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A preliminary assessment of alpha-1 antitrypsin S and Z deficiency allele frequencies in common variable immunodeficiency patients with and without bronchiectasis

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SUMMARY

Common variable immunodeficiency (CVID) is the name given to a clinically heterogeneous group of hypogammaglobulinaemic immunodeficiency states. Bronchiectasis is a feature of this disease and is believed to be the result of recurrent bacterial infection affecting the respiratory tract. Bronchiectasis is also a feature associated with emphysematous changes of the lung in alpha-1 antitrypsin (AAT) deficiency, a serious and relatively common disease, affecting 1 : 2000 in the United Kingdom. This has been demonstrated to result from possession of deficiency alleles, the most clinically important alleles being PI*Z and PI*S. Isolated reports of families with antibody deficiency and AAT deficiency have been published but to date no study has been performed to specifically investigate if AAT deficiency is associated with the lung damage seen in CVID patients. We have developed a PCR genotyping assay that identifies S and Z deficiency alleles and we have used this assay in a preliminary study to investigate the occurrence of these deficiency alleles of AAT in 43 CVID patients. Results of this preliminary study suggest that CVID patients did not have an altered distribution of AAT genes when compared to 70 normal controls. Subgrouping of CVID patients into those with and without bronchiectasis demonstrated a Z allele frequency of 0·077 in those patients with bronchiectasis, which is higher than found in normal controls, namely 0·029 ($P < 0·15$). Due to the relatively small numbers studied, these results are inconclusive in determining whether AAT deficiency may exacerbate lung damage in some CVID patient, the data does however, indicate that a larger multi-centre study involving many more CVID patients may be useful.

Keywords alpha-1 antitrypsin bronchiectasis common variable immunodeficiency S and Z deficiency alleles

INTRODUCTION

Alpha-1-antitrypsin is an acute phase protein produced in the liver, which limits the damage done to self-tissue during an inflammatory immune response. In particular, it has an inhibitory effect on leucocyte elastase produced by activated neutrophils [1]. Deficiency is associated with possession of mutant forms of the gene, the most clinically important forms being PI*Z and PI*S. Homozygosity for the PI*Z allele produces a number of symptoms of the deficiency disease, including emphysema [2] and liver cirrhosis [3]. Possession of the PI*S allele is a risk factor for disease only when the patient has the heterozygous genotype PI*SZ.

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The role of alpha-1 antitrypsin deficiency in the development of bronchiectasis is controversial. A number of studies have suggested that bronchiectasis results from homozygous alpha-1 antitrypsin (AAT) deficiency [4–6]. One of these, a study of 202 patients with bronchiectasis classified according to AAT phenotype, included those with bronchiectasis associated with many diseases (post-tuberculosis, rheumatoid arthritis, respiratory infections during childhood, chronic lymphocytic leukaemia, cystic fibr osis, pulmonary fibrosis and immunoglobulin (Ig) deficiency), but did not look at associations with the individual diseases [5].

Common variable immunodeficiency (CVID) comprises a clinically heterogeneous group of hypogammaglobulinaemic immunodeficiency states. Patients are susceptible to recurrent bacterial infection affecting the respiratory tract [7], have an increased incidence of other infections, lymph node and splenic follicular hyperplasia, autoimmune disorders [8] and

non-caseating granulomas [9]. Attempts at subgrouping the disease have been made but as yet only loose associations have been found based on *in vitro* immunological responses [10–13]. AAT deficiency has been reported in two patients with common variable immunodeficiency [14,15]. Because the development of bronchiectasis is a frequent occurrence in CVID (up to 50% of patients), we have used a recently developed polymerase chain reaction (PCR) genotyping assay (alongside the established phenotyping method) to investigate a possible association between AAT deficiency and CVID with bronchiectasis.

PATIENTS AND METHODS

Patients

Forty-three Caucasian CVID patients, meeting international (PAGID and ESID) diagnostic criteria [16], were ascertained from the immune deficiency clinic in Oxford. None of the CVID patients examined in this study had any other immunodeficiency disorder, such as X linked hyper-IgM syndrome (HIGM1), autosomal recessive hyper-IgM or X-linked agammaglobulinaemia (XLA).

Seventy healthy Caucasian controls were also included. Controls were normal healthy non-immunodeficient adults taken from the tissue typing laboratory and immunology laboratories in Oxford. Non-Caucasian subjects were excluded, as both S and Z phenotypes are rare among non-European populations [17]. An estimate of the statistical power of the study suggested that 40 patients should be suudied, in order to detect a significant (CI 95%) difference between cases and controls in the frequency of the Z gene at the $P < 0{\cdot}05$ value [5].

Genotyping by SSP PCR

AAT genotype, in terms of S and Z alleles, was assessed using a sequence specific primer (SSP)-based PCR technique (ME Sansom, BL Ferry, Z Sherréll, HM Chapel, S Misbah, manuscript in preparation). Briefly, samples were amplified using control and allele specific primers in TDMH with 5% acetamide by 5 μl/ml BioTaq™ DNA polymerase (Bioline, Abingdon, UK). Samples were overlaid with 20 μl of mineral oil (Sigma, St Louis, MO, USA) before being amplified using a MJ Research Inc. (Felsted, UK) PTC-100™ thermocycler. The following cycling conditions were used; 2 min at 96°C, 30 cycles of 25 s at 96°C, 60 s at 64·5°C, 60 s at 72°C and 2 min at 72°C.

PCR primers

Primers were designed based on the *PI*S*, and *PI*Z* gene sequences [18,19], GenBank sequence K02212 (S variant) and J02619 (Z variant). The primers were checked and confirmed for specificity using BLAST (Basic Local Alignment Search Tool). The site of the genetic lesions for the *PI*S* and *PI*Z* alleles are within codon 264 (GAA→GTA) and codon 342 (GAG→AAG), respectively. Two primers for each allele were designed based on the polymorphic site. The base at the 3′ end of these primers determined which allele was recognized as amplification by BioTaq™ DNA polymerase under the stringent conditions of this assay was dependent on an absolute match at this site. The primers produced amplicons of 221 base pairs for the S region and amplicons of 288 base pairs for the Z regions. The primers had the following sequences: sense Z was 5′-GCTGTGCTGACCATCGACA-3′, sense non-Z was 5′-GCTGTGCTGACCATCGACG-3′. antisense Z/non-Z was 5′-CCAGGGATTTACAGATCACATGC-3′. antisense S was 5′-ATGATATCGTGGGTGAGTTCATTTA-3′, antisense non-S was 5′-ATGATATCGTGGGTGAGTTCATTTT-3′ and senseS/non-S was 5′-GAAGTCAAGGACACCGAGGA-3′. Two control primers were included in the primer mix for each reaction well to demonstrate that the PCR was successful. The control band had an amplicon size of 796 base pairs and represented a nonpolymorphic region of an exon 3 sequence in HLA-B [20]. Control primers used were 5′-TGCCAAGTG GAGCACCCAA-3′ and 5′-GCATCTTGCTCTGTGCAGAT-3′. Primers were obtained from Oswel DNA services (Southampton, UK).

Gel electrophoresis of PCR products

Samples were mixed with 5 μl of loading buffer (Orange G) and run in 1% agarose/0·5× TBE gels with 1 μl/100 ml ethidium bromide on a Flowgen submarine electrophoresis system. Gels were electrophoresed for 40 min at 240 V/100 mA and visualized on an ultraviolet transilluminator and photographed with a Polaroid camera [20].

Phenotyping of AAT proteins

Phenotyping was performed by isoelectric focusing based on current protocols used at the Protein Reference Unit, St Georges Hospital, Tooting, UK [17].

Statistical analysis

Statistical analysis was performed using a two-tailed Fisher's exact test (GraphPad Prism® 3·0, GraphPad Software Inc., San Diego, CA, USA). Some of the cell frequencies were small enough!to obviate the use of the χ^2 tert as a reasonable approximation to the exact distribution of the test statistic. The frequency of each allele was calculated by gene counting (number) according to Hardy–Weinberg theory.

Lung status

Bronchiectasis was assessed in CVID patients by expert radiologists at the Department of Radiology, Oxford Radcliffe Hospital, by CT scanning if indicated clinically due to recurrent chest infections ($n = 33$); in other patients without an indication for CT by chest X-ray only ($n = 10$). Serum alpha-1 antitrypsin levels were measured within the routine laboratory, Immunology Department, The Churchill Hospital, Oxford using nephelometry on a Beckman Array™ Protein System.

RESULTS

Characteristics of CVID patients

Table 1 shows details of 43 CVID patients. Table 1 shows the age at presentation and the blood serum presentation levels of IgG, IgA and IgM. In all 43 patients, bronchiectasis was confirmed or excluded by CT scan or X-ray. Thirty-three patients had CT scanning performed and 10 had an X-ray performed. As stated in Materials and methods, patients attending the immunology clinic had CT scanning performed only where it was clinically indicated due to recurrent chest infections. In other patients, a chest X-ray was performed. Twenty-six patients had bronchiectasis and no clinical evidence of bronchiectasis was found in 17 patients. The numbers of smokers/ex-smokers were distributed evenly between bronchiectasis and non-bronchiectasis patients (Table 1).

Table 1. Characteristics of CVID patients

Patient no.	Age at presentation (years)	IgG (g/l)	IgA (g/l)	IgM (g/l)	Smoker[1]	Bronchiectasis	Radiological method[2]	AAT level mg/dL	Genotype[3]	Phenotype[4]
1	32	1·1	0·1	0·3	Cigar	Yes	CT	140	S-Z+	MZ
32	62	3·7	0	0·3	Ex	No	XR	148	S-Z-	MM
35	38	3·1	0·1	0·13	Ex	No	XR	150	S-Z-	MM
17	60	5·3	0·3	1·2	Ex	No	CT	143	S-Z-	MM
41	49	2·1	0·07	0·7	Ex	No	CT	179	S-Z-	MM
23	49	1·8	<0·1	0·3	Ex	Yes	CT	151	S-Z-	MM
14	56	2·4	<0·1	0·1	Ex	Yes	CT	176	S-Z-	MM
7	36	0·35	UNDT[5]	0·16	Ex	Yes	CT	214	S-Z-	MM
16	22	0·8	0·2	0·3	Ex	Yes	CT	120	S-Z+	MZ
25	58	0·2	0	0·2	Ex	Yes	CT	109	S-Z-	MM
5	71	5·1	<0·1	1	Never	Yes	CT	199	S-Z-	MM
0	44	3·9	<0·1	0·9	Never	No	XR	93·5	S-Z-	MM
21	16	0·4	0·4	0·5	Never	No	XR	159	S-Z-	MM
27	54	4·7	1·5	0·55	Never	No	XR	176	S-Z-	MM
33	28	3·5	<0·05	0·3	Never	Yes	CT	188	S-Z-	MM
40	7	5·3	0·13	0·36	Never	No	XR	250	S-Z-	MM
10	42	0·5	UNDT	0·2	Never	No	CT	145	S-Z-	MM
12	39	1·6	0·05	0·3	Never	No	CT	200	S-Z-	MM
30	18	3	0·48	0	Never	No	CT	132	S-Z-	MM
11	34	4·4	<0·1	0·6	Never	No	XR	121	S-Z-	MM
36	35	3·2	<0·5	<0·3	Never	No	XR	155	S-Z-	MM
13	52	<1·0	<0·3	<0·2	Never	Yes	CT	232	S-Z-	MM
24	63	2·3	<0·1	0·17	Never	Yes	CT	259	S-Z-	MM
2	28	1·6	<0·2	0·77	Never	Yes	CT	170	S-Z-	MM
3	42	4·4	UNDT	0·17	Never	Yes	CT	109	S-Z-	MM
6	18	0·3	<0·1	<0·1	Never	Yes	CT	178	S-Z-	MM
8	26	<0·2	UNDT	UNDT	Never	Yes	CT	152	S-Z-	MM
9	8	2·3	0·03	0·12	Never	Yes	CT	146	S-Z-	MM
15	35	4·5	<0·1	0·85	Never	Yes	CT	243	S-Z-	MM
18	5	2·7	0·3	0·4	Never	Yes	CT	80	S-Z+	MZ
22	26	2·7	0	0·2	Never	Yes	CT	124	S-Z-	MM
28	25	0·5	<0·1	0·3	Never	Yes	CT	264	S-Z-	MM
31	50	0·44	0	0·3	Never	Yes	CT	144	S-Z-	MM
37	45	<0·5	<0·1	0·1	Never	Yes	CT	204	S-Z-	MM
39	71	0·3	UNDT	0·1	Never	Yes	CT	52·1	S + Z+	SZ
42	7	4	<0·1	0·1	Never	Yes	CT	196	S-Z-	MM
43	19	1·5	0·2	0·2	Never	Yes	CT	280	S-Z-	MM
29	48	<0·3	<0·07	<0·16	Never	Yes	CT	215	S-Z-	MM
34	27	<0·1	0·4	0·15	Never	No	XR	169	S-Z-	MM
19	33	3·8	ND	0·4	Never	No	XR	74	S+Z-	MS
26	23	<5	0·6	0·5	Yes	No	CT	118	S-Z-	MM
38	54	2·3	0·2	0·4	Yes	Yes	CT	232	S-Z-	MM
4	27	2·7	0·2	0·5	Yes	No	CT	169	S-Z-	MM

Details of 43 CVID patients. Age at presentation (years), presenting serum levels of IgA, IgG and IgM (g/l). Their individual status as a smoker, an ex-smoker or non-smoker is shown. The presence or absence of bronchiectasis in each patient is given and method of detection (CT scan or X-ray). Serum levels of alpha 1 antitrypsin (AAT: mg/dL) are given for each CVID patient together with their genotype and serum phenotype. Where an S or a Z allele was not present, it was defined as not S/not Z and hence is reported here as *S-* or *-Z-* and the allele is assumed to be a normal non-deficiency allele or M allele. Serum or plasma samples were subjected to isoelectric focusing (IEF) gels to obtain the phenotyping results. [1]Smoking status: cigar = occasional cigars only; ex = previous smoker; never = never smoked. [2]Method for detection of bronchiectasis; [3]by PCR and primers for S and Z alleles; [4]by isoelectric focusing. [5]UNDT = undetected by low-level plate.

The occurrence of S and Z alleles within a population of CVID patients

Genotyping was used to test for AAT type in a population of CVID patients and appropriate controls. Genotype and allele frequency for the S and Z alleles were calculated for these groups (Table 2). Statistical analysis using a two-tailed Fisher's exact test did not find any significant differences. The frequencies of the S and Z alleles were calculated using gene counting (Table 2). The distribution of alpha-1 antitrypsin alleles appeared to be inverted with respect to *Pi*S and Pi*Z* in patients with CVID when compared to controls. The frequency of the S allele within the CVID population (0·023) was lower than found in the control

Table 2. Distribution of alpha-1 antitrypsin genotype in CVID patients and controls

	CVID patients $n = 43$		Healthy controls $n = 70$	
AAT genotype	*n*	%	*n*	%
S and Z absent	38	88·37	57	82·43
S heterozygote	1	2·33	9	12·86
Z heterozygote	3	6·98	4	5·71
SZ heterozygote	1	2·33	–	–
SS homozygote	–	–	–	–
ZZ homozygote	–	–	–	–
Allele frequency	*n*	Freq	*n*	Freq
S allele	2	0·023	9	0·064
Z allele	4	0·047	4	0·029

Non-Caucasian results are excluded; *n* is the number of subjects within each group. A corresponding percentage figure is also given. Statistical analysis of AAT genotypes in CVID and control populations using Students' *t*-test did not demonstrate any significant differences between groups. *P*-values comparing different populations were: CVID *versus* healthy controls: S heterozygote, $P < 0{\cdot}09$; Z heterozygote, $P < 1{\cdot}0$, SZ heterozygote, $P < 0{\cdot}38$.

Table 3. Distribution of S and Z alleles in CVID patients subgrouped according to evidence of bronchiectasis

	Alleles from healthy $n = 140$		Alleles from CVID and bronchiectasis $n = 52$		Alleles from CVID no bronchiectasis $n = 34$	
Allele	*n*	Frequency	*n*	Frequency	*n*	Frequency
S	9	0·064	1	0·019	1	0·029
Z	4	0·029	4	0·077	0	0

The frequency of each allele was calculated by gene counting (number) according to Hardy–Weinberg theory; *n* = the number of alleles present. It is assumed that all alleles are independent observations rather than patients being the sampling unit. Where an S or Z allele was not present, it was defined as not S/not Z, which is assumed to be a normal non-deficiency allele. Statistical analysis of S and Z alleles in CVID patients subgrouped according to evidence of bronchiectasis in comparison with control groups, was performed using Fisher's exact test: CVID bronchiectasis *versus* healthy controls: S allele, $P < 0{\cdot}46$; Z allele, $P < 0{\cdot}12$. CVID: no bronchiectasis *versus* healthy controls: S allele, $P < 0{\cdot}69$; Z allele, $P < 0{\cdot}58$.

group, whereas the frequency of the Z alleles within the CVID population was 0·047, raised in comparison to controls. However, statistical analysis using Fisher's exact test again demonstrated no significant difference in S and Z allele frequency between CVID and controls.

Frequencies of the S and Z alleles of AAT in CVID subgroups with or without bronchiectasis

Because the results did not demonstrate a significant association between AAT deficiency alleles and CVID, a search for a clinically significant link was made. The 43 CVID patients were subgrouped according to whether or not there was evidence of bronchiectasis and then analysed according to alpha-1 antitrypsin genotype. Data is shown in Table 1. Analysis revealed that of 38 CVID patients without genotype defined AAT deficiency alleles, 68·4% [21/22] had bronchiectasis and 31·5% [23/22] did not have bronchiectasis (Table 1). Of the three CVID patients who were PiMZ heterozygotes (patients 1, 16 and 18 in Table 1) and one who had a PiSZ (patient 39 in Table 1), all had bronchiectasis. The AAT levels were reduced (35–70% of normal levels when using the median value of 160 mg/dL). The one other PiMS heterozygote CVID patient (patient 19 in Table 1) did not have bronchiectasis, and AAT levels were reduced (~45% of normal levels).

The frequencies of the S and Z alleles of AAT in these CVID subgroups were calculated and statistical analysis (Table 3) was performed. Although the Z allele frequency was increased in the CVID patients with bronchiectasis when compared to controls ($P < 0{\cdot}15$), subgrouping of CVID patients into those with and without bronchiectasis demonstrated no significant difference for either Z or S alleles in the CVID patient subgroups compared to the controls (Table 3).

AAT serum level, AAT Genotyping and AAT phenotyping by isoelectric focusing

Table 1 shows the AAT serum levels (mg/dL) in 43 CVID patients and the corresponding genotyping and phenotyping results. Reductions in AAT levels in CVID patients (Table 1) were in line with levels associated with the AAT deficiency types [17]. Unlike the phenotyping technique, where the products of a number of AAT alleles can reportedly be determined, the AAT PCR technique used in this work detects only S or Z alleles of AAT. Where an S or a Z allele was not present it was defined as not S/not Z, which is assumed to a normal non-deficiency allele or M allele. However, phenotyping and genotyping of the CVID patients showed 100% concordance (Table 1), and in general these different techniques correlate closely (ME Sansom, BL Ferry, Z Sherréll, HM Chapel, S Misbah, manuscript in preparation).

DISCUSSION

Genotyping was used to define the distribution of alpha-1 antitrypsin alleles within a group of patients with CVID and within a control group. Information was also gathered on the radiological status of the lungs and serum AAT levels for the patient group. There are a number of theoretical reasons as to why AAT deficiency might be associated with CVID, including the proximity of the AAT gene (*Pi*) to the immunoglobulin heavy chain gene (*Gm*) [22], overlapping lung disease (emphysema and bronchiectasis) in AAT-deficient patients and CVID patients [13] as well as isolated case reports of patients with hypogammaglobulinaemia and AAT deficiency [14,15]. This preliminary study aimed to clarify if there was an association between CVID and possession of AAT deficiency alleles.

The observed reductions in AAT levels in CVID patients (Table 1) were in line with levels associated with the AAT deficiency types [17]. The results were not thought to be skewed by smoking, as the numbers of smokers/ex-smokers were distributed evenly between bronchiectasis and non-bronchiectasis patients. CVID patients with normal AAT alleles and bronchiectasis had

elevated levels of AAT when compared to patients without bronchiectasis, as would be expected for patients with lung damage [21,23,24].

In this preliminary study, a total of 43 Caucasian CVID patients were genotyped, phenotyped by IEF and AAT levels determined (Table 1). The development of the PCR genotyping method and a comparison between this technique and the established phenotyping method will be reported elsewhere (ME Sansom, BL Ferry, Z Sherréll, HM Chapel, S Misbah, manuscript in preparation); however, our SSP-PCR genotyping showed excellent correlation with the established IEF phenotyping technique.

A total of 43 Caucasian CVID patients were genotyped and compared with a healthy Caucasian control group of 70 subjects (Tables 2 and 3). A higher than expected number of patients with genotypes with Z (one SZ heterozygotes and three Z heterozygotes) was observed in the CVID patient group [5,25]. However, statistical analysis revealed this was not significant.

Analysis of the CVID patient subgroups in terms of bronchiectasis demonstrated a trend towards bronchiectasis in patients with a Z allele (four of four patients). While CVID patients with bronchiectasis had a Z allele frequency of 0·077 (expected value 0·029) [5,25], this was not a significant increase (*P*-value of 0·15) when compared to the control group). The control group was composed of samples from both sexes taken from the Oxford area and was Caucasian in composition.

This study shows a trend towards an association of the Z allele of alpha-1 antitrypsin in a subgroup of patients with CVID who have bronchiectasis. While it is difficult to conclude from this limited study that the Z deficiency allele of AAT plays a role in the development of bronchiectasis in patients with CVID, as suggested by isolated reports suggesting a link between CVID and alpha-1 antitrypsin deficiency [14,15], the data indicate that a larger multi-centre study involving AAT genotyping of larger numbers of bronchiectasis-positive and bronchiectasis-negative CVID patients may be useful.

We recognize that our study has a number of limitations. The Oxford CVID database has been validated and the diagnoses checked against the internationally agreed criteria (no CVID patients met definite or probable criteria for a diagnosis ofXLA, AID and HIGM). The statistical power of these results was limited by the small size of the groups and highlights the need for multi-centre collaboration and disease-specific databases in studies in rare diseases. A larger study could look at multiplexing a number of genes including those for AAT and relevant MHC genes and in addition the ethnic origins of the patients could be studied in more detail. In this study, CVID patients had a CT scan performed only if indicated clinically due to recurrent infections. Ideally, all the CVID patients should be examined for bronchiectasis by high resolution CT scan. A larger multi-centre study could include this as one of the criteria.

An association with alpha-1 antitrypsin deficiency may exacerbate lung damage in CVID patients. If the results from this initial study are corroborated, they may explain why silent and symptomatic changes in pulmonary function may occur in some patients with CVID despite adequate immunologlobulin replacement therapy [26,27]. It is possible that that alpha-1 antitrypsin and immunoglobulin deficiency both contribute to lung damage associated with a subgroup of CVID patients. In the light of this finding the testing of alpha-1 antitrypsin genotype by a SSP PCR-based technique may be indicated for patients with CVID to determine if they have an increased risk of bronchiectasis or whether or not this association accounts for some of the unexplained liver disease in these patients.

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