Cytokine Autoantibody Screening in the Swedish Addison Registry Identifies Patients With Undiagnosed APS1

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Context: Autoimmune polyendocrine syndrome type 1 (APS1) is a monogenic disorder that features autoimmune Addison disease as a major component. Although APS1 accounts for only a small fraction of all patients with Addison disease, early identification of these individuals is vital to prevent the potentially lethal complications of APS1.

Objective: To determine whether available serological and genetic markers are valuable screening tools for the identification of APS1 among patients diagnosed with Addison disease.

Design: We systematically screened 677 patients with Addison disease enrolled in the Swedish Addison Registry for autoantibodies against interleukin-22 and interferon-α4. Autoantibody-positive patients were investigated for clinical manifestations of APS1, additional APS1-specific autoantibodies, and DNA sequence and copy number variations of *AIRE*.

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Abbreviations: 17α-OH, 17α-hydroxylase; 21-OH, 21-hydroxylase; AAD, autoimmune Addison disease; AADC, aromatic L-amino acid decarboxylase; APS1, autoimmune polyendocrine syndrome type 1; CYP1A2, cytochrome P450 1A2; IFN, interferon; IL, interleukin; KCNRG, potassium channel regulator; NALP5, NACHT leucine-rich-repeat protein 5; PCR, polymerase chain reaction; PPV, positive predictive value; SAR, Swedish Addison Registry; SCC, side-chain cleavage enzyme; SOX10, SRY (sex determining region Y)-box 10; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; TPO, thyroid peroxidase.

Results: In total, 17 patients (2.5%) displayed autoantibodies against interleukin-22 and/or interferon-α4, of which nine were known APS1 cases. Four patients previously undiagnosed with APS1 fulfilled clinical, genetic, and serological criteria. Hence, we identified four patients with undiagnosed APS1 with this screening procedure.

Conclusion: We propose that patients with Addison disease should be routinely screened for cytokine autoantibodies. Clinical or serological support for APS1 should warrant DNA sequencing and copy number analysis of *AIRE* to enable early diagnosis and prevention of lethal complications. **(*J Clin Endocrinol Metab* 103: 179–186, 2018)**

A primary insufficiency of adrenal hormones is most often caused by autoimmune destruction of the adrenal cortex, autoimmune Addison disease (AAD) (1). Without early detection and continuous treatment, it can quickly develop into a lethal adrenal crisis (2). The AAD pathogenesis includes autoreactive lymphocytes and autoantibodies against 21-hydroxylase (21-OH), an enzyme essential for the synthesis of cortisol and aldosterone (3–5). Positive 21-OH autoantibodies confirm an autoimmune etiology of primary adrenal insufficiency (5). AAD is generally a disease with complex inheritance and has been associated with multiple human leukocyte antigen haplotypes, such as the coinherited diseases type 1 diabetes and autoimmune thyroid disease (6, 7). On rare occasions, however, it can be caused by monogenic autoimmunity syndromes (8).

Autoimmune polyendocrine syndrome type 1 (APS1) is a rare monogenic disorder (Online Mendelian Inheritance in Man no. 240300). The disease is caused by disruptive mutations in the *AIRE* gene on chromosome 21, encoding the autoimmune regulator protein (9, 10). AIRE acts as a transcriptional regulator in the thymus and promotes ectopic expression of otherwise tissue-specific proteins, which are presented for the maturing T cells to encounter (11). With a dysfunctional AIRE, the expression of self-antigens in the thymus is disrupted, and potentially self-reactive T cells evade the negative selection (11–13). Traditionally defined as a clinical syndrome, APS1 requires at least two of the following three major manifestations for diagnosis: AAD, hypoparathyroidism, and chronic mucocutaneous candidiasis (14, 15). The first signs usually present during childhood, but affected patients acquire various organ-specific autoimmune diseases throughout life (16).

A number of treatable APS1 complications can be fatal if not recognized early, including adrenal crisis in AAD, ketoacidosis in type 1 diabetes, autoimmune hepatitis, and hypoparathyroidism with hypocalcemic convulsive seizures (17, 18). Therefore, the diagnosis of APS1 has prognostic value and warrants a careful follow-up of affected patients to avoid lethal complications (15). With clinical suspicion, sequencing of the *AIRE* gene can confirm the APS1 diagnosis. However, APS1 can be difficult to recognize, not least because many patients first present with only minor manifestations, such as urticarial eruption and intestinal dysfunction, before onset of the classic triad (19). APS1 can easily evade diagnosis as long as only one major component is present. In fact, rare diagnoses such as APS1 can be overlooked even in patients fulfilling the clinical criteria.

Because autoantibodies can precede the onset of disease components, they could potentially be used for identifying individuals with APS1 even before the full syndrome has developed. APS1 is associated with several autoantibodies targeting tissue-specific molecules, and hitherto >15 specific autoantibodies have been described (20–22). Most patients with APS1 also harbor cytokine autoantibodies targeting interferon (IFN)-α4, IFN-ω, and interleukin (IL)-22 (23–26). The high prevalence of cytokine autoantibodies makes them sensitive biomarkers for APS1.

We hypothesized that screening patients with Addison disease for the presence of cytokine autoantibodies could help identify individuals with undiagnosed APS1. The Swedish Addison Registry (SAR) was established in 2008 and has become one of the world's largest Addison disease biobanks. The SAR includes serum samples, whole blood, DNA, and detailed clinical information from >800 patients, representing more than half the estimated number of patients with AAD in Sweden (27). By screening SAR patients for cytokine autoantibodies and by verifying *AIRE* gene mutations in autoantibody-positive individuals, we could assess the positive predictive value (PPV) of cytokine autoantibodies in AAD and evaluate the potential of using these biomarkers for identifying patients with undiagnosed APS1.

Patients and Methods

Patients

In this study, 677 patients consecutively enrolled into the SAR (years 2009 to 2013) were included for investigation of serological, clinical, and genetic aspects of primary adrenal insufficiency and APS1. All patients fulfilled the diagnostic criteria for primary adrenal insufficiency, with low morning serum cortisol and elevated adrenocorticotropic hormone levels or failure to adequately respond to corticotropin stimulation.

Time points for diagnosis, probable etiology, concomitant diseases, medication, and family history of AAD were recorded by the responsible physician. Missing data were complemented with information from medical records. Aliquots of sera and whole blood were stored at −70°C in a biobank until use. The study was approved by the regional ethics committee, permit 2008/296-31/2, and all patients gave their written informed consent.

Autoantibody detection

All patients were screened for autoantibodies against 21-OH, 17α-hydroxylase (17α-OH), side-chain cleavage enzyme (SCC), SRY (sex determining region Y)-box 10 (SOX10), aromatic L-amino acid decarboxylase (AADC), IFN-ω, IFN-α4, and IL-22, thyroid peroxidase (TPO), islet antigen-2, glutamic acid decarboxylase-65, and parietal cells. Patients positive for IFN-α4 or IL-22 autoantibodies were subsequently screened for autoantibodies against a panel of established APS1 autoantigens: NACHT leucine-rich-repeat protein 5 (NALP5), potassium channel regulator (KCNRG), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH), and cytochrome P450 1A2 (CYP1A2). Full-length complementary DNA clones of IFN-α4, IL-22, 21-OH, 17α-OH, SCC, SOX10, AADC, NALP5, KCNRG, TH, TPH, CYP1A2, and IFN-ω in pTNT vectors (L5610; Promega, Madison, WI) were used for *in vitro* transcription and translation of ^{35}S-radiolabeled recombinant proteins (TNT systems; Promega).

Serum samples (2.5 μL) were incubated overnight with radiolabeled protein (>20,000 cpm) in 96-well filtration plates (Merck Millipore, Billerica, MA). Immune complexes were immobilized and precipitated with protein-A sepharose (nProtein A Sepharose 4 Fast Flow; GE Healthcare, Little Chalfont, United Kingdom) before filters were repeatedly washed. After drying, scintillation fluid (OptiPhase SuperMix; PerkinElmer, Waltham, MA) was added and radioactivity measured in a beta counter (Wallac Microbeta 1450; PerkinElmer). For each antigen, serum from a patient with APS1, selected on the basis of well-characterized autoreactivity, was included as a positive standard. Bovine serum albumin (4%) served as a negative control. Index values were calculated as follows: [(cpm in the unknown sample − cpm in negative standard) ÷ (cpm in the positive standard − cpm in negative standard) × 100]. For IFN-α4, IL-22, 17α-OH, SCC, SOX10, AADC, and IFN-ω, samples were first analyzed as single samples, and subsequently positive samples were reanalyzed in duplicates. For 21-OH, NALP5, KCNRG, TH, TPH, and CYP1A2, all samples were analyzed in duplicates. The parallel analyses of duplicate samples enabled us to exclude and reinvestigate samples with discordant signals.

The upper limit of the normal range was defined as the mean index value for blood donors plus three standard deviations. For 21-OH, the limit for positive index values was set on the basis of the results from a recent interlaboratory study (28). To decide which patients with AAD to include in the *AIRE* gene analyses, the upper limits in the IFN-α4 and IL-22 assays were set to visually optimize the discrimination between healthy controls and patients with known APS1. Commercial kits were used to assay antibodies against TPO (RSR Ltd, Cardiff, UK), islet antigen-2 (Medipan GmbH, Berlin, Germany), glutamic acid decarboxylase 65 (Medipan GmbH), and parietal cells (Orgentec, Mainz, Germany).

AIRE gene sequencing and copy number variation analysis

Patients positive for IFN-α4 and/or IL-22 autoantibodies as well as patients with an APS1 diagnosis were investigated for the presence of *AIRE* mutations. Exons and flanking introns of the *AIRE* gene were amplified with polymerase chain reaction (PCR) and were Sanger sequenced using primers and conditions previously described by Wolff *et al.* (29). The next-generation sequencing of the *AIRE* gene was described in detail in our previous AAD sequencing study (7). In brief, genes were targeted by a custom-designed Roche NimbleGen SeqCap EZ Choice XL Library (06266517001; Basel, Switzerland). Exons and 20 bps of the adjacent introns, as well as 5′ and 3′ untranslated regions, and 2 kbps surrounding the transcription start sites were included. DNA was extracted from blood samples by LGC Genomics (Berlin, Germany) and/or QIAamp Blood Midi Kit (51185; Qiagen, Venlo, Netherlands). DNA fragments of 400 bps were bar-coded, and the final library was sequenced with an Illumina HiSEquation 2500 instrument, producing 100 bp paired-end reads. Sequencing reads were mapped to hg19 with the Burrows-Wheeler Aligner 0.7.4 (30) and subsequently processed by Picard tools (http://broadinstitute.github.io/picard) and GATK 3.3.2 (31–33). Effect prediction was performed with SnpEff (34), and detailed sequence investigation was performed with the integrative genomics viewer (35) and the University of California, Santa Cruz, genome browsers (36).

Copy number variation (CNV) calling was made using CODEX software, which is specifically designed to overcome the biases related to exome capture (37). Before accepted as true, bioinformatically suggested CNVs were inspected in IGV and confirmed with custom-designed PCR primers and reactions (Supplemental Table 1). PCR was conducted with iProof HF MasterMix (Bio-Rad), using 50 to 100 ng of DNA and 1 μM of each primer.

Results

We used radioligand binding assays to screen 677 patients in the SAR (38) for the presence of autoantibodies against IFN-α4, IL-22, and IFN-ω. The assays for IFN-α4 and IL-22 gave the most favorable discrimination between patients with known APS1 and healthy controls (Fig. 1; Supplemental Fig. 1) and were therefore used for selecting patients with AAD for *AIRE* sequencing. In total, we found 17 patients (2.5%) who were positive for autoantibodies against IL-22 and/or IFN-α4, 12 of whom were positive for both autoantibodies. Table 1 presents the positive patients' serological, genetic, and clinical data. Interestingly, four patients were not previously diagnosed with APS1 and thereby represented cases of possibly undiagnosed APS1.

To determine whether the 17 patients with cytokine autoantibodies had a genetic susceptibility for APS1, we sequenced all exons in the *AIRE* gene. In all previously known patients with APS1 (n = 9) included at this stage, Sanger sequencing confirmed disease-causing *AIRE* mutations (Table 1). Sequencing also confirmed APS1 in two additional patients (patients 11 and 12) who were

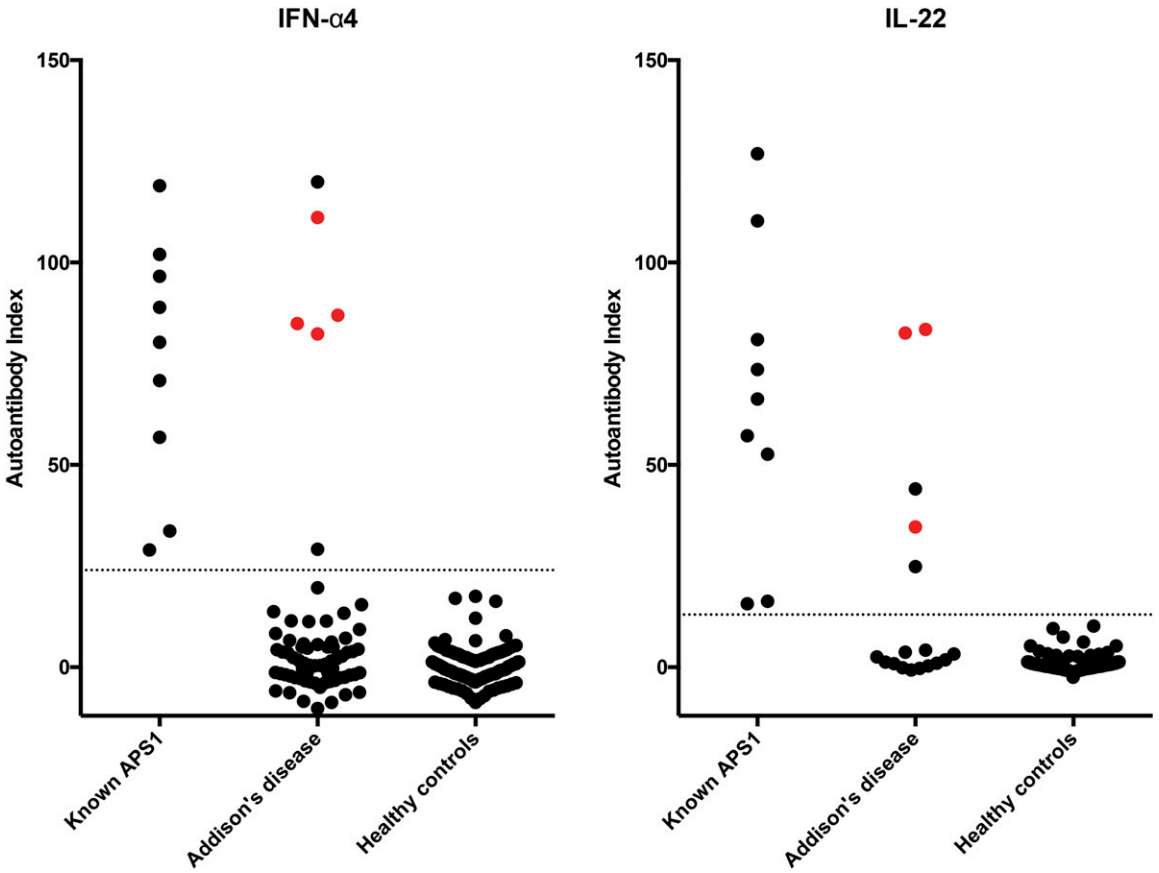

Figure 1. Four patients with Addison disease were positive for IFN-α4 autoantibodies and were later confirmed as having APS1 (red dots). Three of these patients were also found to be positive for IL-22 autoantibodies (red dots). The *y*-axis indicates the autoantibody index. The upper limit of the normal range (dotted line) was set to optimize the discrimination of healthy controls and patients with known APS1.

not previously diagnosed with APS1 but were both found to be homozygous for well-established APS1-causing variants. Patient 13 was found to be a heterozygous carrier of the recessive c.769C>T (p. Arg257ter). At this stage, five patients out of 17 with cytokine autoantibodies did not present with any *AIRE* mutations detected by Sanger sequencing.

In addition to the Sanger sequencing used in clinical practices, we adopted paired-end next-generation sequencing to enable investigation of CNV. Using the CODEX software, we screened our sequenced patients for CNVs on chromosome 21 and found a large deletion affecting the first eight exons of *AIRE* (Supplemental Table 2). The deletion was thought to be present both in homozygosity and heterozygosity in a few of our patients, and that was later confirmed with PCR (chr21: 45701353-45711841; Supplemental Figs. 2–4). Patient 10, in whom Sanger sequencing did not detect any disease-causing variant, was found to be homozygous for the large deletion of the first eight exons. Hence, we also confirmed a genetic basis for disease in this patient newly diagnosed with APS1. Furthermore, patients 7, 8, and 11 were also found to carry the large deletion. Consequently, they were compound heterozygotes with the deletion of either c.967-979del (p. Leu323fs) or c.769C>T (p. Arg257Ter).

All patients with disease-causing APS1 mutations fulfilled at least two of three syndrome components and qualified for a clinical APS1 diagnosis (Table 1). This was also true for all four newly discovered APS1 cases in the SAR. Moreover, all patients with APS1-causing mutations in homozygosity or compound heterozygosity were positive for both IFN-α4 and IL-22 autoantibodies. To complete the serological evaluations, we extended the autoantibody profiling with a panel that included known APS1 autoantigens (Supplemental Table 3). One of the newly diagnosed cases was also found to be positive for autoantibodies against SOX10 and KCNRG. Two newly diagnosed cases were positive for AADC and one for TPO autoantibodies.

The results for IFN-α4 and IL-22 autoantibodies were not concordant in all patients in the SAR. In total, five patients were positive for either IFN-α4 or IL-22 autoantibodies, but not for both. Four of these patients had no pathogenic *AIRE* variant and no additional APS1 manifestations besides AAD. They also had a higher age at onset (range, 32 to 57 years) of the first APS1 manifestation, compared with that of known APS1 cases

Table 1. Serological, Genetic, and Clinical Characteristics of Cytokine Autoantibody-Positive Patients With Addison Disease

	Autoantibodies		*AIRE*	Clinical Criteria				
Patient	IFN-α4	IL-22	Mutations and CNVs[a]	AD	CMC	HP	APS1 Diagnosis	Autoimmune Comorbidity
1	Pos	Pos	c.769C>T (p.Arg257Ter)	Yes	Yes	Yes	Known	EH
2	Pos	Pos	c.769C>T (p.Arg257Ter)	Yes	Yes	Yes	Known	CAG/PA, AA, VIT, EH, ND
3	Pos	Pos	c.769C>T (p.Arg257Ter)	Yes	Yes	Yes	Known	AITD, AA, VIT, EH
4	Pos	Pos	c.769C>T (p.Arg257Ter)	Yes	Yes	Yes	Known	AITD, T1DM, PHG, VIT, ND
5	Pos	Pos	c.769C>T (p.Arg257Ter)	Yes	No	Yes	Known	
6	Pos	Pos	c.769C>T (p.Arg257Ter)	Yes	Yes	Yes	Known	CAG/PA, PHG
7	Pos	Pos	c.769C>T (p.Arg257Ter), Deletion exon 1–8	Yes	No	Yes	Known	
8	Pos	Pos	c.769C>T (p.Arg257Ter), Deletion exon 1–8	Yes	Yes	Yes	Known	CAG/PA, AA, VIT
9	Pos	Pos	c.769C>T (p.Arg257Ter), c.64-69del (p.Val22_Asp23del)	Yes	Yes	Yes	Known	
10	Pos	Pos	Deletion exon 1–8	Yes	No	Yes	New	CAG/PA
11	Pos	Pos	c.967-979del (p.Leu323fs), Deletion exon 1–8	Yes	Yes	Yes	New	
12	Pos	Pos	c.463+2T>C Splice donor variant	Yes	Yes	No	New	
13	Pos	Neg	Heterozygous c.769C>T (p.Arg257Ter)	Yes	No	Yes	New	T1DM
14	Pos	Neg	No mutation	Yes	No	No	No	AITD, CAG/PA, CD
15	Pos	Neg	No mutation	Yes	No	No	No	
16	Neg	Pos	No mutation	Yes	No	No	No	CAG/PA
17	Neg	Pos	No mutation	Yes	No	No	No	T1DM

Abbreviations: AA, alopecia areata; AD, Addison disease; AITD, autoimmune thyroid disease; CAG/PA, chronic atrophic gastritis/pernicious anemia; CD, celiac disease; CMC, chronic mucocutaneous candidiasis; EH, enamel hypoplasia; HP, hypoparathyroidism; ND, nail dystrophy; Neg, negative; PHG, primary hypogonadism; Pos, positive; T1DM, type 1 diabetes mellitus; VIT, vitiligo.

[a]Homozygous when only one allele is presented and nothing else is stated.

(range, 2 to 15 years) (Supplemental Fig. 5). Moreover, they all had autoantibodies against 21-OH.

The clinical diagnostic criteria and *AIRE* sequencing data were consistent in identifying patients with APS1 and served as a gold standard for calculation of a PPV for the occurrence of cytokine autoantibodies. Of the 17 patients who tested positive for at least IFN-α4 or IL-22, 13 were confirmed as having APS1, and thus the PPV corresponded to 76%. Of the 12 patients who tested positive for both IFN-α4 and IL-22, all 12 were confirmed as having APS1, corresponding to a PPV of 100%.

Loss-of-function mutations in *AIRE* are rare in the general Swedish population. In a recent whole-genome sequencing study, all inactivating mutations detected in *AIRE* had allele frequencies of $\leq$0.001, and none of the thousand studied individuals carried any of these alleles in homozygosity (39).

Discussion

Adrenal insufficiency is a major disease component in APS1. We searched the SAR for patients with cytokine autoantibodies as a marker for potential APS1. All patients with autoantibodies against IFN-α4, and/or IL-22 were screened for additional APS1-associated autoantibodies and tested for disease-causing *AIRE* mutations. Using this approach, we were able to identify four hitherto undiagnosed cases of APS1 among the 677 patients in the SAR. These four patients had typical APS1 autoantibody profiles, *AIRE* mutations, and clinical signs of APS1. They also developed autoimmune manifestations at an early age (range, 5 to 17 years), consistent with a diagnosis of APS1.

In our case group, 17 patients had autoantibodies against IFN-α4 and/or IL-22. Of these, 13 fulfilled genetic and clinical criteria for APS1. This left us with four patients who were positive for cytokine autoantibodies but without APS1 manifestations and without disease-causing *AIRE* mutations. The positive autoantibody signals were reproducible in repeated assays and were not caused by background noise. Patients 16 and 17 had IL-22 signals in the range of known APS1 cases, and patient 14 displayed the strongest IFN-α4 signal of all in the study. Nevertheless, besides the cytokine autoantibodies, these patients did not react to many APS1-associated antigens except 21-OH, and

they developed Addison disease later in life than the patients with known APS1.

Alternative causes of IFN autoantibodies include thymomas and monogenic autoimmunity syndromes caused by RAG1/2 mutation (40, 41). Thymomas are thymic neoplasms with insufficient *AIRE* expression and a faulty negative selection of developing T cells (42). The negative selection normally results in apoptosis of T cells reacting strongly with self-peptides. Thymomas are associated with autoantibodies against α-IFNs, IL-1, IL-22, and IL-17A and can present with an APS1-like clinical picture (43). RAG1 and RAG2 proteins are central in V(D)J recombination, the process that initiates the diversification of the B and T cell repertoires. Dysfunction of the RAG genes is associated with severe immunodeficiency and is typically lethal without hematopoietic bone marrow transplantation (44). Milder forms have also been described as accompanied by autoimmune cytopenias, vitiligo, psoriasis, myasthenia gravis, and Guillain-Barré syndrome. RAG deficiency hinders a normal AIRE expression in the thymus and, intriguingly, shares IFN autoantibodies with APS1. However, no inactivating RAG 1/2 mutation was detected in our samples.

Using next-generation sequencing and PCR, we identified and confirmed the presence of a large *AIRE* deletion in four of our patients with APS1. This large deletion could possibly represent the same partially deleted *AIRE* allele as that investigated by Bøe Wolff *et al.* (45) in two Scandinavian patients with APS1. Detailed sequencing data allowed us to narrow the possible regions for the deletion breakpoints in our patients, but the exact range of the deletion suggested by Bøe Wolff *et al.* was not determined in high enough detail to enable a direct comparison. The homozygous carrier of the allele with the large deletion developed APS1. In addition to the deletion, the other *AIRE* mutations we detected had already been described in detail (46). The Finnish mutation (rs121434254, c.769C>T) was the most common mutation among subjects with APS1 in our data (10). We also found a single patient with the pathogenic 13-bp deletion (rs386833675, c.967-979del) common in APS1 case groups in Norway, the United Kingdom, and North America (29, 47–49). This patient was diagnosed with APS1 after our screening, which led to the identification of a sibling who was also confirmed as having APS1 manifested by hypoparathyroidism and candidiasis. Patient 12 was homozygous of a splice donor variant, a T to C substitution at the +2 position of intron 3 (rs786204478, c.463+2T>C), also previously described in APS1 (50–53). Finally, a single patient was heterozygous for a short deletion (rs752303080, c.64-69del) (54). In patient 13, we detected only one mutated *AIRE* allele but could not rule out mutations in introns or regulatory sequences of the gene.

APS1 is a disorder with potentially life-threatening, but treatable, complications. We share the concern of Wolff *et al.* (29) that APS1 is most likely underdiagnosed. With the results from this study, it seems that assorted patients with AAD should benefit from screening for cytokine autoantibodies for early identification of APS1. This screening is especially warranted in pediatric patients who may not have developed more than one component of the syndrome. When a new patient is diagnosed, it is advisable to investigate all siblings for APS1. When the serological profile indicates APS1 in any patient, *AIRE* gene sequencing and copy number analysis should be performed to confirm the diagnosis.

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