CLINICAL STUDY

Detection of a complete autoimmune regulator gene deletion and two additional novel mutations in a cohort of patients with atypical phenotypic variants of autoimmune polyglandular syndrome type 1

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Abstract

Objective: Autoimmune polyglandular syndrome type 1 (APS-1) is characterised by multiple autoimmune diseases. Detection of autoimmune regulator (*AIRE*) gene mutations facilitates timely and precise diagnosis.

Design: *AIRE* mutation detection was performed in a cohort of 11 patients. Two did not meet clinical APS-1 criteria and several started with atypical presentation.

Methods: Sequencing and TaqMan genotyping were used to identify *AIRE* mutations. Complete *AIRE* deletion was confirmed and framed by real-time PCR, long-range amplification and analysis of the microsatellite markers.

Results: Seven different mutations were detected, three were novel: c.892G>A in exon 8, silent mutation c.462A>T in exon 3 most likely affecting splicing, and a complete deletion of a single *AIRE* allele ((?_68)_(1567-14_?)del). Novel (chronic otitis) and rare (systemic juvenile rheumatoid arthritis, autoimmune bronchiolitis, epilepsy) clinical presentations were observed.

Conclusions: *AIRE* mutation detection was valuable in the diagnostics of APS-1 in patients with atypical presentation. Chronic otitis media possibly broadened the cluster of APS-1 manifestations.

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Introduction

Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED; OMIM 240300) or autoimmune polyglandular syndrome type 1 (APS-1) is a rare autosomal recessive disorder, characterised by multiple autoimmune diseases and high levels of autoantibodies against antigens expressed in affected tissues. The classic triad includes chronic mucocutaneous candidiasis, hypoparathyroidism and Addison's disease. At least two of these disorders must be present in a patient to define the syndrome (1). APS-1 also affects other endocrine and non-endocrine organs resulting in diabetes mellitus, gonadal failure, autoimmune thyroid disease, autoimmune hepatitis, vitiligo, pernicious anaemia, exocrine pancreatic insufficiency, alopecia (2) and tubulointerstitial nephritis (3). Asplenia and squamous cell carcinoma can be present (4). The disease usually starts in childhood with additional components appearing throughout life (1).

Individual cases are described to have incomplete clinical presentation (5, 6).

In contrast to most other autoimmune diseases, APS-1 is associated with mutations of a single gene, designated autoimmune regulator (*AIRE*) (7, 8). *AIRE* protein regulates autoimmunity by promoting the ectopic expression of peripheral tissue-restricted antigens in medullary epithelial cells of the thymus (9). To date, more than 70 different mutations of the *AIRE* gene have been described and are distributed throughout the gene. Fifty-nine of them are listed in Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk>), and 13 additional mutations are described in the literature (6, 10–13).

In this report, we present clinical and mutational characteristics of 11 APS-1 patients of different ethnical backgrounds. Three novel mutations, including a complete deletion of a single *AIRE* allele, and atypical clinical manifestations are discussed.

Subjects and methods

Patients

Eleven APS-1 patients from nine unrelated Dutch, Serbian and Slovenian families were identified and included into the study. The study protocol was approved by the National Ethical Committee and written informed consent was obtained by all participants prior to the study.

Mutational analyses of the *AIRE* gene

All 14 exons, exon–intron boundaries and the promoter region (–840 to start codon) of the *AIRE* gene were individually PCR amplified (7, 14). Amplicons were directly sequenced using the BigDye terminator cycle sequencing kit and ABI Prism 310 automated sequencer (Applied Biosystems, Norwalk, CT, USA) and compared with the normal *AIRE* sequence (GenBank accession no. AB006684).

Novel c.892G>A and c.462A>T mutations were verified in 50 unrelated healthy controls by Custom TaqMan single nucleotide polymorphism (SNP) Genotyping Assay, designed by Applied Biosystems, and ABI Prism 7000 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Copy number of c.967_979del13 *AIRE* allele was estimated using The SYBER Green Master Mix System (Applied Biosystems, Warrington, UK) and ABI Prism 7000 HT Sequence Detection System (Applied Biosystems). Primers flanking the c.967_979del13 mutation in exon 8 (5'-GGA GGC GGC CGT GAA T-3' and 5'-GGA CGA GTG TGC CGT GTG T-3') in 300 nM concentrations and DNA in 20, 25 and 30 ng input amount were used. Parameter cycle threshold (C_t) was determined and standard curves were generated by plotting logarithm of template concentration against the corresponding C_t . The slope of the line was used to determine the efficiency of the target amplification (E) using equation $E = (10^{-1/\text{slope}}) - 1$ (15).

AIRE was screened for deletions flanking multiple exons using long-range amplification of the complete gene with Expand Long Template PCR System (Roche) and primers previously used for amplification of exons 1 (forward) and 14 (reverse) respectively. The full length of the amplicon was 11 938 bp and was visualised using 0.6-agarose gel electrophoresis.

Additionally, TaqMan real-time PCR assay and relative quantification were performed to confirm that deletion is spanning the entire *AIRE* gene. *RNaseP* was used as reference single-copy gene (TaqMan *RNaseP* detection reagents; Applied Biosystems) to standardise the amount of template DNA added to the reaction. Before using the comparative C_t method, the validation experiment was performed demonstrating that the amplification efficiencies of target and reference alleles were approximately equal. In order to use the comparative C_t method, the absolute value of the slope

of log input amounts versus ΔC_t (difference between C_t of the target allele and the reference allele) was less than 0.1. Copy number was calculated using the equation $(1 + E)^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is the difference between sample ΔC_t and calibrator ΔC_t . As calibrator, the average C_t of five healthy control samples was used (16). Efficiency was calculated as previously stated (15). The samples were run in duplicate using exon 1 and 14 primers and probes as described in (17). Validation experiments were performed using 5, 2, 1 and 0.5 ng input amounts of healthy control DNA.

Microsatellite length polymorphisms flanking *AIRE* were analysed at the DNA marker loci D21S1912, D21S1979, PFKL and D21S400 using primers described in Gene Databank, where one primer of a pair was labelled with fluorescein. Genomic DNA was PCR amplified and product sizes were estimated using ABI PRISM 310 automated sequencer and GeneMapper analysis software (Applied Biosystems).

Results

Clinical characteristics of patients

Clinical characteristics of individual APS-1 patients are summarised in Table 1. Patients were 8–23 years old, 6 males and 5 females. All but two were fulfilling the clinical diagnostics criteria for APS-1. Patient 4/C was included due to chronic candidiasis since his second year of life. Patient 6/F was included due to Addison's disease and three additional minor manifestations. Nine patients had all three major manifestations, and two patients had only one. Additionally, 12 different minor manifestations were present.

In the Dutch patient 2/A, computed tomography (CT) scan showed calcifications in the subcortex and basal ganglia at the time of diagnosis of hypoparathyroidism. She subsequently presented with complex epilepsy that required treatment. Addison's disease was diagnosed at the age of 8 years requiring treatment with hydrocortisone in combination with 9- α -fludrocortison.

Serbian patient 5/E presented with recurrent episodes of high fever accompanied by rash and arthralgias at the age of 2 years and was diagnosed with systemic form of juvenile rheumatoid arthritis. Fever and arthralgias discontinued a year later. From the age of 3.5 to 8 years, she suffered from repeated episodes of cough, dyspnoea and mild bronchoconstriction. She had been treated for presumed asthma over 4 years without much improvement. According to the course of respiratory disease, autoimmune bronchiolitis was suspected. Treatment with prednisone led to marked improvement. Thereafter, she had no significant respiratory problems. Lung function tests were normal and prednisone was withdrawn. Hypoparathyroidism developed when she was 7.5 years old, adrenal failure at 8 years and ovarian failure at 12 years of age.

Table 1 Clinical characteristics and *AIRE* genotype in analysed autoimmune polyglandular syndrome type 1 (APS-1) patients.

P/F	Sex	YOB	Manifestations (age in years at diagnosis of component)								Additional components	AIRE genotype
			Hp	A	MC	AI	ED	Hg	PA			
Dutch 1/A	M	1993		2	3		3			Chronic/tension head- aches, allergy	c. [967_979del13]+ [(?_68)_(1567-14_?)del] p.[Cys322fs]+(0?)	
2/A	F	1992	5	6	7		8	9		Complex epilepsy, hypothalamic–pituitary dysfunction	c. [967_979del13]+ [(?_68)_(1567-14_?)del] p.[Cys322fs]+(0?)	
3/B	M	1996	6	8	+			?		/	c.[62CT]+[967_979del13] p.[Ala21Val]+[Cys322fs]	
Slovene 4/C	M	1998			2					/	c.[21_43dup23]+[21_43dup23] p.[Arg15fs]+[Arg15fs]	
Serbian 5/E	F	1987	7.5	8	11			12	17	Chronic otitis media with effusion, asthma-like dyspnoea, systemic juvenile rheumatoid arthritis	c.[892G>A]+[769C>T] p.[Glu298Lys]+[Arg257X]	
6/F	M	1996		11		2.5	2			Malabsorption (2)	c.[462A>T]+[769C>T] p.(=)+[Arg257X]	
7/G	F	2000	5	4	1					Vitiligo (1)	c.[769C>T]+[769C>T] p.[Arg257X]+[Arg257X]	
8/H	F	1986	15	16	5				16	Chronic hepatitis (15)	c.[769C>T]+[769C>T] p.[Arg257X]+[Arg257X]	
9/I	M	1988	9	11	6					Malabsorption (9)	c.[769C>T]+[769C>T] p.[Arg257X]+[Arg257X]	
10/J	F	1985	9	10	10	10		18		/	c.[769C>T]+[769C>T] p.[Arg257X]+[Arg257X]	
11/J	M	1989	13	14	11	13			10	Malabsorption (11)	c.[769C>T]+[769C>T] p.[Arg257X]+[Arg257X]	

P, patient; F, family; YOB, year of birth; Hp, hypoparathyroidism; A, Addison's disease; MC, mucocutaneous candidiasis; AI, alopecia; ED, ectodermal dystrophy; Hg, hypogonadism; PA, pernicious anaemia; mutations are described as cDNA change of *AIRE* ORF (GenBank accession no. AB006682) and as affecting the protein coding sequence; novel mutations are presented in bold; numbers represent the age in years at the diagnosis.

Chronic otitis media with effusion was diagnosed when she was 16.5 years old and lasted for 8 months despite the standard treatment. Pernicious anaemia developed when she was 17 years old.

Mutational analyses

Seven different mutations of the *AIRE* gene were detected in 11 APS-1 patients, 3 of which were novel and so far not reported (Table 1). Mutations were named starting the numbering from the translation initiation AUG codon (GenBank accession no. AB006682), and as recommended by den Dunnen and Antonarakis (18). None of them was detected in 50 healthy control subjects.

The novel c.892G>A (p.Glu298Lys) mutation was detected in heterozygous state of maternal origin in patient 5/E (Fig. 1) and was located in exon 8 at the beginning of the first plant homeo domain (PHD).

The second novel mutation in patient 6/F was a substitution of A with T. It is retaining proline at position 154 (Fig. 2). Due to its position at the second nucleotide before the end of exon 3, the mutation was suspected to affect proper splicing. Two splice-site

prediction programs were used, namely Neural Network Splice Site Prediction Tool and FGENESH_SPL, to evaluate the quality of the altered donor site. The wild-type sequence resulted in predicted scores, 0.79 and 0.47 respectively. By contrast, the altered sequence was not recognised as the donor site by any of the used prediction programs.

Figure 1 (A) A novel heterozygous c.892G>A (p.Glu298Lys) mutation and (B) normal exon 8 sequence in patient 5/E.

Figure 2 (A) A novel heterozygous c.462A>T (p.=) mutation and (B) normal exon 3/intron 3 boundary in patient 6/F.

Unusual inheritances of the c.967_979del13 mutation and *AIRE* SNPs were observed in family A (Table 2). Patients 1/A and 2/A did not share the same c.967_979del13 allele with their fathers. They also did not share SNPs in exons 5, 7 and 10 and introns 7 and 9. Existence of a major *AIRE* gene deletion resulting in the loss of at least exons 5–10 in one allele was suggested. The deletion of one allele was confirmed by SYBER Green real-time amplification and detection of exon 8 *AIRE* allele in different concentrations of DNA. The standard curves showed parameter C_t to be one cycle higher in the father and both patients than in the mother in all DNA concentrations (Fig. 3A). Efficiency of the amplification was 67% in patient 1/A, 66% in patient 2/A, 67% in the father and 70% in the mother. Therefore, the difference in the C_t was not due to contamination of the template. Amplification pattern of the complete *AIRE* gene was the same in all family members and in the healthy control subject, excluding deletion limited to only a few exons. TaqMan real-time assay was performed to confirm that the deletion was spanning the entire *AIRE* gene. The validation experiment had fulfilled the criteria and comparative C_t method was applied. Since efficiencies derived from the standard curves were less than 100%, extended formula $(1 + E)^{-\Delta\Delta C_t}$ was used. Factor $(1 + E)^{-\Delta\Delta C_t}$ (relative to

RNaseP) was around 0.5 in patients and their father and 1 in their mother (Table 3) confirming the existence of the one-allele deletion in patients 1/A and 1/B and their father, but not in their mother. The deletion was spanning at least from the annealing sites of the primers from position c.68 in exon 1 and to the position c.1567-14 in intron 13. The deletion was also evident from amplification curves where *AIRE* allele amplicon C_t 's were one cycle higher in patients and their father as in their mother (Fig. 3B). By contrast, *RNaseP* C_t 's were alike. Microsatellite markers D21S1979, PFKL and D21S400 were not informative since all the family members carried the same allele. Analyses of microsatellite marker D21S1912 had shown inheritance of 208 bp paternal and 204 bp maternal alleles. The deletion at this position, 150 kb distant from the *AIRE* gene, was no longer present.

Discussion

APS-1 presents as an extremely variable combination of autoimmune endocrine and non-endocrine disorders. The current clinical diagnosis is based on the presence of two of three of the commonest clinical manifestations and allows early recognition of only a minority of new cases (4). In this report, patients 4/C and 6/F had a clinical picture that did not meet the clinical diagnostics criteria, but were diagnosed with having APS-1 after detection of an *AIRE* mutation. In addition, patient 5/E had extremely unusual clinical presentation with three atypical components. Reports describing less typical clinical cases with late onset (13) or unusual presentation are emerging (4–6, 13, 19), supporting the idea that the diagnostics criteria for APS-1 should be redefined (6). Additionally, diagnostics flow charts defining which patients should be subject to genetic analyses are needed.

Patient 6/F started with malabsorption, ectodermal dystrophy and vitiligo at the age of 2 years, and developed Addison's disease as the only major component when he was 11, fulfilling the criteria for APS-4 rather than APS-1. At that time, mutation detection was performed confirming APS-1. The patient was compound heterozygote for the common Finnish c.769C>T (Arg257X) mutation and a novel

Table 2 Inheritance of the c.967_979del13 mutation, *AIRE* single nucleotide polymorphisms (SNPs) and a major *AIRE* deletion c.(?_68)+(1567-14_?)del in family A (1/A and 2/A are APS-1 patients; M, mother; F, father).

	c.588 C>T (exon 5)	c.834C>G (exon 7)	c.879+101G>A (intron 7)	<i>AIRE</i> mutation	c.995+6G>A (intron 9)	c.1197T>C (exon 10)
1/A	T	C	G	c. [967_979del13]+[(?_68) _(1567-14_?)del]	G	C
2/A	T	C	G	c. [967_979del13]+[(?_68) _(1567-14_?)del]	G	C
M	T/C	C/C	G/G	c. [967_979del13]+[=]	G/G	C/T
F	C	G	A	c.[=]+[(?_68)_(1567-14_?) del]	A	T

Figure 3 Real-time PCR detection of a complete single-allele *AIRE* deletion in family A. (A) SYBER green assay: logarithm of DNA concentration plotted against the corresponding C_t (1/A in 2/A are APS-1 patients; M, mother; F, father). (B) TaqMan assay: amplification plot of exon 1 *AIRE* allele and *RNaseP* allele in patient 1/A and his mother.

c.462A>T (p.(=)) mutation. The c.462A>T mutation emerged *de novo* in this patient. It was located at the end of exon 3 and was part of the classical donor splice site (20). Although the c.462A>T mutation was a silent mutation, it was highly likely to affect proper splicing as was suggested by splice-site prediction tools. Eight different mutations are so far reported to affect splicing, all of them being intronic (12, 13, 21–25). The c.462A>T mutation was the first silent exonic mutation predicted to be pathogenic in APS-1. Therefore, exonic nucleotide substitutions in the *AIRE* gene not resulting in an amino acid substitution should be considered as potentially pathogenic.

Chronic candidiasis was the only manifestation present in patient 4/C. Since candidiasis developed in his early childhood and is frequently the first manifestation of APS-1 (1, 26), the patient was included in the study. Homozygous mutation c.21_43dup23 (R15fs) confirming the diagnosis was found. The c.21_43dup23 mutation was so far reported only in the heterozygous state in one Slovenian (12) and one Hungarian patient (27). Chronic candidiasis detected in early childhood should always be considered a possible manifestation of APS-1. The correct diagnosis is important for the prognosis and anticipation of additional manifestations, especially an adrenal crisis.

A novel c.892G>A (p.Glu298Lys) mutation was detected in patient 5/E. Due to its position, the mutation was expected to disrupt the first PHD domain of the AIRE protein. At least five additional missense mutations in this domain are reported (19, 24, 28, 29). Two of them (c.932G>A and c.977C>A) are

shown to alter intracellular localisation of the protein AIRE and to reduce its stimulating effect on transcription (28). The c.892G>A in heterozygosity with the c.769C>T (Arg257X) mutation was associated with an extremely unusual clinical presentation with three very rare components (Table 1).

Patient 5/E suffered from a systemic form of juvenile rheumatoid arthritis. This is an unusual component, since the pattern of autoimmune diseases in APS-1 shows organ-specific autoimmunity. So far, only two adult patients with rheumatoid arthritis (30) and one with pauciarticular juvenile rheumatoid arthritis (23) are described as having APS-1. Patient 5/E also presented with an asthma-like dyspnoea, which is a rare component of APS-1. One patient was reported to have fatal obstructive lung disease (31) and one bronchitis obliterans organising pneumonia (4). Therefore, autoimmune bronchiolitis should be considered as a rare but potentially life-threatening component of

Table 3 TaqMan real-time assay derived factor $(1+E)^{-\Delta\Delta C_t}$ in family A (1/A and 2/A are APS-1 patients; M, mother; F, father) in exons 1 and 14 (E, efficiency; $\Delta\Delta C_t$, difference between sample ΔC_t and calibrator ΔC_t).

	$(1+E)^{-\Delta\Delta C_t}$	
	Exon 1 ($E=1.28$)	Exon 14 ($E=1.11$)
1/A	0.44	0.52
2/A	0.48	0.65
M	1.31	1.11
F	0.54	0.48

APS-1, as suggested previously (32). In addition, this patient suffered from a chronic otitis media with effusion, which has, to our knowledge, not been previously reported in APS-1. Patients with calcified plaques in the tympanic membrane are not reported to suffer from repeated ear infections (1). The clinical manifestation presented in our patient might represent a novel minor component of the APS-1 and is possibly broadening the wide spectrum of APS-1 clinical manifestations.

All three Dutch patients had the heterozygous c.967_979del13 (Cys322fs) mutation previously described in this population (7, 28). Patient 3/B additionally had a rare c.62C>T (Ala21Val) mutation, so far described in only three patients from Sweden and North America (33). The Dutch siblings from family A additionally had a complete single-allele *AIRE* deletion spanning at least from exon 1 to intron 13 inherited paternally, as confirmed by real-time amplification. Similarly, *AIRE* gene copy number detection in the diagnostics of APS-1 is recently described in two Scandinavian APS-1 patients, where deletion was covering at least exons 2–8 (17). Another large deletion spanning exons 2–4 was described in two patients (3, 27). Large genomic deletions ranging from a single exon to a large chromosomal region can escape conventional mutation analysis, since normal allele PCR products can mask the deletion in the disease-related chromosome (34). Sequencing analysis in patients 1/A and 2/B were misleading and showed homozygosity of c.967_979del13 mutation. Determination of the real *AIRE* genotype could be important, if carrier status of their siblings or relatives had to be determined. Thus, the screening method for larger deletions can be a useful tool in the genetics diagnostics of APS-1.

Patient 2/B had calcifications in the subcortex and basal ganglia and later presented with complex epilepsy. Previously, epilepsy was reported in one Spanish APS-1 patient (35).

The c.769C>T (Arg257X) mutation was the predominant *AIRE* mutation in Serbian APS-1 patients (Table 1). It was present in all patients in at least the heterozygous state. The same was reported for Slovenian (12) and eastern European populations (27), suggesting the clustering of the mutation in this region.

In conclusion, three novel mutations, including a silent exonic mutation possibly affecting the splicing donor site and a complete deletion of a single *AIRE* allele, were described. *AIRE* gene mutation analyses proved useful in establishing the diagnosis in the patient with incomplete or unusual clinical presentation.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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