INTERSTITIAL LUNG DISEASE

Promoter variants in tissue inhibitor of metalloproteinase-3 (TIMP-3) protect against susceptibility in pigeon breeders' disease

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Background: Tissue inhibitors of metalloproteinases (TIMPs) play a major role in extracellular matrix turnover in the lung. However, in chronic lung disorders such as idiopathic pulmonary fibrosis (IPF) and pigeon breeders' disease (PBD), TIMPs may promote an adverse non-degradative environment. We hypothesised that polymorphisms in TIMP-3 could affect susceptibility to IPF and PBD.
Methods: Two promoter variants, −915A>G and −1296T>C, were genotyped in 323 healthy subjects, 94 subjects with IPF, 115 with PBD, and 90 exposed to avian antigen but without PBD. The severity of fibrosis in lung tissue and the clinical outcome after 1 year was determined in the PBD group.
Results: The variants did not influence susceptibility to IPF, but the rare alleles of both variants appeared to be protective against susceptibility to PBD (odds ratio (OR) for carriage of at least one rare allele from either variant 0.48, 95% CI 0.30 to 0.76, p = 0.002). Haplotype analysis of positions −915 and −1296 estimated four haplotypes: *A*T, *G*T, *A*C and *G*C, respectively. Their frequencies differed overall between subjects with PBD and healthy subjects (p = 0.0049) and this was attributable primarily to the *G*C haplotype (OR 0.53, 95% CI 0.36 to 0.77, p = 0.001). The severity of fibrosis correlated with poorer outcome in the PBD group ($r = 0.73$, $p < 0.01$) but no relationship was seen between the *G*C haplotype and outcome or fibrosis. However, PBD subjects with the *G*C haplotype did have proportionally fewer lymphocytes in their bronchoalveolar fluid than those with the common *A*T haplotype (p = 0.029).
Conclusions: TIMP-3 variants appear to contribute to susceptibility to PBD. This may be through the inflammatory reaction rather than the fibrotic reaction.

Idiopathic pulmonary fibrosis (IPF) and pigeon breeders' disease (PBD) are diffuse interstitial lung diseases that may result in pulmonary fibrosis through different mechanisms. IPF is a progressive non-granulomatous disorder of unknown aetiology characterised by epithelial injury and activation, fibroblast proliferation and fibrosis in the lung parenchyma, whereas PBD is a granulomatous inflammatory disease termed "hypersensitivity pneumonitis" that occurs as a result of a hypersensitivity reaction in response to exposure to avian antigen.[1 2]

In IPF, PBD, and several other conditions including rheumatoid arthritis and cancer a marked change in the balance of proteolytic/antiproteolytic processes occurs, particularly in those mediated by matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs).[3–6] MMPs constitute a family of highly regulated zinc dependent proteinases which have a major role in the turnover and degradation of the extracellular matrix (ECM).[6] In adult lung the turnover of ECM is vital for the repair and maintenance of the lung structure.[7] The TIMPs share a common MMP inhibitory action and exert a major controlling influence on ECM turnover. In fibrotic lung, despite increased MMP expression,[8] there is a significant decrease in collagenolytic activity compared with normal lung which suggests that a local non-degrading fibrillar collagen microenvironment prevails.[9] Notably, there is a broader interstitial localisation of TIMP-1, -2, -3 and -4 compared with the collagenases MMP-1, -8 and -13.[4]

There is evidence that gene variants of MMPs and TIMPs influence susceptibility to human disease including cancer, chronic obstructive pulmonary disease, cardiovascular disease and Sorby's fundus dystrophy.[10–12] TIMP-3 is the only one which binds strongly to the ECM[4 13] and has functional variance.[12] Several coding changes in the C-terminus of TIMP-3 have only been found in association with Sorby's fundus dystrophy,[12] although other variants are reported in the promoter of TIMP-3.[14] TIMP-3 is possibly a factor that promotes fibrosis since TIMP-3 null mice develop airspace enlargement but no inflammation or fibrosis, and have reduced amounts of collagen.[15]

We hypothesised that variants which alter gene expression in TIMP-3 will influence susceptibility to chronic lung diseases such as IPF and PBD. In this study the frequency of two common promoter variants in the TIMP-3 gene[14] were determined in Mexican populations with IPF, PBD, and healthy subjects and tested for association with disease. The clinical course of chronic hypersensitivity pneumonitis can result in resolution, stabilisation, or progression to fibrosis, and in approximately 30% of patients the disease evolves into a diffuse fibrotic disorder that is usually lethal.[16] We therefore used a population of PBD subjects to test if the promoter variants are associated with outcome and severity of fibrosis.

METHODS

Subjects

A total of 115 patients with PBD, 94 with IPF, 323 healthy subjects, and 90 subjects exposed to avian antigen but without PBD (EA) were included in the study (table 1). All subjects were Mexican mestizo with at least two generations born in Mexico. The protocol was approved by the ethical committee of the National Institute of Respiratory Diseases in Mexico and the Joint University College London and University College London Hospitals Committees on the

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Table 1 Mean (SD) characteristics of Mexican healthy subjects and those with idiopathic pulmonary fibrosis (IPF), pigeon breeders' disease (PBD), and subjects exposed to avian antigen but without PBD (EA)

	Healthy subjects	IPF	PBD	EA	p value*
Sex (F/M)	78/245	32/62	115/0	71/19	
Age (years)	33.2 (9.8)	62.1 (10.0)	43.9 (13.6)	35.0 (8.7)	<0.001
Time elapsed to first visit (months)†		29.8 (22.8)	21.0 (21.5)		<0.05
FVC (% predicted)		61.8 (19.1)	56.2 (20.3)		<0.05
FEV_1 (% predicted)		65.6 (18.8)	59.6 (19.9)		NS
FEV_1/FVC (%)‡		93.5 (12.5)	91.8 (8.8)		NS
Pa_{O_2} (kPa)¶		6.6 (1.5)	7.1 (1.4)		<0.01
T_{LCO} (%)		49.4 (12.2)	51.7 (18.2)		NS
% BAL macrophages		77.3 (7.9)	35.6 (20.2)		<0.001
% BAL lymphocytes		17.6 (7.0)	62.2 (20.7)		<0.001
% BAL neutrophils		3.4 (3.7)	1.4 (2.8)		<0.01
% BAL eosinophils		1.7 (2.1)	0.8 (1.1)		<0.05

FVC = forced vital capacity; FEV_1 = forced expiratory volume in 1 second; Pa_{O_2} = arterial oxygen tension; T_{LCO} = carbon monoxide transfer factor; BAL = bronchoalveolar lavage fluid.
*IPF group *v* PBD group.
†Time elapsed to first visit is the time elapsed from the beginning of the symptoms to first visit.
‡Absolute ratio.
¶Normal values at Mexico City altitude are 8.9 (0.4) kPa.

Ethics of Human Research in the UK, and written informed consent was obtained from each subject.

The diagnostic criteria for the PBD group have previously been described.[16 17] The EA subjects had between 2 and 31 birds at home, usually parakeets and/or pigeons. This type/intensity/duration of exposure was similar to the PBD cohort. In 72 of 115 patients with PBD the diagnosis was supported by histological evaluation of a surgical lung biopsy specimen. The percentage of fibrosis present in the lung samples was estimated using a semi-quantitative histological assessment as previously described.[18 19] Patients with PBD were followed for at least 1 year and after this time their condition was classified as resolved, improved (>10% increase in forced vital capacity (FVC) plus >0.53 kPa increase in arterial oxygen tension (Pa_{O_2})), stable, worse (>10% decrease in FVC plus >0.53 kPa decrease in Pa_{O_2}), or deceased. Twenty of the 115 patients were lost to follow up.

The diagnosis of IPF was supported by clinical examination, pulmonary function tests, high resolution computed tomography, and bronchoalveolar lavage (BAL) findings, and was supported by surgical biopsy in 53 patients based on the typical morphology of usual interstitial pneumonia.[17 20] In the absence of tissue, patients were required to fulfil the criteria of the ATS/ERS international consensus.[20] Patients with known causes of interstitial lung disease such as collagen vascular disease, drug toxicity, and environmental exposure were excluded. The healthy subjects were sequential unrelated blood donors from the Transfusion Department of the National Institute of Respiratory Diseases, Mexico.

Genotyping

Genomic DNA was extracted from whole blood by salting out methods.[21] The −915A>G and −1296T>C variants in the TIMP-3 promoter were genotyped by enzyme digest of polymerase chain reaction amplification products as previously described.[14] Samples were genotyped blind to phenotype data and checked independently.

Statistical analysis

Allele frequencies were estimated by gene counting. Statistical analysis was performed using SPSS version 11.5 unless otherwise referenced. χ^2 tables were used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium and to compare genotype frequencies between the patient populations and the control groups. The extent of allelic association (D′) between the −915A>G and −1296T>C variants was estimated using log-linear analysis.[22 23] Odds ratios (OR) and 95% confidence intervals (95% CI) were derived from binary logistic regression analysis. Haplotypes were generated using the PHASE program.[24] The relationship between fibrosis and outcome in the PBD population was determined by the Spearman rank correlation coefficient. Linear regression analysis was used to examine the relationship between haplotypes and lung function and BAL fluid cell profile in the PBD population, and ordinal logistic regression was used to examine the relationship between haplotypes and fibrosis in these subjects. For all tests a p value of $\leqslant 0.05$ was considered significant.

RESULTS

Patient characteristics

The baseline characteristics of all subject groups are summarised in table 1. All patients had clinical and functional evidence of interstitial lung disease with variable degrees of dyspnoea, decreased lung capacities, and hypoxaemia at rest that deteriorated during exercise. As expected, the patients with IPF were older than those with PBD. In the PBD group differential cell counts in BAL fluid were characterised by marked lymphocytosis (usually well over 40%), while in patients with IPF most of the BAL fluid inflammatory cells were macrophages.

Frequency and genotype distribution of −915A>G and −1296T>C

There was a significant difference in the genotype distribution across all four subject groups for the −915A>G and −1296T>C variants ($p = 0.016$ and $p = 0.019$, respectively). The genotype distribution and rare allele frequency of the −915A>G and −1296T>C variants in all subject groups are shown in table 2. The rare −915G allele appeared protective against PBD with both allele and genotypic frequencies being significantly lower in the PBD group than in the healthy subjects ($p = 0.009$ and $p = 0.001$, respectively). Similar observations were seen when the PBD subjects were compared with the EA subjects (table 2). The allele and genotypic frequencies were not significantly different between the healthy subjects and the EA subjects so the healthy subjects were subsequently used for comparisons with the PBD subjects (table 2). There was a strong positive allelic association between the −915A>G and −1296T>C

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Table 2 Genotype distribution and rare allele frequency (%) of the −915A>G and −1296T>C variants in Mexican healthy subjects, IPF, PBD and EA populations

Category	n	Rare allele frequency (%) (95% CI)	p value*	Genotype (%) AA	AG	GG	p value†
−915A>G variant							
H	323	0.33 (0.29 to 0.37)		139 (43.0)	155 (48.0)	29 (9.0)	
IPF	94	0.30 (0.23 to 0.36)	0.71	46 (48.9)	40 (42.6)	8 (8.5)	0.591
PBD	115	0.22 (0.17 to 0.28)	0.009 (0.016)	72 (62.6)	35 (30.4)	8 (7.0)	0.001 (0.019)
EA	90	0.35 (0.28 to 0.42)	0.88	39 (43.3)	39 (43.3)	12 (13.3)	0.434
				TT	TC	CC	
−1296T>C variant							
H	323	0.36 (0.32 to 0.39)		133 (41.1)	150 (46.4)	40 (12.4)	
IPF	94	0.33 (0.26 to 0.40)	0.80	42 (44.7)	42 (44.7)	10 (10.6)	0.800
PBD	115	0.23 (0.18 to 0.28)	0.002 (0.016)	70 (60.9)	37 (32.2)	8 (7.0)	0.001 (0.023)
EA	90	0.36 (0.29 to 0.43)	0.99	39 (43.3)	37 (41.1)	14 (15.6)	0.588

H = healthy subjects; IPF = idiopathic pulmonary fibrosis; PBD = pigeon breeders' disease; EA = subjects exposed to avian antigen but without PBD.
All genotypes were checked independently for accuracy. Both −915A>G and −1296T>C variants were in Hardy-Weinberg equilibrium in all four subject groups. χ^2 values for −915A>G were: healthy subjects 2.37 (p = 0.12), IPF 0.03 (p = 0.87), PBD 1.61 (p = 0.21), and EA 0.20 (p = 0.65). χ^2 values for −1296T>C were: healthy subjects 0.05 (p = 0.82), IPF 0.01 (p = 0.92), PBD 0.99 (p = 0.32), and EA 1.07 (p = 0.30).
*Allele and †genotype frequencies compared by χ^2 test with healthy subjects (PBD subjects have also been compared by χ^2 test with EA subjects and the p values are shown in parentheses).

variants (D′ = 0.97, p<0.0001). Carriage of at least one rare allele from either variant, being heterozygote for both variants or homozygote for the rare allele for both variants, showed a significant risk for PBD subjects but not IPF subjects (table 3).

The healthy subjects were not well matched for age and sex with the IPF and PBD subjects and there is significant evidence of an interaction between age and genotype on risk when comparing the PBD and healthy subjects (p<0.005). Splitting the data by the median age of the data set (34 years) indicated a stronger effect of genotype on risk in older subjects (for carriage of at least one rare allele from either variant: subjects <34 years, OR 1.04 (95% CI 0.49 to 2.21), p = 0.93; subjects >34 years, OR 0.32 (95% CI 0.18 to 0.58), p<0.001). Similar results were obtained using different age cut off points such as the median age of the PBD subjects. There was no difference in genotype frequencies between the sexes in the healthy subjects (78 women, 245 men) to confound the relationship between genotype and disease (p = 0.261). An effect of genotype between PBD and healthy subjects was still observed after the analysis was stratified by sex (OR for carriage of at least one rare allele from either variant 0.50 (95% CI 0.28 to 0.89), p = 0.019), providing no evidence that sex modifies the relationship between genotype and disease.

Haplotype analysis of positions −915 and −1296 estimated four haplotypes: *A*T, *G*T, *A*C and *G*C, respectively. Their frequencies differed overall between the PBD and healthy subjects (p = 0.0049) and this was attributable primarily to the *G*C haplotype having a protective effect (table 4). The haplotype frequencies did not differ between IPF and healthy subjects (p = 0.44).

TIMP-3 haplotypes against lung function, BAL findings, poorer outcome, and fibrosis in PBD

There was no difference in lung function by TIMP-3 haplotype within the PBD group (table 5). However, the BAL cell profile appeared to be influenced by TIMP-3 haplotype. Subjects with the *G*C haplotype had proportionally fewer lymphocytes in their cell count than the common *A*T haplotype (table 5). Of the 115 PBD subjects, 95 were followed up for at least 1 year and five categories of outcome were then assigned. Two of these categories reflected poorer outcome (worse/died) and so were combined (n = 39) to determine whether the *G*C haplotype conferred a protective influence on poorer outcome compared with the remaining subjects with outcome data (resolved/improved/stable; n = 56). The *G*C haplotype had no effect on outcome (OR 1.04 (95% CI 0.72 to 1.51), p = 0.83, table 5). Of the 95 genotyped PBD subjects with outcome data, 60 had an estimate of fibrosis in their lungs. The severity of fibrosis correlated with poorer outcome in these subjects ($r = 0.73$, p<0.01). There was no evidence to suggest a relationship between haplotypes and fibrosis (ordinal logistic regression adjusted for age and smoking, p = 0.38).

DISCUSSION

In this study healthy subjects and those with IPF, PBD, and EA were investigated for the frequency of two common TIMP-3 promoter variants, −915A>G and −1296T>C. Our results suggest that these TIMP-3 variants do not contribute to susceptibility to IPF. By contrast, the rare alleles of these variants appear to protect against the risk of developing PBD. The EA subjects represent a more defined control population for the PBD subjects but, as there was no difference in the

Table 3 TIMP-3 promoter variants −915A>G and −1296T>C and risk of pigeon breeders' disease (PBD)

	Double homozygote for rare allele			Heterozygote for both variants			Carriage of at least one rare allele from either variant		
	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
H	1.0			1.0			1.0		
IPF	0.49	0.10 to 2.39	0.38	0.54	0.21 to 1.39	0.20	0.57	0.24 to 1.36	0.21
PBD	0.35	0.13 to 0.91	0.031	0.47	0.28 to 0.759	0.004	0.48	0.30 to 0.76	0.002

H = healthy subjects; IPF = idiopathic pulmonary fibrosis; PBD = pigeon breeders' disease.
OR values are adjusted for age.

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Table 4 Association of −915 and −1296 TIMP-3 haplotypes with IPF and PBD

Allele at position		Haplotype frequency					Haplotype frequency			
−915	−1296	H	IPF	OR	95% CI	p value	PBD	OR	95% CI	p value
A	T	0.638	0.670	1.0			0.761	1.0		
G	T	0.006	0.000	–			0.008	1.05	0.16 to 6.82	0.96
A	C	0.033	0.032	1.35	0.24 to 7.67	0.74	0.018	0.25	0.06 to 1.10	0.07
G	C	0.323	0.298	0.65	0.33 to 1.26	0.20	0.213	0.53	0.36 to 0.77	0.001

H = healthy subjects; IPF = idiopathic pulmonary fibrosis; PBD = pigeon breeders' disease.
OR values are adjusted for age.

TIMP-3 genotype distribution between this group and the healthy subjects, the healthy subjects were used throughout the study to calculate risk.

As with other studies in interstitial lung disease, the controls and cases used were not well matched for age and sex. Odds ratio calculations have been adjusted for age, but it is possible that the healthy subjects include individuals who are at risk of PBD and who in later life may develop disease. The presence of such individuals would reduce the ability to detect an effect of genotype on the risk of PBD. The fact that all the subjects with PBD were female is due to domestic exposure to pigeons. In Mexico pigeons are mainly kept as domestic pets, sharing the same living space as their owners. Women are likely to spend more time at home than men and consequently have greater exposure. The difference in the effect of genotype on subjects with PBD and healthy subjects was still observed after the groups were stratified by sex, but we cannot exclude the possibility that there may be a difference in genotype by sex in the disease group.

The relatively low incidence of PBD in the antigen exposed population suggests that there are some host susceptibility and/or environmental conditions that modify the immune response. These can be protective or promoting factors and include genetic susceptibility associated with the major histocompatibility complex and the presence of a recent viral respiratory infection.[2 17 25] In this context, this is the first report of an allelic association between molecules involved in ECM turnover in the lung and PBD, suggesting that some TIMP-3 promoter variants may confer resistance to the development of PBD. However, the association needs to be confirmed in a separate PBD population.

A strong positive allelic association was observed between −915A>G and −1296T>C in the healthy Mexican subjects, similar to that in a white Czech population in which higher allele frequencies for −915G and −1296C (0.39 and 0.40, respectively) were found.[14] For this reason, haplotypes across the −915 and −1296 positions were generated revealing the *G*C haplotype as the primary cause of the association with PBD. Despite a positive protective effect of this haplotype against susceptibility to PBD, it did not appear to influence the clinical outcome in this group after follow up at 1 year. The absence of an effect of the haplotypes on fibrosis in subjects with PBD is consistent with the results in IPF. IPF is an important comparator for PBD in this study because all the IPF subjects had clinical evidence of pulmonary fibrosis whereas relatively few patients with PBD are likely to develop pulmonary fibrosis. Although the *G*C haplotype appears to protect against the development of PBD, it does not prevent the development of fibrosis once the disease is established, suggesting that the association in PBD is likely to relate to earlier inflammatory processes in PBD rather than to the fibrotic reaction. Furthermore, the percentage of lymphocytes in the BAL fluid of subjects with PBD appears to be strongly genotype dependent. Lymphocytosis is a recognised feature of PBD and the percentage of BAL lymphocytes may reflect the degree of lung tissue inflammation. Immediately after antigen challenge there is an influx of neutrophils into the alveoli and this is followed by an influx of activated T cells, activation of alveolar macrophages, and the release of proinflammatory cytokines including tumour necrosis factor (TNF)-α and interleukin 1.[2] Subjects with the *G*C haplotype appear to have proportionally fewer lymphocytes in their cell count than those with the common *A*T haplotype. TIMP-3 may therefore act in the earlier inflammatory stages of PBD and possibly through an action that is not by inhibition of MMPs.

TIMP-3 is the only TIMP that binds tightly to the ECM,[4 13] regulating movement through the basement membrane. Interestingly, it has recently been suggested that a balance between MMPs and TIMPs determines the migratory

Table 5 Relationship of TIMP-3 haplotypes with lung function, BAL cell profile, and outcome in subjects with pigeon breeders' disease (PBD)

	TIMP-3 haplotype		
	*A*T	*G*C	p value
Age	44.1 (13.3)	43.5 (14.5)	0.77
FEV_1 (% predicted)	59.5 (20.0)	61.3 (19.7)	0.49
FVC (% predicted)	56.4 (20.8)	56.8 (19.4)	0.75
Pa_{O_2} (kPa)	7.1 (1.4)	7.0 (1.3)	0.70
BAL lymphocytes (%)	63.9 (20.3)	55.0 (21.4)	0.029
BAL macrophages (%)	33.6 (20.1)	43.0 (20.3)	0.016
BAL neutrophils (%)	1.4 (2.5)	1.2 (3.4)	0.49
BAL eosinophils (%)	0.8 (1.0)	0.7 (1.2)	0.65
% of subjects with haplotype having a poorer outcome	41.1	41.0	0.83

FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; Pa_{O_2} = arterial oxygen tension; BAL = bronchoalveolar lavage.
Lung function and BAL cell profile data are expressed as mean (SD). The effect of haplotype on the data was determined using stepwise regression analysis with age and smoking as covariates.

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capacity of human dendritic cells and, moreover, that TIMP-3 is significantly increased in mature dendritic cells.[26] Since these and other antigen presenting cells are essential in the initiation of the immune response, changes in the MMP/TIMP balance may affect this reaction. TIMP-3 is also capable of inhibiting members of two groups of enzymes within the adamalysin family. Examples include the enzyme TACE (TNF-cleaving enzyme, ADAM 17)[27] and some members of the ADAMTS (ADAM with thrombospondin-like repeats) group responsible for aggrecan degradation in cartilage.[28] Inhibiting TACE promotes cell apoptosis through stabilisation of the TNF-α receptors.[27] Overexpression of TIMP-3 also induces Fas-associated apoptotic cell death.[29] Patients with hypersensitivity pneumonitis exhibit increased levels of Fas and Fas ligand in BAL fluid and an upregulation of both proteins in lung tissues.[30] We have previously shown that a promoter variant in TNF-α (–308G>A) influences the susceptibility to PBD.[17] The −308A allele, associated with raised constitutive and inducible levels of TNF-α,[31] was significantly increased in frequency in subjects with PBD compared with healthy subjects. It is possible that the TIMP-3 variants affect susceptibility to PBD by acting on the TNF-α converting enzyme. This remains to be confirmed as the functional cause of the TIMP-3 variants associated with PBD has yet to be determined.

In summary, this study provides evidence that genetic variants in TIMP-3 contribute to susceptibility to PBD. The effect is not confounded by fibrosis so the probable cause of the association probably relates to earlier inflammatory processes in this disease.

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