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ORIGINAL ARTICLE

Stromelysin-1 polymorphism as a new potential risk factor in progression of chronic obstructive pulmonary disease

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ABSTRACT: ***Stromelysin-1 polymorphism as a new potential risk factor in progression of chronic obstructive pulmonary disease. P. Santus, F. Casanova, M.L. Biondi, F. Blasi, F. Di Marco, S. Centanni.***

Background. **Chronic obstructive lung disease (COPD) is characterised by partially reversible usually progressive airflow limitation caused by inflammation and remodelling. Stromelysin-1 (MMP-3) has regulatory activity on other matrix-metalloproteinases. Altered MMP-3 activity has been described in different diseases. We investigated the role of a promoter MMP-3 polymorphism in determining susceptibility and severity of COPD.**

Methods. **We studied 147 patients with COPD in stable conditions and distinguished two groups based on FEV_1 values. In 100 patients functional modifications across a two-year period were noted. 133 healthy subjects were used as controls. Genotyping for the –1171 5A/6A MMP-3 polymorphism was performed using nucleotide sequencing.**

Results. **No difference was noted in the genotype distribution between COPD patients and controls. However, among patients with severe disease 6A/6A genotype and 6A allelic frequency were significantly more represented than among mild-moderate patients ($p < 0.05$). The 6A/6A genotype was also associated with a higher FEV_1 decline over time.**

Conclusions. **Our data suggests that –1171 6A allele does not represent a risk factor for the development of COPD while it is associated with more severe disease and different functional decline. We hypothesise that a disregulation of MMP-3, possibly caused by the –1171 5A/6A polymorphism or other linked variants, may lead to different progression in COPD.**

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Keywords: COPD, candidate genes, Stromelysin-1, Matrix-metalloproteinases, Genotype, Genetic polymorphisms.

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Introduction

According to the guidelines described by the Global Initiative for the diagnosis, management and prevention of chronic Obstructive Lung Disease (GOLD), chronic obstructive pulmonary disease (COPD) can be defined as "a disease state characterised by airflow limitation that is not fully reversible" [1]. Such limitation is usually progressive and associated with an abnormal inflammatory response of the lung to different stimuli.

Although cigarette smoking is the most important risk factor for COPD, only a small percentage of smokers will present the disease [2], thus indicating the presence of genetic factors in determining susceptibility. From a pathological standpoint, inflammation and structural changes, referred as remodelling of the lung parenchyma and airways (particularly of small caliber), are considered the main contributors to the airflow limitation and to the decline of FEV_1 [3].

Beside the typical emphysematous lesions Vlahovic *et al* [4] reported an infiltration of the alveolar septa accompanied by specific connective tissue changes characterised by an increase of alveolar wall thickness due to deposition of both elastin and collagen [5]. Similarly, inflammation and peribronchiolar fibrosis leading to a disarrange of the alveolar-bronchiolar attachments and consequent loss of elastic recoil are commonly detected in small airways [6].

Given such evidence we can assume that the remodelling of the connective tissue, and in particular of the extra cellular matrix (ECM), may play a key role in the development of COPD. In this contest, growing interest in the literature has been raised by metalloproteinase (MMPs), zinc dependent proteolytic enzymes interplaying in the dynamic homeostasis of the ECM, highly regulated at different levels [6].

Experimental evidence indicates their potential role in the pathogenesis of the emphysema; in particular MMP-9 [7-14], MMP-12 [14-17] and MMP-1 [14, 15, 18, 19] have been extensively studied.

Stromelysin-1 (MMP-3) is a key member of the MMPs family as it activates other proteases

[20], responds to cytokines, growth factors, oncogenic products [21], cotinine and nicotine [22], and presents a broad spectrum of cleavage activity on ECM components.

A role for MMP-3 in the induction or progression of multifactorial diseases was first described in the atherosclerosis process [23] with a functional polymorphism of the promoter region (–1171 5A/6A) associated with different risks of progression of coronary disease [24]. Interestingly, Ye and colleagues [25] demonstrated *in vitro* that the 6A allele was associated with a lower gene expression when compared to 5A. Since these observations, the same polymorphism has been associated to a broad spectrum of complex diseases [26-33].

We herein describe the prevalence of the –1171 5A/6A MMP-3 polymorphism in a population of patients with COPD and controls, and its possible role in the determination of disease susceptibility and progression.

Methods

Study population

We enrolled 147 outpatients affected by COPD who consecutively attended our Respiratory Unit between January 2004 and May 2004. The inclusion criteria consisted of diagnosis of COPD according to GOLD guidelines [1]; absence of clinical worsening in the previous six weeks; and no registered variation of ongoing medical therapy during the previous three months.

All patients underwent respiratory functional tests including spirometry and arterial blood gas analysis breathing room air at rest. As for the ongoing medical treatment, 10 patients were receiving beta-2 long acting agonist (either salmeterol or formoterol), 49 anticholinergic (oxitropium bromide) and 88 both medications; moreover 28 patients were also receiving theophylline at therapeutical dosage. The overall characteristics of the population in the study are shown in table 1.

We further subdivided the population with COPD in two groups based on disease severity. Patients with FEV_1 values, expressed as percentage of predicted, higher or equal to 50% were considered as having mild to moderate COPD and those with FEV_1 lower than 50% as presenting severe disease. Table 1 shows the characteristics of patients included in the two groups. In a subgroup of 100 patients FEV_1 modifications over a two-year time span since first enrolled, expressed as absolute values, were noted.

133 healthy subjects age and geographically matched with patients, with neither clinical nor functional evidence of COPD, were used as controls (see table 1).

The study protocol was approved by Ethics Committee and written consent was obtained for each subject after being informed about the nature of the study; the ethical guidelines of the 1975 Declaration of Helsinki and subsequent modifications was respected.

Table 1. - Clinical and functional features of the study population at the time of blood sample

	COPD subjects	**COPD with $FEV_1 \geq 50\%$ (*n = 72*)**	**COPD with $FEV_1 < 50\%$ (*n = 75*)**	**Healty subjects (*n = 133*)**
Age (*years*)	69.3 ± 8.3*	68.8 ± 8.4* NS	70.2 ± 8.3*	67.5 ± 8.8*
Sex (*n*)	121 M (82%) - 26 F (18%)	58 M (81%) - 14 F (19%) NS	63 M (84%) - 12 F (16%)	107 M (80%) - 26 F (20%)
Current smokers (*n*)	71 (48%)	33 (46%) NS	38 (51%)	56 (45%)
Former smokers (*n*)	76 (52%)	39 (54%) NS	37 (49%)	77 (55%)
Pack years (*n*)	53.8 ± 28.3*	51.3 ± 26.3* NS	56.7 ± 30.2*	15.8 ± 5.4*
FEV_1 (*%*)	50.3 ± 16*	63.3 ± 9.1* p<0.05	36.3 ± 8.1*	98.6 ± 12*
PaO_2 (*mmHg*)	75 ± 8*	76 ± 8.1* NS	72.7 ± 8.3*	93 ± 5*
$PaCO_2$ (*mmHg*)	40 ± 4.9*	39.5 ± 4.4* NS	41.5 ± 5.1*	37 ± 3.4*

* Values expressed as average ± standard deviation; NS = Non Significant (this value is calculated in COPD with $FEV_1 \geq 50\%$ vs COPD with FEV_1 <50%). The functional parameters were collected in basal condition and values of arterial blood gases were obtained at rest while breathing room air.

–1171 5A/6A MMP-3 genotyping

Whole blood (3 ml) from patients and controls was collected into potassium EDTA. DNA was prepared with the Istagene Matrix extraction kit (Bio-Rad Laboratories). The polymerase chain reaction for MMP-3 was performed in a total volume of 25 µL with 5 µL of extracted genomic DNA, 100 µmol/L of dATP, dGTP, dTTP, and dCTP, 1.5 mmol/L of $MgCl_2$, and 1 U of Taq polymerase, with the 2 primers, forward and reverse, each at a concentration of 80 nmol/L. The primers were designed with Primer Express software. The MMP-3 primer sequence is as follows: forward: 5'-TCCTCATATCAATGTGGCCAAA-3'; reverse: 5'-CGGCACCTGGCCTAAAGAC-3'. The polymerase chain reaction starts with 5 minutes of incubation at 94°C to activate the enzyme, followed by 35 cycles of 20 seconds at 94°C, 20 seconds at 55°C, and 30 seconds at 72°C. The amplification was verified on an agarose gel (2%) followed directly by sequencing with an automatic sequencer in fluorescent DNA capillary electrophoresis (ABI Prism 310; Applied Biosystems).

Analyses

The *chi*-square and Fisher's exact tests were used for the analysis of categorical variables. In the case of continuous variables, the Mann-Whitney test and the *t* test were used to compare two groups, and the Kruskal-Wallis non-parametric one-way analysis of variance test to compare more than two groups. The statistical comparisons were made using Stata Statistical Software (Stata Corporation, College Station, TX). All of the analyses were two-sided, and *P* values of < 0.05 were considered statistically significant.

Results

The characteristics of the population of patients with COPD, overall and after stratification, according to disease severity, are summarised in table 1.

The allelic frequencies observed in patients with COPD were not different from those found in control subjects (6A allele frequency: 0.524 *vs*. 0.507) (table 2). We used the control population (table 1) just to evaluate the frequency distribution of MMP3 polymorphism in general population. Our results showed that COPD subjects have the same MMP3 frequency genotype distribution compared to control subjects and it follows that COPD does not represent a particular population "*a priori*".

However, among patients with severe disease 6A/6A genotype and 6A allelic frequency were both significantly higher compared to mild-moderate patients (6A allelic frequency 0.573 *vs*. 0.472; $p < 0.05$), see table 3. The two subgroups did not significantly differ in age, sex, smoking status, pack years and current therapy. Accordingly, when the patients were arrayed by genotype the FEV_1 values among patients carrying the 6A/6A genotype were significantly lower (mean 44% *vs*. mean 51% in patients with 5A/5A 5A/6A genotype; $p < 0.05$), see table 4. The 6A/6A genotype also represented a risk factor for more severe disease compared to the 5A/5A 5A/6A ones (Odds Ratio 2.57 95% Confidence Interval 1.13 – 5.83).

Table 2. - Distribution of the –1171 5A/6A genotypes and alleles frequency in patients with COPD and control population

	Patients ***(n = 147)***	Controls ***(n = 133)***	p
–1171 5A/5A ***(n)***	**25 (17%)**	**36 (27%)**	**NS**
–1171 5A/6A ***(n)***	**90 (61%)**	**59 (44%)**	**NS**
–1171 6A/6A ***(n)***	**32 (22%)**	**38 (29%)**	**NS**
–1171 6A allelic frequency	**0.524**	**0.507**	**NS**

NS = not statistically significant.

Table 3. - Distribution of the –1171 5A/6A genotypes and alleles frequency in the two subgroups of patients with COPD

	$FEV_1 \geq 50$ ***(n = 72)***	$FEV_1 < 50$ ***(n = 75)***	p
–1171 5A/5A ***(n)***	**14 (19%)**	**11 (15%)**	**NS**
–1171 5A/6A ***(n)***	**48 (67%)**	**42 (56%)**	**NS**
–1171 6A/6A ***(n)***	**10 (14%)**	**22 (29%)**	**NS**
–1171 6A allelic frequency	**0.472**	**0.573**	**< 0.05**

NS = not statistically significant.

Table 4. - ΔFEV_1 (from 2004 to 2006) in all patients and in the current and former smokers group distributed according the –1171 5A/6A genotype

	Total *(n = 100)* ΔFEV_1 (ml)	**Current smokers (55.1 ± 28.9 Pack year)** *(n = 41)* ΔFEV_1 (ml)	**Former smokers (50.4 ± 25.4 Pack year)** *(n = 59)* ΔFEV_1 (ml)	**Mean FEV_1 among two genotypes** FEV_1 (%)#
–1171 5A/5A + –1171 5A/6A	-8 ± 19,3	-7,8 ± 19,5	-5,7 ± 18	51
–1171 6A/6A	-18 ± 23,6*	-21,5 ± 22,7	-23,3 ± 27,2*	44*

All values expressed as mean ± standard deviation.
FEV_1 is expressed as percent of predicted value.
* $p < 0.05$ *vs.* 5A/5A+5A/6A.

When either FEV_1 values or the distribution of the genotypes were confronted with age, sex, smoking status and pack years no significant association was noted.

Similarly, no correlation was found between genotype and other functional parameters such as arterial partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$).

In 100 patients with COPD (41 current [55.1 ± 28.8 pack/year] and 59 former smokers [50.4 ± 25.4 pack/year]) data on changes of FEV_1 after a two-year follow up was available. We noted no variations of the smoking status across the two years and while the two subgroups did not differ for age, sex (data not shown) or smoking history. Overall, patients with COPD carrying the 6A/6A genotype showed a significantly higher decline in FEV_1 expressed as absolute values (-18 ml ± 23,6 *vs.* – 8 ml ± 19 in the 5A/5A 5A/6A genotypes; $p < 0.05$), as shown in table 4. Similarly, when we considered individually the former and current smokers subgroups of patients, the same association was noted in both of them, although in the latter the difference was not statistical significant (-21,5 ml ± 22,7 *vs.* -7,8 ml ± 19,5).

Discussion

COPD is currently defined as a disease state characterised by partially reversible airflow limitation [1]. Hystopathologically, two different processes can be recognised, namely an early and persisting inflammatory infiltration and a progressive parenchymal remodelling consisting in disruption of alveolar septa, thickening of the alveolar wall and fibrosis of the small-caliber airways [4]. The interaction of these structural changes is believed to account for the resulting airflow limitation.

The relationship and relative role of these processes as well as their possible expression in response to acute or chronic injury induced by cigarette smoking are poorly understood. Similarly, it is not known which steps lead to the change of physiological into pathological process. An imbalance between protease and antiprotease enzymes, possibly induced by direct action of smoking or mediated by inflammatory cells has been hypothesised. In particular, MMPs and their potential effects in induction or progression of COPD constitute a recently developed field of research, with a number of observations supporting the role of MMP-1, MMP-9 and MMP-12 [7-19].

Among these proteases, MMP-3 is considered a key enzyme, as it recognises a broad spectrum of substrates, can activate other MMPs (in particular MMP-9) and it can be responsible for the release of growth factors. Since the early evidence obtained in atherosclerosis process [23], a wide spectrum of disease has been associated with MMP-3 expression [24]; in similar fashion, a functional promoter gene –1171 5A/6A polymorphism has been identified and associated with atherosclerosis [25] and other complex diseases such as primary sclerosing cholangitis [26], scleroderma [30], primary biliary cirrhosis [31] and cancer invasivity [32, 33].

The results described herein indicate that –1171 5A/6A polymorphism of MMP-3 does not represent a risk factor for susceptibility to COPD, since no difference in allelic frequency distribution between patients with COPD and controls could be found. On the other hand, when patients affected by COPD were subdivided according to clinical severity into two groups, using an observed FEV_1 of 50% of predicted as threshold, data showed a significant association between the 6A/6A allele and a more severe disease. Concerns might be raised by the use of FEV_1 values as the only index of disease severity, since COPD staging should consider the impairment of the respiratory function as well as the impact of the disease on quality of life and its complications. However, at the current stage of knowledge, FEV_1 represents the only reliable tool available for evaluation of outpatients, as suggested by GOLD guidelines. The choice of the FEV_1 value used in this study was moreover derived from the clinical observation that patients with FEV_1 below 50% of predicted presented a significant higher risk of exacerbations, known episodic factors responsible for the impairment in quality of life and eventually changes in natural history of the disease [34-37].

Interestingly, when the patients with COPD were stratified according the MMP-3 genotype, the association between 6A/6A genotype with lower FEV_1 values was confirmed, suggesting

once again that the lower expression of MMP-3 can be one of the factors involved in the progression of the disease. Furthermore, when we considered changes of FEV_1 values in a two-year time span, an association between the 6A/6A genotype and a higher decline of FEV_1 values was observed among all patients, including both former and current smokers, although the difference was not statistical significant; probably these results could be related to a small number of patients (table 4).

Results described herein indicate for the first time a possible role of common genetic variants of MMP-3 in natural history of COPD, possibly by modifying MMP-3 expression. Such observations appear intriguing since possibly in contrast with the finding that enhanced enzymatic activity of MMPs (MMP-12, MMP-1 and MMP-9) are associated to susceptibility or progression of COPD [8, 9, 15, 17]. On the contrary, this finding is consistent with findings from several previous studies that the 6A/6A genotype is associated with more rapid progression of coronary atherosclerosis [24, 38, 39] and with greater intima-media thickness [40-42]. Matrix accumulation in the arterial wall is enhanced in individuals carrying the transcriptionally less active 6A allele of the MMP3 gene predisposing to progressive fibrosis.

It is now accepted that the elements that determine the airflow limitation and its progression are the disruption of the alveolar septa, the progressive deposition of elastin and collagen in the interstitium of the alveolar wall and the progressive fibrosis of the small airways, although at varying degrees [5]. MMP-3 plays different roles or a different role in maintaining the dynamic balance of the ECM, being responsible for cleavage activity on ECM components with potential release of growth factors (particularly those involved in fibrogenesis) [43-45], and regulating the activity of a number of other MMPs. Given our results, we hypothesise that a deregulation of MMP-3, possibly secondary to a lower expression associated with the –1171 6A/6A polymorphism, may determine an imbalanced remodelling of the ECM with consequent tendency to fibrosis both directly or mediated by others MMPs.

It is important to note, however, that complex mechanism regulate the gene expression and eventually the protein synthesis and the activity both to cellular and tissue level. Further studies are warranted in order to fully understand the impact of our observation in the pathology of CODP, possibly using molecular analysis of MMP-3 expression and activity in the pulmonary tissue, in bronchoalveolar lavage (BAL) or induced sputum.

Moreover we should assume that polymorphisms of the MMP-3 gene, located in the 11q22-q23 region, are in linkage disequilibrium with other polymorphisms, such as those of MMP-1 or MMP-12, located in the same region and previously indicated as involved in determining COPD [14]. Joos and colleagues [14] observed a significant correlation between MMP-1 and MMP-12 genotypes and FEV1 decline in a smokers' population. Interestingly, such correlation was found related to combined presence of determined variants on both genes, while neither MMP-1 nor MMP-12 were by themselves sufficient to explain functional decline. Our results appear to implement such observation, given the mapping of MMP-3 gene between MMP-1 and MMP-12. The same authors in fact suggested that another gene in the immediately chromosomal vicinity to MMP-1 and MMP-12 could be the real responsible for the association [14]. Numerous candidate genes that could be linked to disease pathogenesis have been implicated in COPD genetics. However, the candidate gene approach is often limited by inconsistent results in other populations. In the future, ongoing exact phenotype definition, combination of several approaches, genome-wide association studies will lead to new insights into the genetics of COPD. In conclusion, only a genetic linkage study on a large population of representative families will allow the possibility of defining the role of MMP-3 and other polymorphisms in the natural history of COPD. As observed in other diseases, sharing the same multifactorial pathogenesis as COPD, a "multi-hit" model seems to apply also to COPD, in which environmental exposition and separate genetic factors may lead to disease development (first or initial "hit") and rate of progression (second or subsequent "hit").

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