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ARTICLE

An alpha$_1$-antitrypsin enhancer polymorphism is a genetic modifier of pulmonary outcome in cystic fibrosis

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Lung disease is the direct cause of death in over 90% of cystic fibrosis (CF) patients. Excess neutrophil elastase is an important determinant of pulmonary disease in CF. α_1-antitrypsin (AAT), also known as α_1-proteinase inhibitor (α_1PI) is a major modulator of elastase activity. We investigated the hypothesis that an enhancer polymorphism in the AAT gene would contribute to pulmonary prognosis in CF. Respiratory function, chest X-ray scores, bacterial colonisation and infective exacerbation were assessed to evaluate pulmonary disease severity in the CF group. Sixteen patients were found to have the 1237A allele, and 108 the more frequent G allele. Contrary to expectation, the patients with the 1237A allele were found to have better indices of pulmonary disease progression than those without, as indicated by less change in X-ray score (1237A: 0.2$\pm$0.1; 1237G: 1.2$\pm$0.1; *P*=0.002) and fewer infective exacerbations (1237A: 2.8$\pm$0.6; 1237G: 4.6$\pm$0.3; *P*=0.03) over the preceding 2 years. Also, a higher proportion of the 1237A (25%) than the 1237G (6.5%) were not colonised by Pseudomonas Aeruginosa (*P*=0.04). Prospective monitoring of infections for a further 2 years confirmed a lesser propensity to infection in patients with the 1237A allele. These trends were also observed in a tightly matched sub-set of CF genotypes of similar age and sex, thus confirming that these effects were independent of the CF genotype. These results indicate that this AAT enhancer polymorphism is associated with better pulmonary prognosis in CF. Though the number of CF patients with the polymorphism is small, and these data need to be confirmed in larger studies, they suggest that a cautious approach should perhaps be taken to treatment of CF patients with supplemental AAT. *European Journal of Human Genetics* (2001) **9,** 273–278.

Keywords: α_1-antitrypsin enhancer polymorphism; pulmonary outcome; cystic fibrosis

Introduction

Lung disease is the direct cause of death in over 90% of cystic fibrosis (CF) patients.[1] The disease is typically characterised by chronic low grade infection with acute exacerbations. During infections the inflammatory response is dominated by an influx of neutrophils, and this results in the release of large quantities of neutrophil elastase (NE). NE is thought to be a major contributor to the lung damage observed, although it may have a beneficial effect in acute infections.[2] There is significant variation in lung disease severity among CF patients and studies suggest that genetic factors unrelated to mutations within the cystic fibrosis transmembrane regulatory (CFTR) gene locus are likely contributors to this variation.[3]

α_1-antitrypsin (AAT) inhibits several serine proteinases, including neutrophil elastase (NE), its principle target. The inhibitor is a true acute phase reactant, with plasma

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concentrations increasing 2–3-fold following inflammation.[4] The main site of synthesis is the liver, but during inflammation it is also produced by activated inflammatory cells, including blood monocytes and tissue macrophages.[5]

The well-described association of genetic deficiency of AAT to early onset emphysema[6] demonstrates an important physiological role for AAT in protecting the lower respiratory tract from NE induced injury. This has led to the development and trial of supplemental AAT therapy not only for emphysema but also for other lung diseases in which excessive NE activity is implicated, particularly cystic fibrosis (CF).[7]

A G→A polymorphism present 1237 bases downstream from the end of the last exon has previously been shown by transfection studies to result in diminished reporter gene expression in response to the acute phase cytokine, interleukin-6.[8] These data, together with studies investigating the interactions between transcription factors in the enhancer where the mutation is located suggest that the mutation may compromise the normal AAT acute-phase response, and these individuals would have an impaired response to infection.[8]

Against this background, the primary aim of the current study was to examine the hypothesis that the AAT 3′ enhancer polymorphism is associated with greater pulmonary disease severity in CF.

Materials and methods

Blood samples were collected from 124 white Caucasian adult CF patients (mean age=23.6, range 17–40 years; 64 M/60 F) who had no evidence of acute deterioration requiring back-up antibiotic therapy at the time of the sampling. The patients were therefore all stable with respect to their disease. All of the CF group were non-smokers with the exception of two individuals. Samples were also collected from 114 healthy control subjects matched to the CF group with respect to age (22.6, 20–32 years), sex (51 male) and ethnic background. With the exception of 13 individuals in the control group who were current smokers, all the remainder were non-smokers and had no family history of CF. All participants gave their informed consent and the study was approved by St. Vincent's Hospital ethics committee.

Assessment of pulmonary disease severity

Pulmonary function was assessed by spirometry and expressed as per cent predicted FEV1 (forced expiratory volume in 1 s) and FVC (forced vital capacity) normalised for age and sex. Severity of disease as indicated by chest roentgenogram (CXR) was assessed using the Brasfield scoring system[9] in which the highest score of 25 indicates a clear CXR and low scores indicate significant pulmonary disease. Retrospective blind analysis of pulmonary function and CXRs from 2 years prior to the reference visit was undertaken by two reviewers and the number of infective exacerbations of pulmonary disease which required supplemental antibiotic therapy over that 2 year period was recorded from patient files and confirmed by direct patient interview. The presence of pancreatic disease and diabetes mellitus was also scored to reflect the severity of disease. Prospective monitoring for a further 2 year period was carried out in 14 of the 16 patients with the 1237A allele and in 84 of the 1237G subjects. The remaining subjects in both groups were not available for prospective follow up but all of the subjects were included in the retrospective analysis. Recording of the follow-up data was done by a clinician who was not aware of the genotype status of the patients.

Genotyping for the 1237A polymorphism

DNA was extracted from peripheral blood leukocytes using QIAGEN QIA amp blood kits (Qiagen Ltd., Crawley, UK) by standard techniques. Amplification of the region containing the 3′ enhancer polymorphism was carried out using 30 pmol of each oligonucleotide primer (forward: 5′-AGTGCCACGGCAGGATGTT and reverse: 5′-GGCTTGGTGATGGCCATATCTT) to produce a fragment of 431 bp.

The 50 μl amplification reaction also contained dATP (200 μM), dCTP (200 μM), dGTP (200 μM), dTTP (200 μM), Taq polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KC1, 0.08% Nonidet P40), $MgCl_2$ (2.5 mM) and Taq polymerase (1 unit). PCR was conducted under the following conditions: 95°C (denaturation) for 1 min, 62°C (annealling) for 1 min and 72°C (extension) for 1 min, for a total of 35 cycles.

The size of the resulting PCR product was verified on 1.5% agarose gels with ϕX 174 markers. The 431 bp product was then digested with the *Taq*I restriction enzyme (T_CGA) overnight, resulting in a specific banding pattern dependent on the sample genotype. The 1237G allele produced two fragments of 275 bp and 156 bp, whereas the 1237A allele produced a single, undigested, 431 bp fragment. Digestion fragments were separated and visualised by ethidium bromide staining on 2.5% agarose gels.

Genotyping for the PIS and PIZ was also carried out with the following primers: SF (forward) 5′-CCTGATGAGGGGAAACTACAGCACCTCG and SR (reverse) 5′-CAGTCCCAACATGGCTAAGAGGTG for the S variant, producing a 146 bp product and ZF (forward) 5′-GCCGTGCATAAGGCTGTGCTGACCATCGTC and ZR (reverse) 5′-TTGAGGAGCGAGAGGCAGTT for the Z variant, producing a PCR product of 210 bp (in each instance the base underlined indicates the site which differentiates S/Z mutants from those with the normal PI M genotype). Fifty-microlitre PCR reactions were run containing the following components: DNA (50 ng), forward primers (35 pmole), reverse primers (35 pmole), dATP (200 μM), dCTP (200 μM), dGTP (200 μM), dTTP (200 μM), Taq polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P40), $MgCl_2$ (1.5 mM) and Taq polymerase (1 unit). PCR was conducted under the following conditions: 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, for a total of 35 cycles. The respective PCR products were then digested overnight at 55°C, with *Taq*I restriction endonuclease (8 units) in *Taq*I

buffer (1 mM Tris Hcl pH 8.5, 1 mM $MgCl_2$, 10 mM KCl). The resulting digests with 100 bp markers were separated on a 3% agarose gel as previously described.[10] The genotyping yielded 119 bp and 27 bp products for the M allele and the S allele yielded a single, undigested, 146 bp product. The Z genotyping yielded 181 bp and 29 bp products for the M allele and an undigested 210 bp product for the Z allele.

CF genotyping

This was undertaken by the Clinical Genetics Department, City Hospital, Nottingham, UK, using a commercially available kit to detect up to 20 mutations (Cellmark Diagnostics, Abingdon, UK).

Plasma AAT

Plasma AAT was measured by enzyme-linked immunosorbent assay as described previously.[11]

Statistical analysis

The genotype frequencies were tested for Hardy-Weinberg equilibrium. All data is expressed as mean $\pm$ SEM with ranges where appropriate. The Mann-Whitney non parametric *U* and Fisher's exact tests were used to compare characteristics between groups. The characteristics of interest were CXR score, Pseudomonas colonisation and FEV% predicted at time of sampling; changes in CXR score, FEV% predicted and number of infections over the preceding 2-year period and changes in FEV% predicted and number of infections in the subsequent 2-year period. Multiple regression analysis was carried out to assess the impact of age, sex, CFTR genotype, presence of pancreatic disease, liver disease and diabetes on each of these outcomes. In addition to comparisons made within the full study group, statistical analysis on measures of pulmonary disease was also carried out between CF patients with and without the 1237A polymorphism tightly matched for age, sex and CFTR genotype, and all of whom had no history of cigarette smoking. In all instances a two-tailed *P* value <0.05 was considered significant.

Results

The overall prevalence of the enhancer 1237A polymorphism in the CF group (*n*=16, 13%; 2AA, 14GA) did not differ from that in the matched control group (*n*=17, 15%; 2AA, 15GA), and in both groups the genotypes did not deviate from Hardy-Weinberg equilibrium. The prevalence of the 1237A polymorphism did not differ with respect to age or sex in either the control or CF populations. Within the CF group there was no significant difference in the prevalence of the DF508 CFTR genotype between those with and without the 1237A polymorphism (Table 1) nor was the occurrence of any other CFTR allele more or less frequent in either group. To further exclude CFTR genotype effects on pulmonary outcome, a sub-set analysis of tightly controlled CF genotypes was undertaken (see below). All except two of the study patients were pancreatic enzyme insufficient, and the two exceptions were homozygous G for the enhancer polymorphism. There was no significant difference between the patient groups with respect to the presence of liver disease or diabetes mellitus.

Table 1 Characteristics of control and CF groups with A and G variants of the AAT 1237 3′ enhancer polymorphism

	1237G group	*1237A group*
Control group		
N (%)	97 (85%)	17 (15%)
Mean age (range) years	22.6 (20–30)	22.7 (20–32)
Gender	55F/42M	8F/9M
CF group		
N (%)	108 (87%)	16 (13%)
Mean age (range) years	23.9 (17–40)	21.8 (18–29) NS
Gender	50F/58M	9F/7M
CFTR genotype:		
Homozygous DF508	67 (62%)	7 (44%)
Heterozygous DF508[a]	39 (36%)	8 (50%)
Other[a]	2 (2%)	1 (6%)

[a]The differences in CFTR allele frequency between the polymorphism groups was not significant. Non DF 508 alleles in the two groups were: 1237A group: G551D (3); N1303K (2); R117H (1); R560T (1); Unknown (3). 1237G group: G551D (10); R117H (3); R560T (3); D1507 (2); E60X (2); N1303K (1); 1717-1 (1); 621H (1); G542X (1); POL 400 (1); R352Q (1); RT0F (1); 621+G>T (1); Unknown (15). NS=not significant.

Lung disease progression, as assessed by change in Brasfield chest X-ray score over 2 years prior to sampling, was markedly lower in the 16 patients with the 1237A polymorphism compared to the 1237G group (*P*=0.002, Table 2). This was also reflected in a difference in chest X-ray score between the two groups at time of sampling, although this did not quite reach statistical significance when adjusted for effects of age, sex, CFTR genotype, liver disease and diabetes. Patients with the 1237A polymorphism also had a significantly lower number of clinically relevant respiratory infections over the same 2-year period, compared to the 1237G group (*P*=0.03). Even though this was an adult CF poplulation, 25% of those positive for the 1237A polymorphism were not colonised with Pseudomonas aeruginosa compared to only 6.5% of the 1237G group (*P*=0.04). At the reference visit, pulmonary function did not differ significantly between the groups, although the tendency was towards better function in those carrying the 1237A polymorphism (Table 2).

Assessment of the full study group indicated that the differences observed between the 1237A and 1237G groups were not attributable to the CFTR genotype (Table 1). To further examine this, comparison of CXR, pulmonary function and number of infections was also carried out in the 16CF patients with the 1237A polymorphism and a tightly-matched sub-set of the 1237G group, matched for age, sex and CFTR genotype relationship between the

enhancer polymorphism and pulmonary function, CXR score and number of infections a comparison was also carried out in the 16 CF patients with the 1237A polymorphism and a tightly-matched sub-set of the 1237G group, matched for age, sex and CFTR genotype. Over a 2-year period two patients were not available for the 4-year follow-up, and comparison for the latter were therefore restricted to 14 patients in each group. For subjects heterozygous for DF508, the second allele was matched as closely as possible and included the following: G551D, N1303K, R117H and R560T. All the differences attributable to the effect of the polymorphism on analysis of the full study group were confirmed in the tightly matched sub-set (Table 3).

When assessed prospectively 2 years following the reference visit, per cent predicted FEV1 was significantly better in patients with the 1237A polymorphism (Figure 1). During the follow-up monitoring period, the difference in number of infective exacerbations between the two groups was also maintained. In all, over the 4-year evaluation period, those with the 1237A polymorphism had an average of 4.5

Table 2 Measures of pulmonary disease severity on CF patients with and without the AAT-3′ enhancer polymorphism

	1237G group (n=108)	*1237A group* (n=16)	*Actual* P *value*[a]	*Adjusted* P *value*[b]
CXR				
Score at sampling	15.4±0.3 (9–23)	17.5±1.0 (9–23)	0.04	0.055
Change in score over 2 years	1.2±0.1 (0–5)	0.2±0.1 (0–1)	0.0004	0.002
Infection				
Patients not colonised with *Pseudomonas Aeruginosa*	7 (6.5%)	4 (25%)	0.04[c]	
No. of infections over 2 years	4.6±0.3 (0–20)	2.8±0.6 (0–10)	0.02	0.03
Pulmonary function				
FEV_1 % predicted	58.6±2.4 (18.5–123)	67.5±5.5 (28–109)	NS	NS
Change in FEV_1 % over 2 years	10.7±1.4 (0–63)	9.3±3.5 (0–49)	NS	NS

Values are expressed as means±SE (range). [a]Actual *P* values were assessed by Mann Whitney test except where otherwise indicated. [b]Adjusted *P* values were assessed by multiple regression adjusting for age, sex, liver disease and diabetes. [c]Fisher's Exact test was used to assess differences in occurrence of Pseudomonas infection between groups. NS=No significant difference between groups.

Table 3 Characteristics of matched CF groups with G and A variants of the AAT 1237 3′ enhancer polymorphism

	1237G group	*1237A group*	P *value*
n	16	16	
Mean age (range) years	21.9 (18–28)	21.8 (18–29)	
CFTR genotype:			
Homozygous DF508	7	7	
Heterozygous DF508[a]	8	8	
Other[a]	1	1	
Change in CXR score	1.5±0.3	0.2±0.1	0.002
No. infective exacerbations			
over 2 years	4.7±0.7	2.8±0.6	0.03
over 4 years[b]	10.5±1.8	4.5±1.1	0.006
FEV1 % predicted at reference visit	55.5±7.4	67.5±5.5	NS
2 years post reference visit[b]	53.1±8.5	68.4±5.5 (*n*=14)	0.09 (NS)

[a]Non DF 508 alleles in the two groups were: 1237A group: G551D (3); R117H (1); R560T (1); N1303K (2); Unknown (3); 1237G group: G551D (4); R117H (1); R560T (1); 621+G>T (1); Unknown (3). [b]Fourteen of the 16 patients in the 1237A Group were available for follow-up. For 4-year comparisons, 14 patients with 1237A were matched with 14 patients with 1237G.

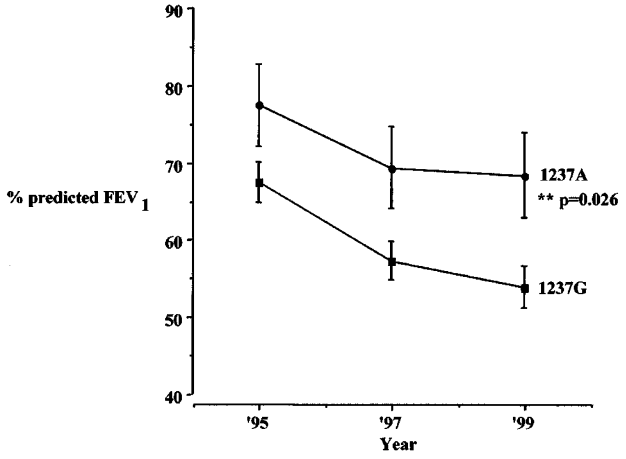

Figure 1 Change in % predicted FEV in 84 CF patients with the G and 14 A variants of the 1237 3′ enhancer polymorphism over a 4-year period. On final evaluation % predicted FEV was significantly better in patients with the 1237A polymorphism. **P=0.026.

($\pm$1.1) exacerbations, compared to 10.5 ($\pm$1.8; P=0.006) in the 1237G group. This decrease in pulmonary symptoms was not, however, associated with an increase in overall survival as one patient with the 1237A polymorphism (the eldest of the group) died during the 2-year period of prospective monitoring.

There was no difference in plasma AAT concentrations between CF patients with and without the 1237A polymorphism at the time of sampling (1237G group: mean $\pm$ SD 1.7 $\pm$ 0.1, range 0.4–3.3 gm/l; 1237A group: 1.6 $\pm$ 0.1, 1.1–2.6 gm/l. Assay c.v. 9.8%) confirming that these patients did not have an acute infection when sampled. On analysis for AAT deficiency genotypes, 12 CF patients were found to have the PI MS genotype and two were PI MZ. The prevalence and distribution of AAT PI variants was no different from that in the control group where 16 individuals had either an S or Z mutation, comprising 11 MS, two MZ, two SZ and one SS genotype. Within the CF group, two of the 16 patients with the 1237A polymorphism had an MS genotype and the rest were PI MM. Similarly, two control individuals with the 1237A polymorphism also had PI deficiency genotypes, one MS and one MZ. No association was observed between PI deficiency (MS and MZ) genotypes and severity of respiratory disease in the CF population. As expected, serum AAT concentrations were significantly lower in those with PI MS and PI MZ deficiency genotypes (1.0 $\pm$ 0.1; 0.6 $\pm$ 1.4 g/l) compared to those with PIMM genotype (1.7 $\pm$ 0.05; 0.35–3.3 g/l; P<0.0001).

Discussion

In this study we show that the presence of an AAT 3′ enhancer polymorphism is associated with better pulmonary prognosis in CF. The trend was observed for a number of indices, including deterioration in chest X-ray score, frequency of infective episodes and decline in lung function. As the prevalence of the 1237A polymorphism in the CF group and in a control group matched for age, sex and ethnic background are in Hardy-Weinberg equilibrium it is unlikely that the association between the polymorphism and less progressive pulmonary disease in CF is influenced by a selection bias for or against the polymorphism in CF. Our control and CF populations were carefully matched, as the allele frequency of the AAT 1237A enhancer polymorphism shows significant variation in different population groups, ranging from 0.025 to 0.235.[12] The association between the 1237A polymorphism and better pulmonary prognosis was also observed on analysis of a sub-set of the CF group matched for age, sex and CFTR genotype.

An association between AAT PI S and Z deficiency mutations and less severe pulmonary disease in CF has also recently been reported.[13] These authors also screened for the 3′ enhancer polymorphism and found no relationship between it and static estimates of pulmonary disease severity, although a trend towards improved respiratory function in patients with the 1237A polymorphism was noted. This is consistent with our findings which indicate that measures of disease progression over time are those most strongly associated with the enhancer polymorphism. Given the individual variation in pulmonary disease within the CF population, static or single time-point measurements are less likely to detect influencing factors than sequential measurements on the same patients over time. Differences between the two studies may also reflect differences in the CF groups studied. All patients in our study were recruited from a single CF centre and, to minimise the major influence of age on disease in CF, only adult patients were selected. In the study by Mahedeva *et al*[13] the cohort of 147 patients ranged in age from 3 to 50 years.

We were unable to confirm the association between better pulmonary function and PI S/Z mutations noted by Mahedeva *et al* in our CF population, either as static measurements or as disease progression. This may also be related to the different groups studied. In selecting only adult patients for our study it is probable that, overall, our patients represent a population with milder disease than that studied by Mahedeva *et al* and that, as a result, the influence of the deficiency mutations is not as observable. Larger studies spanning the full range of CF lung disease are required to comprehensively address this issue. It is of note, however, that both studies show that decreased levels of AAT are not associated with more severe disease in CF, as might be expected in light of the proposed deleterious effect of NE in CF lung disease. This is also consistent with another study of CF patients by Doring *et al* which found that those with mild to moderate AAT deficiency arising from PIS or PIZ mutations had no increased risk of more severe lung disease.[14] As in the

present study the initial hypothesis in this study was based on a more severe outcome.[14]

Although overall survival is not increased, our results do suggest that the enhancer polymorphism 1237A is associated with better pulmonary prognosis in CF. However, larger studies are needed to confirm these observations and it is theoretically possible that the polymorphism is in linkage disequilibrium with a mutation in a region flanking the AAT gene. Transfection studies indicate that presence of this polymorphism results in a reduced ability to upregulate AAT production in response to IL-6 stimulation.[8] Preliminary *in vivo* and *in vitro* studies also suggest that the effect of the native 1237A variant is to decrease the AAT response to infections and stimulation by other cytokines[15] and (unpublished observations).

While the explanation for an association between the 1237A polymorphism and better pulmonary prognosis in CF requires further study it is of interest to speculate that decreased AAT production may reflect a beneficial role for NE in the host response to acute infection.

That NE can play such a role is supported by a recent report that NE knock-out mice have an impaired host defence against gram negative bacterial sepsis.[16] A previous study on CF patients also noted a potential beneficial effect on NE in acute infections.[2] A direct bactericidal effect of NE has been demonstrated[17] which, in the case of *E. Coli*, has recently been shown to involve proteolytic cleavage of SpA from the bacterial cell wall.[18] There may, therefore, be a dual effect of NE on lung disease in CF, where its activity is beneficial in clearing bacteria during acute infection while its presence during the chronic phase of disease may exacerbate ongoing lung damage. It is also theoretically possible that the 1237A polymorphism is in linkage disequilibrium with a region of flanking DNA, and another neighbouring gene may influence this outcome.

In summary, this study demonstrates that CF patients who carry the AAT 3′ enhancer polymorphism 1237A, have an improved pulmonary prognosis. This would suggest that treatment of CF patients with supplemental AAT should be cautiously assessed, particularly in the context of acute infection.

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