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AUTOIMMUNE POLYENDOCRINOPATHY-CANDIDIASIS-ECTODERMAL- DYSTROPHY (APECED) IN SICILY: CONFIRMATION THAT R203X IS THE PECULIAR AIRE GENE MUTATION

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ABSTRACT. Background: Autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED), also known as autoimmune polyendocrine syndrome type 1 (APS-1) (OMIM 240300), is a very rare disease. Accepted criteria for diagnosis require the presence of at least 2 of 3 major clinical features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison's disease (AD). Aim: We analyzed AIRE gene mutations and genotype-phenotype correlation in APECED patients originating from Sicily and in their relatives. Subjects and methods: In 4 patients, clinical evaluations, genetic analysis of AIRE, and APECED-related autoantibodies were performed. Results: Two patients carried the mutation R203X in homozygosis on exon 5. One had the mutation R203X combined with R139X. The fourth had the R203X mutation in heterozygosis with R257X. Expression of the disease showed wide variability of clinical manifestations. Analysis of relatives allowed the identification of 10 heterozygotes for AIRE gene mutations. None of these subjects presented major findings of APECED. Three of the 4 patients were positive for autoantibodies to interferon- γ . Conclusions: In Sicily, R203X is confirmed to be the typical recessive and prevalent AIRE gene mutation on exon 5. Genotype-phenotype correlation failed to reveal a relationship between detected mutations and clinical expression. Mutations in heterozygosity in AIRE gene are not associated with major findings of APECED.

Introduction

Autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED), also known as autoimmune polyendocrine syndrome type 1 (APS-1) (OMIM 240300), is a very rare disease (1). Accepted criteria for diagnosis require the presence of at least 2 of 3 major clinical features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and primary adrenocortical insufficiency (Addison's disease) (AD) (2, 3).

APECED has low overall global prevalence and incidence (<1:100,000/yr) (1) with about 400 cases described worldwide (4). The female-to-male ratio varies from 0.8:1 to 2.4:1, thus revealing a slight female preponderance (1). However, a high prevalence among some populations such as Finns (1:25,000) (2, 5) and Iranian Jews (1:9,000)

(6) has been demonstrated. Unlike most other autoimmune diseases, APECED is a monogenic disorder and the gene responsible is named AIRE, mapped on chromosome 21 (7, 8). APECED is inherited as an autosomal recessive disease (9), but even the possibility of a dominant pattern of inheritance has been suggested (10). To date, more than 60 mutations of the AIRE gene have been identified in APECED patients from different ethnicities (3, 11). Nonsense and missense mutations, deletions and small insertions have been detected (9, 12-14). Some specific mutations were described in conformity with the geographical distribution of patients and interestingly the hot spots of incidence already mentioned correspond to isolated populations, thus suggesting a potential founder effect. The most common and recurrent mutation is R257X in exon 6, recognized in the largest series of patient with APECED in Finland (15) and also in Eastern Europe and Northern Italy (4, 16). The other typical mutation is Y85C in Iranian Jews (6). Furthermore, a 13bp deletion (1094-1106del) has also been observed in other patients of different origin (16).

In Italy, 3 hot spot areas have already been acknowledged: one in Sardinia (1:14,400) (17), another in Apulia (1:35,000) (18), and the third in the Venetian region (1:4,400) (19). Furthermore, in this country, different specific mutations have been demonstrated in the 3 hot spot areas. In APECED patients from Sardinia the peculiar mutation proved to be R139X in exon 3 (17), in patients from Apulia there were 2 different mutations W78R in exon 2 and Q358X in exon 9, and finally in the Venetian region, R257X mutation and del13 were the most frequent (16). Recently, 6 patients with APECED that originated from

Campania a region in the south of Italy, were identified, and mutations in exon 1 peculiar to this region were identified (20).

Three Sicilian patients with APECED have already been described, identifying a peculiar AIRE mutation: R203X in exon 5 (16, 21). This very rare mutation has been detected in 2 brothers associated with R257X and in the third case in homozygosis. These findings led us to hypothesize that R203X mutation can be typical of Sicilian patients with APECED.

In this paper, we report the clinical, immunological, and genetic data of 3 new Sicilian patients affected by APECED. The fourth patient presented in this paper has already been described, but only genetically in a previous paper (16), and is described now from the clinical and immunological point of view, so that the description is now complete.

MATERIALS AND METHODS

Analysis of AIRE gene

Genomic DNA was extracted from peripheral blood. All 14 exons of the AIRE gene were amplified with the use of primers located on the respective flanking introns and were analyzed by direct sequencing using the ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA). The analysis included sequencing of the donor/acceptor sites of all the introns as previously reported (16).

Autoantibodies

Autoantibodies against the following antigens were performed by classical indirect immunofluorescence technique or enzyme-linked immunosorbent assay: thyroglobulin (TgAb), thyroid microsomal (TMAb), thyroperoxidase (TPOAb), parietal cells (PCA), intrinsic factor (IFA), islet cells (ICA), adrenal cortex (ACA), glutamic acid decarboxylase (GADA), steroid-producing cells (StCA), melanin producing cells (MPCAb), and tissue transglutaminase (tTgAb) as previously described (20).

Specifically 21-hydroxylase (21-OHAb) were tested using a kit from RSR Ltd as previously reported (22), 17 α -hydroxylase (17 α -OHAb), side-chain cleavage (sccAb) as previously reported (23), Aromatic-L-Aminoacid decarboxylase (AADC), tryptophan hydroxylase (TPHAb) as previously reported (24), and autoantibodies to interferon- γ (INF γ Ab) were tested at FIRS Laboratories RSR Ltd in Cardiff (UK) using a standard immunoprecipitation assay based on 35S-labeled antigens produced in an in vitro transcription/translation system, as previously described (25).

RESULTS

Patient 1

The first patient is a 27-year-old female with no autoimmune diseases reported in her family history. Her parents were consanguineous as they were first cousins and both were of Sicilian origin. The patient's eldest brother died of an acute unidentified liver disease at 8 months and no autopsy findings were available to determine the cause of his death.

No signs or symptoms of APECED were evident in the proband until the age of 8 yr, when she suffered from a grand mal type seizure. For this event, the patient was admitted to hospital and successfully treated with an anti-epileptic drug. Further tests revealed very low serum calcium and PTH levels, which required supplemental calcium and vitamin D therapy. Thus, diagnosis of CH was made and the previous anti-epileptic therapy was interrupted. Cranial magnetic resonance imaging (MRI) revealed diffuse cranial calcifications.

At the age of 11 yr, the patient developed alopecia areata, which became increasingly severe with complete loss of hair. At 14 yr of age the absence of menarche and the lack of any sign of pubertal development revealed hypogonadotropic hypogonadism, causing primary amenorrhea. Hormone replacement therapy with estrogen and progesterone hormones was necessary in order to induce menarche and to develop and maintain secondary sexual characteristics. A pelvic ultrasound scan was performed and all the pelvic organs examined were normal. The karyotype was not investigated.

At 25 yr, the patient developed Graves' disease with positivity for TPOAb, TgAb, and TSH-Receptor autoantibodies (TRAb). She was treated with methimazole for 24 months, until complete remission of the disease was achieved. At 25 yr of age, a complete evaluation of the clinical components of APECED (Table 1, Patient 1) and autoantibody screening were performed.

CMC affecting the foot nails was evident and the patient denied that these manifestations had been present long before. At 25 yr, she started to suffer from diarrhea with negativity for tTGAb, TPHAb, and PCA. Only IFA were found to be positive. Esophagogastroduodenoscopy (EGDS) revealed an esophageal candida albicans infection with normal gastric pattern and a duodenal biopsy showed chronic inflammation of the lamina propria with lymphocytic intraepithelial infiltration. Colonoscopy was performed without revealing any pathological findings. Diarrhea slowly decreased till complete remission.

The autoantibody screening revealed positivity for autoantibodies against TPOAb and TgAb with normal thyroid function. Persistent normocytic mild anemia was present. INF α -Ab were found to be positive. ACA and 21-OHAb were negative without clinical signs of adrenal insufficiency.

Evaluation of the AIRE gene revealed the presence of the mutation R203X/R203X in homozygosis in exon 5. This mutation was present in heterozygosis in the parents, while the brother was negative for this mutation. Neither of them suffered from any illness related to APECED or other clinical autoimmune diseases. The autoantibody screening was performed in all first-degree relatives and only in the father, affected by a multinodular normofunctional goiter, TgAb were detected.

Patient 2

The second patient is a 36-yr-old female affected by APECED since childhood. Her family history was negative for autoimmune diseases and her parents were not consanguineous. Her father was of Sicilian and her mother of Sardinian origin.

CMC affecting the hand nails was the first manifestation diagnosed at 1 yr, followed by CH at 5 yr of age (Table 1, Patient 2). At 5 yr APECED was diagnosed when the patient was hospitalized because of grand mal type seizures. CH and grand mal type seizures are still both present, as the attempt to reduce the dosage of antiepileptic drugs led the patient to a grand mal type seizure. This finding suggests that grand mal type seizure is not due to low calcium levels, as it persists despite the therapy with calcium and vitamin D. Her somatic and pubertal development was regular, with menarche at the age of 14 yr.

The diagnosis of autoimmune hepatitis (AIH) was performed at 30 yr on the basis of occasional increment of transaminases and with a percutaneous liver biopsy. For AIH, she was treated with different immunosuppressive therapies (azathioprine, cyclosporine, mycophenolate). Unfortunately, none of these drugs was completely effective in inducing clinical and laboratory remission of AIH. Currently AIH is controlled with corticosteroids.

A radiographic examination of the vertebral column performed in 2009 showed loss of the cervical lordotic curve and signs of cervical arthrosis and spondyloarthropathy affecting the dorsal and lumbosacral column, associated with the presence of small marginal osteophytes. Moreover, the patient suffers from osteochondrosis dissecans of both the lateral femoral condyles, at 22 yr, and was edentulous from the age of 18.

Interestingly, in this patient, the AIRE gene harbors two different mutations in compound heterozygosity, R203X in exon 5 and R139X in exon 3 (Table 1, Patient 2).

AIRE gene screening demonstrated the presence of the R203X mutation in her father (Sicilian origin) and the R139X mutation in her mother (Sardinian origin). Her 29-yr-old sister is heterozygous for R203X. In this patient, the autoantibody screening was negative, including INF α Ab. Furthermore, the parents and sister were not affected by clinical autoimmune diseases and also the autoantibody screening was negative.

Patient 3

The third APECED patient is a 38-yr-old male with negative family history for autoimmune diseases, whose parents were both from Sicily without consanguinity.

The first autoimmune manifestation of APECED was CH, diagnosed at the age of 4 yr; from this period therapy with calcium and vitamin D was started. At the age of 15 yr the patient began to suffer from oral CMC infection, now controlled with periodical treatment with specific drugs. AD became evident with an acute adrenal crisis at 25 yr and from diagnosis the patient started adrenal steroid replacement therapy.

At 27 yr routine laboratory examination showed hyper-

transaminasemia: on this occasion liver-kidney microsomal autoantibodies were negative but some years later the patient was found to be positive for AADC, and AIH was diagnosed (Table 1, Patient 3). The autoantibody screening performed at 38 yr, in addition to AADC, revealed TPHAb, a marker of an autoimmune gastrointestinal disease (26), but this patient does not manifest gastrointestinal dysfunction. Furthermore, INF α Ab were found to be positive.

The AIRE gene evaluation revealed the presence of a compound mutation constituted by R203X mutation in exon 5 and R257X in exon 6 (Table 1, Patient 3).

The genetic study of his family revealed a heterozygous state for R203X in his father and sister, and R257X in heterozygosis in the mother.

Autoantibody screening revealed positivity for TPOAb only in the father, with no biochemical or ultrasound alterations of the thyroid gland.

Patient 4

The fourth patient is a 32-yr-old female born in Turin. Her parents were not consanguineous; her mother and her paternal grandmother were of Sicilian origin. None of them suffered from any autoimmune disease.

A sister died of an unidentified acute cardiac disease at the age of 5 months. Another sister is alive and suffers from CMC of the vagina and primary autoimmune hypothyroidism requiring hormone replacement therapy. At the age of 6 yr she developed vitiligo on her hand and from the age of 12 it became diffuse.

The first manifestation of APECED was CH, diagnosed at the age of 10 yr during hospitalization because of frequent painful muscular spasms. From the age of 12 yr, the patient suffered from enamel dysplasia. From the same age, she developed pernicious anemia with autoimmune gastritis confirmed by EGDS and by autoantibody profile. Primary amenorrhea with hypergonadotropic hypogonadism was diagnosed at the age of 14 yr, because of the absence of menarche and the lack of any sign of pubertal development. She was successfully treated with hormone replacement therapy with estrogen and

AIRE gene mutation in APECED patients in Sicily

progesteron hormones. The pelvic organs examined with ultrasound scan were normal. The karyotype was not investigated, but autoantibody screening performed many years after demonstrated the presence of sccAbs, considered markers of lymphocytic oophoritis (27).

AD was biochemically diagnosed at the age of 22 yr during a complete hormonal and biochemical evaluation, without clinical manifestations, and 21-OHAb were found to be positive. Afterwards, the patient received glucocorticoid and mineralocorticoid replacement therapy. At the age of 27 yr, gastric pain and severe steatorrhea appeared. To identify the cause of these manifestations in the absence of tTGAb, but, in the presence of TPHAb, an EGDS was performed and revealed an esophageal candida albicans infection and lymphocytic infiltration of the antrum, body, and fundus of the stomach. However, lymphocytic infiltration was also present in the duodenum without villous atrophy. In this patient, serotonin-producing cells were completely absent in the stomach and duodenum, suggesting that the gastrointestinal dysfunction may be due to an autoimmune disease of APUD cells (24, 26). Among relevant organ- and non organ-specific autoantibodies, in addition to the above-mentioned autoantibodies the patient was found to be positive for ADDC without any alteration of liver function, as well as for ICA and GADAb without alteration of the oral glucose tolerance test, and also INF α Ab were found to be positive.

The AIRE gene evaluation of this patient revealed the presence of R203X mutation in homozygosis in exon 5 (Table 1, Patient 4).

This mutation was present in heterozygosis in the parents. Autoantibody screening performed in the parents showed the positivity of thyroid autoantibodies in the father without any ultrasound thyroid alteration and normal thyroid function; in the mother, it showed PCA positivity, with autoimmune gastritis confirmed by EGDS.

DISCUSSION

Three Sicilian patients with APECED were previously described (16, 21). Two of them were sibs with R203X mutation in exon 5, combined with R257X mutation in exon 6 (21), and the third patient was homozygous for R203X (16). On the basis of these findings we hypothesized that R203X could be the peculiar mutation of APECED in Sicily.

In this paper, we report the clinical, immunological and genetic data relating of 3 new cases of APECED patients of Sicilian origin. The fourth case has already been published as case number 24 in a previous paper (16) and in this paper we describe the clinical and immunological features. R203X mutation of the AIRE gene was found in homozygosis in the first and fourth case. The second case had the combination of the AIRE gene mutation R203X with R139X. The mutation R203X was inherited from the father, who was of Sicilian origin, and the R139X mutation was inherited from the mother, who was of Sardinian origin. According to previously reported data R139X is known to be the typical mutation of Sardinian patients with APECED (17). The third case was characterized by the R203X mutation combined with R257X, as in the case of the two brothers previously published (21). The present data confirm our previous hypothesis (16, 21) that Sicilian patients with APECED share a typical and peculiar mutation: R203X in exon 5.

The R203X mutation (c.68C>T) determines a substitution of arginine with a stop codon. In addition to the typical mutations already discovered in Sardinia (17), Apulia (16, 18), Campania (20), and the Venetian region (16, 19), we now suggest including the R203X mutation as typical of patients from Sicily. This genetic heterogeneity of APECED in Italy can be related to the different founder effect for different populations. We are now searching for patients with APECED originating from other Italian regions in order to complete the genetic map of APECED in Italy.

As regards, the clinical phenotypic pattern of Sicilian patients with APECED, all the patients reported on in this paper were affected by CMC and CH, but only 50% were affected by AD. The frequency of AD is lower in comparison with APECED patients from other Italian regions, where AD was diagnosed in 82-83%. Specifically, in Sicilian patients CH developed at mean age 6.5 yr, CMC at mean age 13.5 yr and AD at mean age 23.5 yr. Only CH appeared at the same mean age as in other Italian APECED patients, while CMC and AD developed at an older age (16-18). Moreover, the absence of ACA and/or 21-OHAb in the 2 cases without AD indicates that the risk of AD is not imminent.

In order to explain why the 2 cases without adrenal autoantibodies are still free of disease, it is reasonable to hypothesize they will develop AD in the near follow-up. Of course, they will be strictly followed for both adrenal autoimmunity and specific biochemical pattern of AD. Recently, IFN γ Ab have been recognized as specific APECED markers (3). These antibodies were found to be positive in 3 of our patients and the only 1 negative case developed APECED more than 30 yr before.

Other autoimmune disorders such as hypergonadotropic hypogonadism, alopecia, vitiligo, atrophic gastritis with or without pernicious anemia, autoimmune hepatitis, thyroid autoimmune diseases and Type 1 diabetes mellitus, and autoantibodies directed against target organs are very frequently found in APECED patients (15, 27-30). These autoantibodies can also be demonstrated before the onset of the respective disorder (3, 4, 29), thus allowing early predictive diagnosis.

In our patients, these disorders or many autoantibodies without respective clinical disease were also present. It appears to be important to remember that the frequency of thyroid autoimmune diseases greatly varied in patients with APECED, being present in 0% in Sardinia (17), 11% in Veneto (29), 36% in Apulia (31), and 1% in Finland (15). Furthermore, Graves' disease is exceptional as it was demonstrated in only 1 of the 54 patients with APECED described so far in Italy (29) and in none of the 91 Finnish patients (15). It is to be stressed that the only Sicilian patient with autoimmune thyroid disease, was affected by Graves' disease.

Idiopathic gastrointestinal dysfunction (GID), with chronic diarrhea or stypsis, was described in APECED patients in association with TPHA (26). For this reason TPHA were investigated in the four Sicilian patients and were found to be positive in 2. One of these 2 had GID (Table 1, Case 4) and an EGDS revealed the presence an autoimmune disease directed against APUD cells (24, 26). The same condition was also present in the 2 previously reported cases with APECED from Sicily (21),

indicating that this autoimmune disease too could be more frequent in the presence of the Sicilian AIRE gene mutation.

APECED is a recessive inherited disease, and in general parents are heterozygotic for the mutations found in the probands. The parents and some first-degree relatives of our APECED Sicilian patients were also studied for AIRE gene mutations and clinical and serological autoimmunity. We identified 10 first-degree relatives bearing heterozygosity for R203X or for other mutations such as R257X and R139X, but they did not show any clinical manifestation typical of APECED, thus confirming that these are recessive mutations as demonstrated for other AIRE mutations (16).

Our confirmation of a common Sicilian APECED mutation, in addition to facilitating its genetic diagnosis, points to regional specificity and separate founder effects in Italy and provides new clues for further investigating the involvement of AIRE in autoimmune diseases.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1 - *Clinical manifestation and age at presentation in the 4 cases from Sicily.*

| Clinical manifestations | Case 1 Age at onset (yr) | Case 2 Age at onset (yr) | Case 3 Age at onset (yr) | Case 4 Age at onset (yr) |
|-----------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Chronic mucocutaneous candidiasis | 24 | 1 | 15 | 12 |
| Chronic hypoparathyroidism | 8 | 5 | 4 | 10 |
| Addison's disease | - | - | 25 | 22 |
| Hypergonadotropic hypogonadism | 14 | - | - | 14 |
| Alopecia areata | 11 | - | - | - |
| Vitiligo | - | - | - | 6 |
| Autoimmune hepatitis | -- | 30 | 27 | - |
| Graves' disease | 25 | - | - | - |
| Gastrointestinal dysfunction | 25 | - | - | 27 |
| Grand mal type seizures | - | 5 | - | - |
| Nail dystrophy | - | - | - | - |
| <i>AIRE</i> gene mutations | R203X/R203X | R203X/R139X | R203X/R257X | R203X/R203X |
| INF ω Ab | Positive | Negative | Positive | Positive |

INF ω Ab: interferon ω autoantibodies.