofia University, Sofia, Bulgaria

ABSTRACT

The remodeling of the bronchial walls is an important process of the pathophysiology of COPD as the matrix metalloproteinase-2 (MMP-2) is shown to play an important role in this process.

The aim of the current study was to elucidate the possible role of *MMP2 -1306C>T* promoter polymorphism as a risk factor for COPD. We genotyped by PCR-RFLP 84 patients with COPD and 71 control individuals.

The genotype, but not the allele distribution, differed between COPD patients and controls (p=0.021 and 0.602, respectively). Carriers of the variant *T* allele genotypes *(CT+TT)* tended to have 1.64-fold higher risk for COPD (OR (Odds ratio) =1.64, 95% CI (confidence interval): 0.82-3.26, p=0.164) than those with *CC* genotype, as that risk was significant in the subset of individuals older than 65 years (OR=4.24, 95% CI:1.31-13.57, p=0.019). Patients with *T* containing genotypes *(CT+TT)* had a later onset of the disease (64.1±7.1 years) than those with a CC genotype (59.7±9.5 years, p=0.045). The risk for COPD of *T* carriers *(CT+TT)* was also significant in those individuals without diabetes as co-morbidity.

In conclusion, our results suggest that the carriers of T allele genotypes *(CT+TT)* of *MMP2 -1306C>T* SNP may have a higher risk for COPD in advanced age.

Keywords: *COPD, matrix metalloproteinases, polymorphism*

INTRODUCTION

The guidelines of Global Initiative for Chronic Obstructive Lung Disease (GOLD) define COPD as a disease state characterized by airflow limitation that is not fully reversible, usually progressive, and is associated with an abnormal inflammatory response of the lungs to inhaled noxious particles or gases. Subsets of patients may have dominant features of chronic bronchitis and/or emphysema. The result is airflow obstruction that is not fully reversible (1). This pathology is associated with an airway inflammatory process characterized by an accumulation of inflammatory cells such as macrophages and neutrophils (2).

Systemic inflammation is often associated with COPD. There has been a growing recognition that co-morbidities such as cardiovascular disease, diabetes mellitus, cachexia, anemia, osteoporosis, or depression may be present in a greater proportion of

Address for correspondence:
Tanya Tancheva
Dept. Chemistry and Biochemistry,
Medical Faculty, Trakia University
11 Armeiska St.
Stara Zagora
e-mail: tanyatacheva@abv.bg

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patients with COPD than in the general population (3,4).

The most important risk factor for the development of COPD is smoking. However, only 10-20% of chronic heavy smokers develop symptomatic COPD, which indicates that a difference in susceptibility to tobacco smoke injury possibly exist and may be related to genetic factors (5). Various groups of genes are involved in the development of COPD. These are some protein-encoding genes with proteolytic and anti-proteolytic activity (matrix metalloproteinases, α1-antitrypsin and α1-antichymotrypsin, α2-macroglobulin), proteins responsible for the increased reactivity of the airways, the alveolar surfactant proteins, mediators involved in inflammatory response and enzymes involved in the metabolism of toxic substances in cigarette smoke and antioxidant enzymes (5-10). The panacinar form of emphysema is usually associated with α1 anti-trypsin deficiency (11).

The development of emphysema usually reflects a relative excess of cell-derived proteases that degrade the connective tissue of the lung and a relative decrease of anti-proteolytic defenses. This theory is often referred to as the "protease/antiprotease imbalance" hypothesis and involves mainly serine proteases such as neutrophil elastase and matrix metalloproteinases (MMPs) (initially characterized by their capacity to degrade components of the extracellular matrix, ECM) (2,9).

Depending on substrate specificity, amino acid similarity and identifiable sequence modules, the MMP family can be classified into distinct subclasses: collagenases (MMP- l, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), membrane-type MMP (MMP-14 to MMP-25), matrilysin (MMP-7), and macrophage metalloelastase (MMP-12). MMP are synthetized as zymogens (pro-enzymes). The MMP activity is tightly regulated on the level of transcription, activation and inhibition by the tissue inhibitors of MMPs (TIMPs: TIMP-1, TIMP-2, TIMP-3, and TIMP-4) and α2-macroglobulin (2,12-15).

MMP-2 (gelatinase A) is primarily expressed in mesenchymal cells (mainly fibroblasts) during development and tissue regeneration. It is also synthesized by neutrophils, macrophages and monocytes – cells that participate in the development of the pathological changes in the lungs in COPD. Due to the airway remodeling (synthesis and degradation of ECM) there are structural changes, in addition to the inflammatory response (16,17).

There are many promoter polymorphisms in MMP genes that lead to altered gene expression. The expression of MMP-2 is influenced by several promoter SNPs, including *MMP2 -1306C>T*, however, data concerning this SNP as a predisposing factor for COPD, are quite limited and with controversial conclusions.

In this study we aimed to elucidate the possible role of *MMP2 -1306C>T* promoter polymorphism for the risk of COPD.

MATERIALS AND METHODS

Patients

In this study we enrolled 84 patients with COPD with different stages of the disease according to GOLD (GOLD II, III and IV) and 71 non-affected control individuals from the central Bulgarian region of Stara Zagora. In both groups the age of inclusion in the study and the smoking status were noted, and in the patients' group age of diagnosis, duration and the stages of the disease were reported, too. The data of the patients and controls are presented in Table 1.

The study was approved according to the rules in the Medical Faculty, Trakia University, Stara Zagora for work with humans in scientific projects (No 4/2014). Informed consents were obtained from the patients before the study.

Isolation of DNA

Genomic DNA was isolated from 0.2 ml of whole blood using a commercial kit for isolation of genomic DNA from blood (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma, USA).

Genotyping

The genotyping for MMP2 -1306 C>T (rs243865) was performed via PCR-RFLP-based methods as it was described earlier (18). Each reaction with a total volume of 15μl, contained 1.5 μl of 10x PCR buffer (with 25 mM MgCl2) (STS Ltd, Bulgaria), 1.2 μl of 2.5 μmol/l dNTPs (Fermentas), 0.6 U of Taq DNA polymerase (STS Ltd. Bulgaria), 7.6 pmol of each primer and 100 ng of DNA. The sequences of the primers were as it follows: MMP2F: 5'-CTT CCT AGG CTG GTC CTT ACT GA-3'; MMP2R: 5'-CTG AGA CCT GAA GAG CTA AAG AGC T-3'.

Table 1. Demographic and clinical data of the patients with COPD and controls.

Characteristics	Patients with COPD	Controls
Number	(n=84)	(n=71)
males	67 (80%)	27 (38%)
females	17 (20%)	44 (62%)
Age at the inclusion in the study		
mean±SD (years)	66.8±8.59	50±10.72
median(range) (years)	66 (45-84)	51.5 (30-67)
Age at the diagnosis of the disease		
mean±SD (years)	61.32±8.98	
median (range) (years)	61 (34-83)	
Duration of the disease		
mean±SD (years)	4.70±4.37	
median (range) (years)	4 (0-19)	
Smoking status	(n=80)	(n=63)
non - smokers	14 (17%)	47 (75%)
ex-smokers	50 (63%)	4 (6%)
current smokers	16 (20%)	12 (19%)
Smoking habits (packs/year)		
mean±SD (years)	31.53±14.34	21.5±11.35
median(range)	30 (5-70)	20 (10-50)
Stage of COPD	(n=84)	
GOLD II	41 (49%)	
GOLD III	37 (44%)	
GOLD IV	6 (7%)	

The temperature profile of the PCR reactions included primary denaturing of template DNA for 5 min at 94˚ C, followed by 30 cycles of denaturation for 30 sec at 94˚ C, annealing for 30 sec at 64˚ C and polymerization for 30 sec at 72˚ C. The PCR reaction was terminated by a final extension for 1 min at 72˚ C and for 1 min at 25˚C.

The restriction reaction of 4 µl of each PCR product was carried out with 5U *Xsp*I (Fermentas) in a mix with a final volume of 15 µl for 16 h at 37˚ C. The fragments obtained after restriction reactions were analyzed on 10% Polyacrylamide gel electrophoresis (PAGE).

The electrophoresis was performed in 1xTBE buffer with a field gradient of the electric current of 10-20 V/cm for 3.5 hours. The gels were silver-stained and documented with a Gel documentation system (Syngene, Synoptics Ltd, UK).

Statistical Analyses

Statistical analyses were performed using SPSS 16.0 for Windows (SPSS Inc.). The ANOVA test was applied for comparing the continuous variables in independent groups. The Odds ratios (OR) were calculated by using an interactive Online Software Package at the web site http://statpages.org/#Package (http://statpages.org/ctab2x2.html). Factors with $p<0.05$ were considered statistically significant.

RESULTS

The PCR product amplified with the primers for *MMP2 -1306 C>T* SNP was of 193 bp in length. The *Xsp*I digestion resulted in 2 fragments with a length of 188 bp and 5 bp for allele C and in 3 fragments with a length of 162 bp, 26 bp and 5 bp for allele T (Figure 1).

The genotype, but not allele distribution of *MMP2 -1306C>T*, differed between COPD patients and controls (p=0.021 and p=0.602, respectively, Figure 2, Table 2). The genotypes containing the variant *T* allele (*CT+TT*) tended to be more common in COPD patients (35.7%) than in controls (25.4%, p=0.164), determining non-significantly 1.64-fold higher risk for COPD (OR=1.64, 95% CI: 0.82-3.26, Figure 3, Table 2).

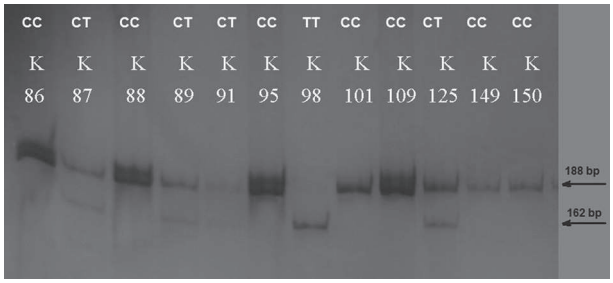

Figure 1. PAGE for visualization of PCR-RFLP products and genotyping for MMP2 -1306C>T. The homozygous CC carriers were determined with one visible band of 188 bp (41, 46, 49, 56, 57, 90, 97); the heterozygous CT carriers with two visible bands of 188 bp and 162 bp (28, 48), and the homozygous TT carriers – with one visible band of 162 bp.

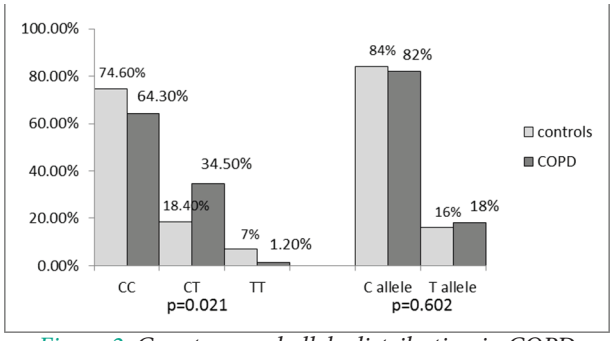

Figure 2. Genotype and allele distribution in COPD patients and controls

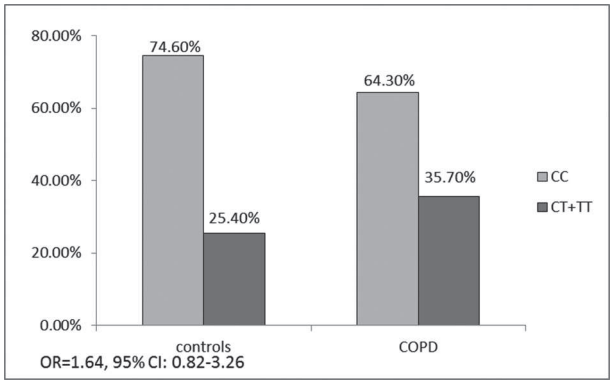

Figure 3. Genotype distribution in COPD patients and controls

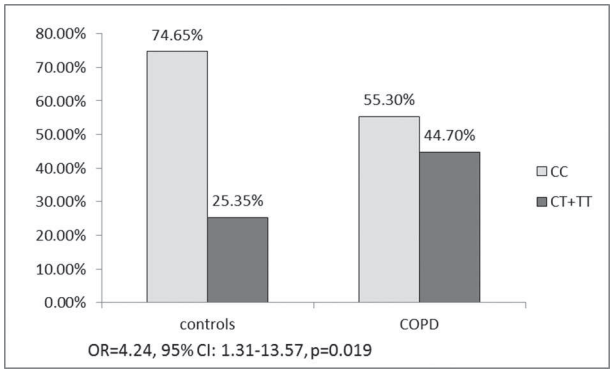

Figure 4. Genotype distribution in patients with COPD above 65 years

The risk for COPD of *T* genotypes' carriers was significant and even higher in the subset of older individuals (more than 65 years) (OR=4.24, 95% CI: 1.31-13.57, p=0.019, Figure 4) and in those without diabetes as a co-morbidity (OR=3.48, 95% CI: 1.24-9.72, p=0.023, Figure 5). Such relation was not observed among individuals with diabetes (OR=0.481, 95% CI: 0.102-2.360, p=0.427)

Table 2. Genotype and allele frequencies of -1306C>T MMP2 SNP in COPD patients and control individuals.

-1306C>T MMP2 SNP	COPD patients		Controls		OR (95% CI), p-value
	n	frequency	n	frequency	
	n = 84		n = 71		
Genotype frequency					
CC	54	0.642	53	0.746	1.0 (referent)
CT	29	0.345	13	0.183	2.189 (1.04-4.62), p=0.045
TT	1	0.012	5	0.070	0.196 (0.03-1.33), p=0.207
Genotype frequency					
CC	54	0.642	53	0.746	1.0 (referent)
CT+TT	30	0.357	18	0.254	1.636 (0.82-3.26), p=0.164
Allele frequency					
-1306C	137	0.815	119	0.838	1.0 (referent)
-1306T	31	0.185	23	0.162	1.171 (0.65-2.11), p=0.654

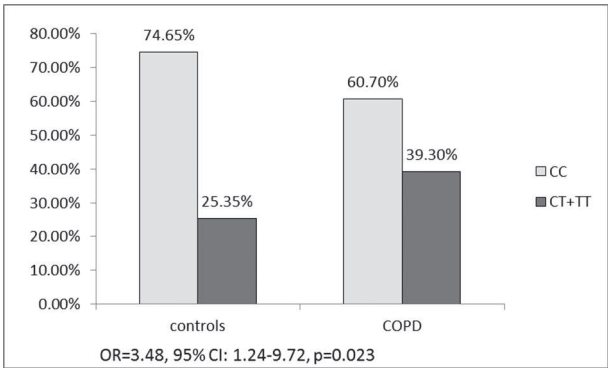

Figure 5. Genotype distribution in patients with COPD without diabetes

T genotypes (*CT+TT*) were associated with a later onset of the disease (mean of 64.11±7.12 years) than *CC* genotypes (59.74±9.54 years, p=0.045). No relations were found between the *MMP2 -1306C>T* genotypes and lung function indexes.

DISCUSSION

Smoking is the major risk factor for the development of chronic obstructive pulmonary disease (COPD), and smoking cessation is the only effective way to slow down the disease progression (19-21).

Cigarette smokers develop some degree of lung inflammation, but the COPD patients develop a far greater degree of inflammation that progresses rapidly with the advance of the disease, often accompanied by systemic inflammation and inflammation in the heart, blood vessels and skeletal muscle. The fact that only 10-20% of smokers develop severe decline of the lung function and pathological changes characteristic of COPD indicates participation of other etiological factors and the presence of predisposing genetic mechanisms of this disease (22). One of the main hypotheses for the development of pathological changes in COPD is protease-antiprotease imbalance. According to this hypothesis the increased number of cells which produce proteolytic enzymes (for example neutrophils and macrophages) in the lung and as well as the decreased synthesis of antiproteases, lead to uncontrolled proteolysis of the ECM and emphysema (8,23).

Matrix metalloproteinases, are zinc-dependent endopeptidases that participate in ECM degradation (2,14). In addition to the degradation of ECM, MMPs are involved in epithelial repair mechanisms and in the control of cytokine and chemokine activity. These features indicate an important role for MMPs in inflammatory processes. To date, 24 MMPs have been identified in mammals of which 23 are found in humans. On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups: Collagenases, Gelatinases, Stromelysins, Matrilysins, Membrane-Type MMPs, and other MMPs, which are not classified in the above categories (14,24).MMP-2 together with MMP-9 is in the group of Gelatinases. MMP-2 (gelatinase-A) is constitutively expressed by many cell types including fibroblasts, keratinocytes, endothelial cells, chondrocytes. Gelatinases degrade a broad spectrum of ECM molecules such as collagen types I, IV, V, VII, X, IX, elastin, fibronectin, aggrecan, vitronectin, laminin (25), but also many non-ECM molecules including pro-TNF-α, transforming growth factor (TGF)-β, pro-IL-1β, pro-IL-8 and monocyte chemoattractant protein (MCP)-3. MMP gene expression is primarily regulated at the transcriptional level, which usually results in low basal levels of these enzymes in normal physiological conditions (26-28). It has been found that in COPD patients alveolar macrophages, interstitial cells, and epithelial cells express gelatinase-A. Moreover, increased gelatinase-A was noticed in BAL fluid from COPD patients (29).

Several polymorphisms potentially affecting gene expression have been described in promoter regions of MMP. *MMP-2 -1306C>T* is a functional polymorphism and has been described to alter the transcriptional levels (18). Two DNA-protein complexes were detected as binding to the *C* allele, but not the *T* allele, and additional competition experiments combined with supershift analysis identified the protein binding to this region as Sp1. Transient transfection experiments showed that reporter gene expressions driven by the *C* allele were greater than reporter gene expressions driven by the T allele. So, the *C>T* polymorphism located at nucleotide -1306 site disrupts an Sp1 regulatory element and thus, the *T* allele has a strikingly lower promoter activity compared with the *C* allele (30).

In the present study we found that the genotype, but not allele distribution, differed between COPD patients and controls, as genotypes containing the variant T allele tended to be more common in

the group of patients. The carriage of the T allele determines non-significantly 1.64-fold higher risk for developing COPD, as this risk turned out to be significant in older individuals (over 65 years). These findings may lead to the hypothesis that other factors may play role in the gene expression of MMP-2. Even more immunohistochemical studies on human lung tissue show an increased expression of MMP-2 in COPD patients (24,29). While it is well recognized that MMP gene expression is mainly regulated at the transcriptional level, recent evidence, however, suggests that post-transcriptional mechanisms are also involved in the control of MMP expression in response to certain signals. As an example, TGF-β increases MMP-2 and -9 levels, mainly by extending the half-life of MMP mRNAs (26). Another important point is that the oxidative stress, thought to be important in COPD, may also contribute to an ongoing stimulus for MMP activation. The fact that sputum neutrophilia is increased in advanced COPD and is associated with the presence of an accelerated decline in lung function should not be underestimated (22,31).

On the other hand, COPD may develop not only due to emphysema, or increased destruction of ECM, but also due to airway obstruction. The chronic airflow limitation, characteristic of COPD, is caused by a mixture of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from person to person (20). The extent of inflammation, fibrosis, and luminal exudates in small airways is correlated with the reduction in FEV1 and FEV1/FVC ratio, and probably with the accelerated decline in FEV1 characteristic of COPD (20). Perhaps the lower promoter activity of the *T* allele may lead to increased deposition of connective tissue in the lungs and development of airflow obstruction, which possibly is the reason for increased risk for development of COPD in older individuals.

The majority of studies have indicated that collagen content increases with age. There is also a change, with age, of the amount of the collagen types: decrease of type III collagen and increase of type I collagen. The observed alterations of the collagen content have been shown to be concomitant with a decrease in MMP-1 and MMP-2 activity suggesting that depression of the degradative pathway may be partly responsible for age-related variations in the collagen in the lung (32). There has also been described an increase in matrix deposition in the adventitial compartments of the small airways and fibrosis accompanied by an accumulation of fibroblasts and myofibroblasts in those compartments (22). The presence of fibrillar collagen raises the possibility that the collagen is contracted and leads to fixed airflow limitation by preventing the complete relaxation of the airway smooth muscle during hyperinflation or pharmacologically induced smooth muscle relaxation (22).

The results of our study showed that the carrying of T-containing genotypes (CT+TT) of *MMP-2 -1306C>T* SNP may increase the risk of COPD in non-diabetic individuals, while such association was not found in those with diabetes as co-morbidities. There are no reported data in the literature supporting such observations. We suppose that in diabetic individuals the expression of MMPs is influenced by many other factors involved in the course of the disease which may confound the effect of genetic factors. That might be the explanation of the observed result that the studied SNP was a predisposing factor for COPD only in non-diabetic individuals.

CONCLUSION

In conclusion, our results suggest that the carriers of T allele genotypes (*CT+TT*) of *MMP2 -1306C>T* SNP may have higher risk for COPD in advanced age.

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