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Characterization of mutations in patients with autoimmune polyglandular syndrome type 1 (APS1)

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Abstract Autoimmune polyglandular syndrome type 1 (APS1), also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), is an autosomal recessive disorder characterized by the failure of several endocrine glands as well as nonendocrine organs. The autoimmune regulator (*AIRE*) gene responsible for APS1 on chromosome 21q22.3 has recently been identified. Here, we have characterized mutations in the *AIRE* gene by direct DNA sequencing in 16 unrelated APS1 families ascertained mainly from the USA. Our analyses identified four different mutations (a 13-bp deletion, a 2-bp insertion, one nonsense mutation, and one potential splice/donor site mutation) that are likely to be pathogenic. Fifty-six percent (9/16) of the patients contained at least one copy of a 13-bp deletion (1094–1106del) in exon 8 (seven homozygotes and two compound heterozygotes). A nonsense mutation (R257X) in exon 6 was also found in 31.3% (5/16) of the USA patients. These data are important for genetic diagnosis and counseling for families with autoimmune endocrine syndromes.

Introduction

Autoimmune disease of endocrine glands occurs most often in only a single organ, but involvement of multiple endocrine glands and nonendocrine organs has been well described. These disorders are known as the autoimmune polyglandular syndromes (APS). To date, three types of APS have been documented. APS type 1 (APS1) is also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). The syndrome is defined by the presence of two of three conditions: (1) adrenocortical failure (Addison disease), (2) hypoparathyroidism, and (3) mucocutaneous candidiasis (Neufeld et al. 1980, 1981; Ahonen et al. 1990). Several other disorders, such as gonadal failure, alopecia, malabsorption, vitiligo, chronic active hepatitis and keratopathy, may also be present in APS1 patients. APS2 is the coexistence of immune-mediated adrenocortical insufficiency (Addison disease) with thyroiditis and/or type 1 diabetes mellitus. APS3 is defined as thyroiditis without adrenocortical insufficiency, but associated with diabetes mellitus, pernicious anemia, vitiligo, or alopecia (Neufeld et al. 1980).

APS1 is a rare disease that affects both sexes equally and the age of onset varies from 1–60 years (Ahonen et al. 1990). The syndrome is more common in Iranian Jews (1:6500 to 1:9000) (Zlotogora and Shapiro 1992) and Finns (1:25,000) (Norio 1981). APS1 is an autosomal recessive disease caused by a single gene on chromosome 21q22.3 as determined by linkage analysis in the Finnish families (Aaltonen et al. 1994). The gene has recently been cloned by two independent groups of investigators (Finnish-German APECED Consortium 1997; Nagamine et al. 1997). The *AIRE* (for autoimmune regulator) gene responsible for APS1 consists of 14 exons and a 2445-bp mRNA. The AIRE protein (545 amino acids) has two PHD finger motifs, a proline-rich region, and four LXXLL motifs, suggesting that the AIRE protein might act as a transcriptional regulator (Finnish-German APECED Consortium 1997; Nagamine et al. 1997). Eight different germ-line mutations have been reported in the *AIRE* gene in APS1 patients from European countries (Finnish-German APECED Consortium 1997; Nagamine et al. 1997; Scott et al. 1998). A nonsense mutation (257Arg→Stop) in exon 6 was found in APS1 patients from several populations and is believed to be a major mutation in Finnish APS1 patients (Finnish-German APECED Consortium 1997; Scott et al. 1998). In this study, we have searched for mutations in the entire coding region and splice/donor/acceptor sites of the *AIRE* gene using direct sequencing of polymerase chain reaction (PCR)-amplified products in APS1 patients from 16 families ascertained from the USA.

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Table 1 APS1 patients and clinical features. (*HP* hypoparathyroidism, *AD* Addison disease, *MC* mucocutaneous candidiasis, *AL* alopecia, *OV* ovarian failure, *HEP* chronic active hepatitis, *HG* hypogonadism, *HT* Hashimoto's thyroiditis, *VT* vitiligo)

Patients	Sex	HP	AD	MC	AL	OV	HEP	HG	HT	Other diseases
101A	M	+	+	+	+	–	–	–	+	VT
105A	M	+	+	+	–	–	+	+	–	
105B	F	+	+	+	–	–	–	–	–	
105C	F	+	+	+	–	–	+	–	–	
106A	M	+	+	–	+	–	–	–	+	
107A	M	+	+	+	–	–	–	–	+	
109A	F	+	+	+	+	–	–	–	+	VT
113A	F	+	+	+	–	–	–	–	–	
114A	M	+	+	+	+	–	+	–	–	
115A	F	+	–	+	+	–	–	–	–	Malabsorption
116A	M	+	+	+	–	+	+	+	+	
117A	F	+	+	+	–	–	–	–	–	
118A	F	+	+	–	+	+	–	–	–	
118B	M	+	+	–	+	–	–	–	–	
118C	F	+	+	–	+	–	–	–	–	
119A	F	+	+	+	–	–	–	–	–	
130A	F	+	+	+	–	–	–	–	–	
131A	F	+	+	+	–	–	–	–	–	
133A	M	+	+	+	–	–	–	–	–	
214B	F	+	+	+	–	–	+	–	–	

Table 2 The sequences of primers used in this study for amplification of individual exons in the *AIRE* gene. (T_m optimum annealing temperature for polymerase chain reaction)

Exon	5′→3′ Sequences	Locations	Size (bp)	T_m (°C)
1	F: CGT GGT CGC GGG GGT ATA A	4532–4550	363	58
	R: GGA CTA TCC CTG GCT CAC A	4894–4876		
2	F: AGC TCC ACC CTC TAG TCA TG	5171–5190	326	58
	R: CTG GGC TGA GCA GGT GAC A	5496–5478		
3	F: GGC CAA GGT GTC CAG TTC T	5594–5612	275	58
	R: GAG ACC CTG GCT GGC TTC	5868–5851		
4	F: CCG GCA CTC ACC CCC ACT	6160–6177	190	58
	R: CCT GAC CCC TTC CCC CTG	6349–6332		
5	F: ATA GAG TAT GTG CTT GGG AAC	6981–7001	232	58
	R: CAT CTT GGA GCC TGG GTC T			
6	F: TCT GCT AGA CCC CAC CCT G	8268–8286	327	58
	R: GCC CCC AGC AGA GCC ACT	8594–8577		
7	F: TGC CGA GAG ACG CCT GGT G	8605–8623	239	58
	R: AAA GGC AGA GGC AGC AGG AC	8843–8824		
8	F: GGA GTT CAG GTA CCC AGA GA	9682–9701	352	60
	R: TGA CTC AGA ACC CCT TTC CA	10,033–10,014		
9	F: CAT GTC TCT GAC TGG TGG ACA	10,972–10,992	182	62
	R: GTG GCC ATG TGG ACA GGA G	11,153–11,135		
10	F: CAG TCA CTG ACT CCT GGG TG	11,623–11,642	323	60
	R: GGT GAA TTC ATC CGC CCC GT	11,945–11,926		
11	F: CTC GGG TTC GGG TTC AGC TA	12,422–12,441	322	62
	R: GAG TGT AGG GTG TGG GTG C	12,783–12,765		
12	F: GGA GGT GGC ACT CCT GCT C	13,044–13,062	292	60
	R: TGG TCA GTG CGT CTG GTG C	13,335–13,317		
13	F: CCT GCG GCC TCT GTA CCC	15,013–15,030	209	61
	R: AGA GTG GGG AGC CTG GGT G	15,221–15,203		
14	F: AGG TTC TCA CCG TCA CTC TGT	16,327–16,347	219	58
	R: ACT GAC AAG AGG TGG CGC TGT	16,545–16,525		

Table 3 Mutations and polymorphisms found in APS1 patients

Mutation ID	Exon/intron	DNA		Function		No. of
		Position[a]	Change	Amino acid no.	Change	alleles
Pathogenic mutations						
5835T/C	Intron 3	5835	T→C	–	Splicing	1
R257X	Exon 6	896	C→T	257	Arg→Stop	7
1094–1106[b]	Exon 8	1094–1106	13-bp deletion	323–327	Frameshift	18
1422insAC[c]	Exon 11	1422	2-bp insertion	432	Frameshift	1
Polymorphisms						
L196P	Exon 5	715	C→T	196	Leu→Pro	10
7175G/T	Intron 5	7175	G→T	–	Intronic	1
808C/T	Exon 6	808	C→T	–	Silent	2
S278R	Exon 7	961	C→G	278	Ser→Arg	2
9961C/A	Intron 8	9961	C→A	–	Intronic	1
11,107G/A	Intron 9	11,107	G→A	–	Intronic	1
1324C/T	Exon 10	1324	T→C	–	Silent	5
13,072G/C	Intron 11	13,072	G→C	–	Intronic	1
1705T/C	Exon 14	1578	T→C	–	Silent	7

[a] Nucleotide positions for cDNA and genomic DNA were based on accession numbers AB006682 and AB006684, respectively
[b] 13-bp deletion: CTG TCC CCT CCG C

Materials and methods

Patients and controls

We ascertained 16 pedigrees containing 20 APS1 patients and 45 unaffected family members from the USA. Informed consent was obtained from all subjects. All families are of Caucasian origin. Two of these families have three affected children and all other families have only one affected child. All patients have at least two of the three diagnostic diseases (mucocutaneous candidiasis, hypoparathyroidism, and/or Addison disease). Most of them also have additional component diseases (Table 1). Therefore, the patients are typical index cases for APS1. All patients with APS2 and healthy controls were also Caucasians from the USA.

DNA preparation and PCR amplification

Peripheral blood mononuclear cells were isolated using an ammonium chloride red-cell lysis buffer and DNA was then purified using a standard phenol/chloroform extraction method. Fourteen sets of primers were designed within flanking introns to amplify individual exons (Table 2). The PCR was carried out in a 50-µl volume containing 50 mM KCl, 10 mM TRIS-HCl, pH 8.3, 1.5 mM $MgCl_2$; 60 µM of each dNTP, 20 ng of genomic DNA, 2 pmol of each primer and 1.5 U *Taq* DNA polymerase. Samples were subjected to 35 cycles of 30 s at 94°C for denaturing, 30 s at optimum annealing temperature, and 30 s at 72°C for extension, using a Perkin-Elmer-Cetus 9600 thermocycler.

Direct sequencing of PCR products

After PCR amplification, products (50 µl) were electrophoresed on a 2.0–2.5% agarose gel. The expected bands were excised from the gel and transferred into 1.5-ml Eppendorf tubes. After adding 5 µl of H_2O, the tubes were frozen at –20°C for 5–10 min. The gels were smashed while frozen and then incubated in a 50°–60°C waterbath for 10–15 min. After a brief vortex, the PCR products were eluted out of the gels by a brief centrifugation. The supernatant was centrifuged again to remove any remaining agarose. An aliquot of products (30 ng) was then used directly for cycle sequencing using the ABI Prism BigDye terminator (Applied Biosystems) according to the manufacturer's instructions. The sequencing reactions were then precipitated in 3 vol. 100% ethanol and 0.1 vol. 3 M sodium acetate, pH 5.2. The pellets were washed with 250 µl of 70% ethanol, dried in a vacuum dryer and then dissolved in 20 µl of template suppression buffer (Perkin Elmer). DNA sequencing was carried out using an ABI 310 automated sequencer (Applied Biosystems).

Results

To identify mutations in our APS1 patients, we first screened for the major mutation in Finnish APS1 patients (R257X, Arg to Stop at amino acid 257) by PCR amplification and *TaqI* digestion (Table 3). Individuals with the mutation were then confirmed by sequencing analysis of PCR products. This mutation was found in 5 of 16 unrelated patients (31.3%). Two of these patients were homozygous for the mutation and the three others contained one copy of this mutant allele (Table 4). We then screened for a 13-bp deletion in exon 8 (1094–1106del), previously identified in a number of patients from European countries (Finnish-German APECED Consortium 1997; Nagamine et al. 1997; Scott et al. 1998). Surprisingly, this mutation was found in 9 of the 16 unrelated patients (56.3%). Seven of these patients were homozygous for the mutation. Their homozygous status was confirmed by flanking polymorphic markers (Table 4 and unpublished data). Two other patients had one copy of the mutant allele. Since this is a common mutation in APS1 patients, we searched for the mutation in 22 APS2 patients and 50 normal controls. This mutation was not found in any of these subjects.

Table 4 Haplotypes for APS1 patients. (*NI* not identified) No mutations were found in patients 107A and 116A

Patients	*D21S1912*	*AIRE* mutation	*PFKL*	*D21S171*
101A	5	1094–1106del	5	5
	5	1094–1106del	5	7
105A, B, C	6	1094–1106del	5	5
	8	1094–1106del	7	5
106A	5	1094–1106del	5	5
	5	1094–1106del	5	5
114A	4	1094–1106del	5	5
	7	1094–1106del	5	5
115A	6	1094–1106del	5	5
	6	1094–1106del	5	5
117A	4	1094–1106del	5	5
	1	1094–1106del	5	5
119A[a]	5	1094–1106del	5	5
	4	1094–1106del	7	7
113A	6	R257X	7	5
	6	R257X	7	5
133A	6	R257X	7	5
	7	R257X	7	6
109A	6	R257X	7	5
	6	1094–1106del	5	5
131A	7	R257X	9	9
	5	1094–1106del	5	5
130A	6	R257X	7	5
214B	5	1422insAC	5	5
118A, B, C	4	5835T/C	5	7

[a] The haplotypes for patient 119A cannot be determined because no information from the parents is available

To identify other mutations responsible for APS1, we carried out direct sequencing of PCR products for all 14 exons of the *AIRE* gene in all five patients who had none or only one copy of the 1094–1106del and/or the R257X mutation. We also sequenced all exons for three patients who were homozygous for the 1094–1106del mutation and two patients who were homozygous for the R257X mutation. We identified nine normal polymorphisms (three silent, four intronic and two nonsynonymous mutations). We also identified two new potentially pathogenic mutations including a 2-bp insertion in exon 11 (1422insAC) and one splice/donor site mutation (5835T/C) (Table 3). The 2-bp insertion is a frameshift mutation and is a likely pathogenic mutation. The 5835T/C mutation is a T to C subsititution at the +2 site of intron 3. The mutation changes the first two nucleotides of intron 3 from GT to GC. The mutation was not found in 30 sequenced chromosomes of healthy controls.

To investigate whether 1094–1106del and R257X are recurrent mutations or are due to a founder effect, we constructed haplotypes for the region surrounding the *AIRE* gene using microsatellite markers *D21S1912*, *PFKL* and *D21S171*. As shown in Table 4, the 1904–1106del mutation is found on multiple haplotypes defined by these markers.

Discussion

We have identified four putative pathogenic mutations and nine normal polymorphisms in the *AIRE* gene in APS1 patients from the USA. The 1094–1106del mutation is a 13-bp deletion encompassing codons 323–326 and the first nucleotide of codon 327 in exon 8 of the *AIRE* gene. This deletion creates a premature termination codon at position 371, resulting in a polypeptide that is 73 amino acids shorter. Exon 8 encompasses codons 294–332 and contains the first PHD finger motifs (codons 294–340) of the AIRE protein. As more than 90% of the PHD-1 finger motif is deleted in the mutant allele, this deletion should disrupt the function of the protein. This 13-bp deletion is the most frequent mutation in our collection of patients as it is present in 56.3% (9/16) of the US patients or 50% (16/32) of the mutant alleles. The same mutation has also been reported in APS1 patients from several European countries (Finnish-German APECED Consortium 1997; Nagamine et al. 1997; Scott et al. 1998).

The second major mutation (R257X) is a C→T transition at nucleotide 896 of the mRNA (257Arg→Stop) in exon 6 of the *AIRE* gene. As it is present in 82% of the APS1 patients in the Finnish population, the mutation is believed to be a major Finnish mutation (Finnish-German APECED Consortium 1997). This mutation introduces a premature stop codon at position 257 and results in a protein that is 288 amino acids shorter. The premature protein is probably unable to function normally. The mutation was found in 5 of 16 families examined in this study. Two patients were homozygous for this mutation and three other patients contained one copy of this mutation. The R257X and the 1094–1106del mutation together account for 72% of mutant alleles in the USA APS1 patients.

The high frequency of these two mutations in the USA APS1 patients may be explained by two nonalternative hypotheses: a founder effect or recurrent mutation. It has been suggested that these are recurring mutations as they were found on several different haplotypes defined by microsatellite markers flanking the *AIRE* gene (Scott et al. 1998). Similarly, the 1094–1106del mutation was found in multiple haplotypes defined by *D21S1912–PFKL–D21S171* in this study (Table 4). However, it is important to point out that 14 haplotypes with the 1094–1106del mutation are associated with allele 5 of *PFKL*, which is only a few kilobases downstream of the *AIRE* gene (Finnish-German APECED Consortium 1997; Nagamine et al. 1997). The other two 1094–1106del haplotypes are associated with allele 7 of *PFKL* (Table 4). In contrast, the 1094–1106del mutation is associated with several different alleles of *D21S1912*, which is about 150 kb upstream of the *AIRE* gene. Therefore, it is possible that the *D21S1912* alleles on the haplotypes with the 1094–1106del mutation could have resulted from recombinational events. Furthermore, the high mutation rate of microsatellite markers may also introduce new alleles on ancestral haplotypes. Therefore, it is possible that the 1094–1106del mutation on some haplotypes may be from a common ancestral haplotype. To address this question,

polymorphic markers in very close proximity to the *AIRE* gene need to be identified and analyzed.

Our direct sequencing analyses also identified two new mutations that may be pathogenic. One of these mutations is a 2-bp insertion in exon 11 found in one patient and results in a premature termination codon at amino acid 479 and a protein that is 66 amino acids shorter. The second new mutation is a T→C transition at position +2 of intron 3. This is a putative splice/donor site mutation as the position is conserved in the majority of introns and plays a significant role in RNA splicing (Fishel et al. 1993). All introns of the *AIRE* gene follow the GT-AG rule. The 5835T→C transition at the +2 site changes GT to GC and therefore may affect RNA splicing. In addition, sequencing of 30 normal chromosomes from the same population did not reveal this mutation. However, the functional significance of the mutation will have to be tested using RNA, which was unfortunately not available from the patient.

Among the nine nucleotide substitutions that we considered as normal variations in the *AIRE* gene, the T→C transition at position 715 in exon 5 changes a leucine to proline at amino acid 196. We believe that this mutation is not pathogenic because it is also found in two APS1 patients who had two copies of pathogenic mutations and the same mutation was found in two of the three sequenced normal controls. The S278R substitution in exon 7 changes Ser to Arg. This substitution was also found in 1 of 30 normal chromosomes sequenced. In addition, the same substitution was found in the patients with two pathogenic mutations (Scott et al. 1998). Therefore, it is not a pathogenic mutation. The 11107G/A substitution is at the +6 position of intron 9 and this position could influence RNA splicing (Fishel et al. 1993). Since the substitution is found in patients with two pathogenic mutations (Scott et al. 1998), it is an unlikely candidate for pathogenic mutation. Three variations were in introns far away from the exon/intron junctions. Each of these intronic mutations was found in one patient who had two pathogenic mutations, suggesting that the intronic mutations are not pathogenic.

Despite our great efforts, no mutation was identified in two patients and only one mutant allele was found in three other patients. There is currently no evidence for genetic heterogeneity for APS1. Identification of mutations by sequencing of PCR products in heterozygous samples was easily accomplished because of the good quality of sequences obtained in this study. Therefore, we believe that the chance of our missing mutations in the sequenced regions is very low although a 100% detection rate cannot be guaranteed. However, our sequencing analyses did not cover the promoter region and the intronic sequences far away from the exon/intron junctions. It has been shown that mutations in intronic sequences distant from exon/intron junctions may affect RNA splicing (Fishel et al. 1993). Our mutation analyses were not able to detect deletions of complete exons and primer-binding regions of the introns or large insertions within exons in the patients with only one copy of such mutations.

APS1 appears to be a genetically homogenous syndrome caused by the same gene in different patients from various populations. However, the phenotype of the syndrome is extremely heterogeneous. The components of diseases and their sequence of appearance are different from patient to patient, even between affected siblings in the same family. To determine the possible phenotype/genotype correlation, we compared the phenotypes between patients with the 1094–1106del mutation and those with other mutations. As no apparent correlation was found, our results suggest that the phenotypic heterogeneity may be related to background genes, particularly genes involved in immune regulation and autoimmunity.

This study identified two common mutations (1094–1106del and the R257X nonsense mutation) that accounted for 72% of the mutant alleles in the USA APS1 patients. The 13-bp deletion can be easily detected using PCR amplification from genomic DNA and 4% agarose gel electrophoresis. The nonsense mutation can be quickly assayed by *Taq*I digestion of PCR products and agarose gel electrophoresis. The easy detection and high frequency of these mutations offer an excellent tool for the diagnosis of APS1 in relatives of APS1 patients and those with one APS1 component disease. Additional APS1 component diseases may only occur many years after the onset of the first disease and it is often difficult to distinguish APS1 from other APS patients before the appearance of diagnostic component diseases. Therefore, screening for mutations in the *AIRE* gene in autoimmune endocrine patients has significant implications for the diagnosis, prognosis and care of these patients.

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