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 diagno- sis require the presence of at least 2 of 3 major clinical fea- tures: chronic mucocutaneous candidiasis (CMC), chronic hy- poparathyroidism (CH), and Addison's disease (AD). Aim: We analyzed AIRE gene mutations and genotype-phenotype cor- relation 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 car- ried 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. Expres- sion of the disease showed wide variability of clinical mani- festations. 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-□. 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 relation- ship between detected mutations and clinical expression. Mutations in heterozygosity in AIRE gene are not associat- ed with major findings of APECED.

Introduction

Autoimmune polyendocrinopathy-candidiasis-ectoder- mal-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 fea- tures: chronic mucocutaneous candidiasis (CMC), chron- ic 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 world- wide (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 autoim- mune diseases, APECED is a monogenic disorder and the gene responsible is named AIRE, mapped on chro- mosome 21 (7, 8). APECED is inherited as an autosomal recessive disease (9), but even the possibility of a domi- nant 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 eth- nicities (3, 11). Nonsense and missense mutations, dele- tions and small insertions have been detected (9, 12-14). Some specific mutations were described in conformity with the geographical distribution of patients and inter- estingly the hot spots of incidence already mentioned correspond to isolated populations, thus suggesting a potential founder effect. The most common and recur- rent 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 oth- er typical mutation is Y85C in Iranian Jews (6). Further- more, a 13bp deletion (1094-1106del) has also been ob- served in other patients of different origin (16).

In Italy, 3 hot spot areas have already been acknowl- edged: 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 spe- cific mutations have been demonstrated in the 3 hot spot areas. In APECED patients from Sardinia the peculiar mu- tation 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 iden- tified (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 de-

tected in 2 brothers associated with R257X and in the third case in homozygosis. These findings led us to hy- pothesize 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 ex- ons of the AIRE gene were amplified with the use of primers lo- cated on the respective flanking introns and were analyzed by di- rect 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 re- ported (16).

Autoantibodies

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

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

RESULTS

Patient 1

The first patient is a 27-year-old female with no autoim- mune 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 autoptic 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 an- ti-epileptic drug. Further tests revealed very low serum calcium and PTH levels, which required supplemental cal- cium and vitamin D therapy. Thus, diagnosis of CH was made and the previous anti- epileptic therapy was inter- rupted. Cranial magnetic resonance imaging (MRI) re- vealed diffuse cranial calcifications.

At the age of 11 yr, the patient developed alopecia area- ta, 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 hy- pergonadotropic hypogonadism, causing primary amen- orrhea. Hormone replacement therapy with estrogen and progesteron hormones was necessary in order to induce menarche and to develop and maintain secondary sexu- al characteristics. A pelvic ultrasound scan was performed and all the pelvic organs examined were normal. The caryotype was not investigated.

At 25 yr, the patient developed Graves' disease with pos- itivity for TPOAb, TgAb, and TSH-Receptor autoanti- bodies (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 infec- tion 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 au- toantibodies against TPOAb and TgAb with normal thy- roid function. Persistent normocytic mild anemia was pre- sent. INF□-Ab were found to be positive. ACA and 21- OHAb were negative without clinical signs of adreno- cortical 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 normo- functional goiter, TgAb were detected.

Patient 2

The second patient is a 36-yr-old female affected by APECED since childhood. Her family history was nega- tive for autoimmune diseases and her parents were not consanguineous. Her father was of Sicilian and her moth- er 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 pa- tient 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 cal- cium 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 per- formed 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 ef- fective in inducing clinical and laboratory remission of AIH. Currently AIH is controlled with corticosteroids.

A radiographic examination of the vertebral column per- formed 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. More- over, 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□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 neg- ative family history for autoimmune diseases, whose par- ents 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 microso- mal 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, re- vealed TPHAb, a marker of an autoimmune gastroin- testinal disease (26), but this patient does not manifest gastrointestinal dysfunction. Furthermore, INF□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 het- erozygosis in the mother.

Autoantibody screening revealed positivity for TPOAb only in the father, with no biochemical or ultrasound al- terations 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 fre- quent painful muscular spasms. From the age of 12 yr, the patient suffered from enamel dysplasia. From the same age, she developed pernicious anemia with au- toimmune gastritis confirmed by EGDS and by autoanti- body profile. Primary amenorrhea with hypergonadotro- pic 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 treat- ed with hormone replacement therapy with estrogen and

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

AD was biochemically diagnosed at the age of 22 yr dur- ing a complete hormonal and biochemical evaluation, without clinical manifestations, and 21-OHAb were found to be positive. Afterwards, the patient received gluco- and mineralcorticoid replacement therapy. At the age of 27 yr, gastric pain and severe stypsis appeared. To iden- tify the cause of these manifestations in the absence of tTGAb, but, in the presence of TPHAb, an EGDS was per- formed and revealed an esophageal candida albicans in- fection and lymphocytic infiltration of the antrum, body, and fundus of the stomach. However, lymphocytic infil- tration was also present in the duodenum without villous atrophy. In this patient, serotonin-producing cells were completely absent in the stomach and duodenum, sug- gesting 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 pa- tient was found to be positive for ADDC without any al- teration of liver function, as well as for ICA and GADAb without alteration of the oral glucose tolerance test, and also INF□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 par- ents. Autoantibody screening performed in the parents showed the positivity of thyroid autoantibodies in the fa- ther without any ultrasound thyroid alteration and nor- mal thyroid function; in the mother, it showed PCA pos- itivity, with autoimmune gastritis confirmed by EGDS.

DISCUSSION

Three Sicilian patients with APECED were previously de- scribed (16, 21). Two of them were sibs with R203X muta- tion 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 homozy- gosis 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 fa- ther, who was of Sicilian origin, and the R139X mutation was inherited from the mother, who was of Sardinian ori- gin. 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 pre- sent 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 or- der to complete the genetic map of APECED in Italy.

As regards, the clinical phenotypic pattern of Sicilian pa- tients 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 com- parison with APECED patients from other Italian regions, where AD was diagnosed in 82-83%. Specifically, in Si- cilian 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 au- toantibodies 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□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 hypergonadotrop- ic hypogonadism, alopecia, vitiligo, atrophic gastritis with or without pernicious anemia, autoimmune hepatitis, thy- roid 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 al- lowing 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 frequen- cy of thyroid autoimmune diseases greatly varied in pa- tients with APECED, being present in 0% in Sardinia (17), 11% in Veneto (29), 36% in Apulia (31), and 1% in Fin- land (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 af- fected by Graves' disease.

Idiopathic gastrointestinal dysfunction (GID), with chron- ic 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 au- toimmunity. We identified 10 first-degree relatives bear- ing 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 muta- tion, 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 in- volvement 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 per- ceived 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	-	-	-	-
AIRE gene mutations	R203X/R203X	R203X/R139X	R203X/R257X	R203X/R203X
INFωAb	Positive	Negative	Positive	Positive

INFωAb: interferon ω autoantibodies.