

Original

Absence of Heterozygous K83E and R257X Mutations of the AIRE-1 Gene in 46 Children with Type 1 Diabetes and 44 Children with Graves' Disease

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Abstract. Type 1 diabetes mellitus (DM) and Graves' disease are autoimmune diseases, and a number of genetic factors, including HLA and CTLA-4 genes, have been reported to contribute to their etiology. The gene responsible for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) has been cloned and named the autoimmune regulator-1 (*AIRE-1*) gene. AIRE-1 protein is thought to be a transcription regulatory protein and to have a role in the maintenance of immunological tolerance. The aim of this study was to determine whether heterozygous *AIRE-1* gene mutations are associated with childhood-onset type 1 diabetes and Graves' disease in the Japanese population. We investigated 46 children with type 1 DM (29 females and 17 males; age at the time of diagnosis, 0.5–16 yr) and 44 children with Graves' disease (34 females and 10 males; age at the time of diagnosis, 3–16 yr) for the presence of the K83E mutation in exon 2 and the R257X mutation in exon 6 of the *AIRE-1* gene. The alleles were identified by polymerase chain reaction of genomic DNA and restriction fragment-length polymorphism analysis (PCR-RFLP) with endonuclease *TaqI*. Since no patients with type 1 DM or Graves' disease were found to carry the K83E or the R257X heterozygous mutation, we concluded that neither the K83E nor the R257X heterozygous mutation in the *AIRE-1* gene seem to be the cause of the more common isolated endocrinopathies, i.e., type 1 diabetes mellitus and Graves' disease, in Japanese children.

Key words: *AIRE*, type 1 diabetes mellitus, Graves' disease, children

Introduction

Type 1 diabetes mellitus (DM) and Graves' disease are autoimmune diseases, and a number of genetic and environmental factors are thought to be involved in their etiology. We have demonstrated an association between both HLA class II genotype and *CTLA-4* (cytotoxic T lymphocyte antigen-4) gene polymorphism and

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childhood-onset type 1 DM (1, 2). The incidences of HLA-DRB1*0405, *0901, and DQB1*0303, *0401 were found to be significantly higher in children with type 1 DM than in controls, whereas the incidences of DRB1*0803, *1501, *1502 and DQB1*0301, *0601, *0602 were significantly lower (1). The G allele in the *CTLA-4* A/G polymorphism at position 49 was more frequent in type 1 DM children than in controls (2). We have also found an association between both HLA class II genotype and *CTLA-4* gene polymorphism and childhood-onset Graves' disease in Japanese patients (submitted for publication).

In 1994 Aaltonen *et al.* assigned the disease locus in Finnish families with APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) to chromosome 21q22.3 based on the results of a linkage analysis between two markers, D21S49 and D21S171 (3), and in 1997 a novel gene was isolated from this region and named the *AIRE-1* (autoimmune regulator-1) gene (4, 5). The *AIRE-1* gene is composed of 14 exons. *AIRE-1* protein is expressed in the thymic medulla, where T-cell immune tolerance is established, as well as in the lymph nodes, spleen, and fetal liver, but it is not expressed in the affected organs of APECED patients. Kogawa *et al.* recently found that *AIRE-1* protein is also restrictively expressed in peripheral CD14-positive monocytes and in differentiated dendritic cells (6).

APECED is an autosomal recessive disease that is especially frequent in Finnish and Iranian Jews, and is characterized by the simultaneous presence of at least two of three major diseases in the same individual: hypoparathyroidism, Addison's disease, and chronic mucocutaneous candidiasis. It may also be associated with clinical manifestations of autoimmune thyroid disease, type 1 DM, and gonadal dysfunction. APECED causes multiple organ dysfunction in a wide variety of endocrine and extra-endocrine organs, and production of autoantibodies against the affected organs and lymphocyte invasion of the affected organs have frequently been

demonstrated.

A high percentage of patients with type 1 DM produce autoantibody against the thyroid gland as well as against the β cells of the pancreas and have autoimmune thyroid disease, including Graves' disease and Hashimoto thyroiditis (7, 8). The patients with Graves' disease may not only have autoantibodies against thyroid epithelial cells or the TSH receptor, but antinuclear antibodies and antineutrophil cytoplasmic antibodies (ANCA) that are not specific for endocrine organs (9).

These findings suggest that patients with type 1 DM and Graves' disease tend to have autoimmune reactions not only against specific target organs but against several other organs and that they may possess heterozygous *AIRE-1* mutations. In this study we examined Japanese children with isolated type 1 DM or Graves' disease for the presence of heterozygous *AIRE-1* mutations. As a first step we analyzed the R257X heterozygous mutation in exon 6 and the K83E heterozygous mutation in exon 2 which can be easily analyzed. The R257X mutation is the most common mutation in APECED which has been reported in many ethnic groups and in exon 2 many kinds of missense mutation have been reported.

Subjects

Forty-six unrelated children with type 1 DM (17 males and 29 females; age at the time of diagnosis, 0.5–16 yr) and 44 unrelated children with Graves' disease (10 males and 34 females; age at the time of diagnosis, 3–16 yr) were the subjects of this study.

Type 1 DM was diagnosed according to the criteria of the WHO Study Group (10). The age of the subjects with type 1 DM at the time of diagnosis ranged from six mo to 16 yr. The interval between the time of diagnosis and examination for mutations ranged widely, from 0 to 18 yr. Twenty-one of the 39 DM patients tested were positive for anti-GAD antibody, and 20 of the 34 DM patients

tested were positive for IA-2 antibody.

Graves' disease was diagnosed on the basis of the presence of biochemical hyperthyroidism, the presence of TSH receptor antibodies (TRAb), and the presence of clinical evidence, such as palpable diffuse goiter, exophthalmos, or tachycardia, plus the absence of other causes of hyperthyroidism. The age of the Graves' disease patients at the time of diagnosis ranged from 3 to 16 yr. TRAb was detected in 38 of the 44 Graves' disease patients. The microsome test (MCHC) was positive in 37 of the 44 Graves' disease patients, and the thyroid test (TGHA) was positive in 10 of the 44.

None of the patients had a past history or family history of suspected APECED.

This study was carried out in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects and/or their parents.

Method

In this study we analyzed *AIRE-1* genes for an A>G transition in exon 2 (K83E mutation) and a C>T transition in exon 6 (R257X mutation). DNA was extracted from peripheral blood leukocytes with Dna Quick II (Dainihonsei-yaku, Japan). The *AIRE-1* gene mutations were typed by PCR of genomic DNA and restriction fragment-length polymorphism analysis (PCR-RFLP) (4).

The primers used for the K83E mutation were 5'-TCCACCACAAGCCGAGGAGAT-3' and 5'-ACGGGCTCCTCAAACACCACT-3'. The PCR was performed in a thermocycler at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 68°C for 30 sec, and a final step at 68°C for 10 min. The amplified products were dissolved with restriction enzyme *TaqI* (Roche, Germany) and run on a 2% agarose gel. The A allele corresponds to the 424-base pair (bp) uncleaved fragment with no *TaqI* site and the G allele corresponds to the presence of 261-bp and 163-bp cleaved fragments generated by *TaqI* dissolution.

The primers used for the R257X mutation

were 5'-GCGGCTCCAAGAAGTGCATCCAGG-3' and 5'-CTCCACCCTGCAAGGAAGAGGGGC-3'. The PCR was performed in a thermocycler at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec, and a final step at 72°C for 10 min. The amplified products were dissolved with restriction enzyme *TaqI* and run on polyacrylamide gel. The C allele corresponds to the presence of three cleaved fragments (222 bp, 61 bp, and 55 bp), and the T allele corresponds to the presence of two cleaved fragments (283 bp and 55 bp) generated by *TaqI* dissolution (4).

Results

The results are shown in the Fig. 1. Analysis of the A>G transition in exon 2 (K83E mutation) revealed the 424-bp uncleaved fragment in all of the type 1 DM and Graves' disease subjects, and analysis of the C>T transition in exon 6 (R257X mutation) revealed three cleaved fragments (222 bp, 61 bp, and 55 bp) in all of the subjects. None of the childhood-onset type 1 DM or Graves' disease subjects had the K83E or R257X heterozygous mutation in the *AIRE-1* gene.

Discussion

Meyer *et al.* examined 139 patients with Graves' disease, 224 with type 1 DM, 83 with Addison's disease, and 75 with Hashimoto's thyroiditis, in Germany, for an association between heterozygous *AIRE-1* mutations and isolated autoimmune endocrinopathy (11). The R257X mutation was found in one patient with Hashimoto's thyroiditis in its heterozygous form, but not in any patients with type 1 DM, Graves' disease, or Addison's disease. It was noteworthy that the patient who had the R257X mutation in its heterozygous form later developed other endocrine diseases.

In the present study in Japanese patients we did not find any K83E or R257X heterozygous mutations of the *AIRE-1* gene in 46 children with

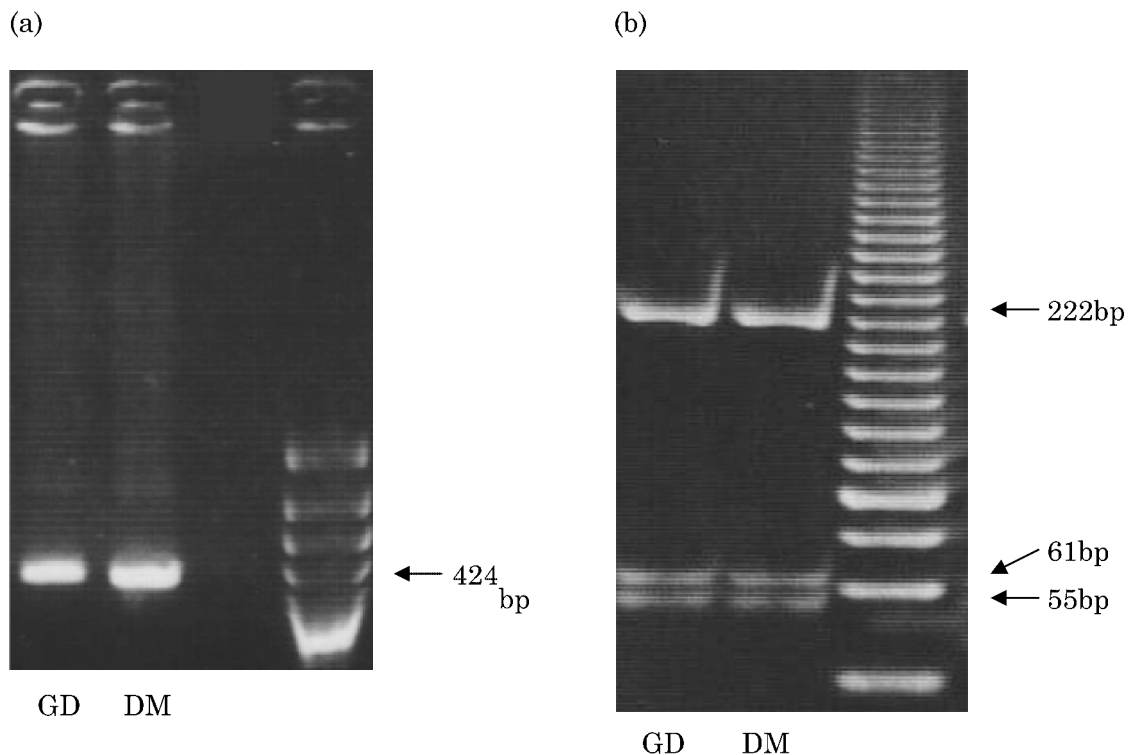


Fig. 1 Electrophoretograms showing the results of digestion with TaqI. (a) Analysis for the A>G transition (K83E mutation) in exon 2. Figure (a) shows the 424-bp uncleaved fragment in two representative samples of type 1 DM and Graves' disease (GD) subjects. (b) Analysis for the C>T transition (R256X mutation) in exon 6. Figure (b) shows three cleaved fragments (222 bp, 61 bp, and 55 bp) in two representative samples of type 1 DM and Graves' disease (GD) subjects.

type 1 DM and 44 children with Graves' disease. These results suggest the absence of any association between K83E or R257X heterozygous mutations and isolated type 1 DM and Graves' disease in Japanese children.

Although APECED is very rare in Japan, the following mutations have been discovered in Japanese patients: L28P in exon 1 and IVS9-1G>C (12), missense mutation R15C in exon 1 (13), insertion of a cytosine at nucleotide 29635 in exon 10, and a deletion of guanine at nucleotide 33031 in exon 13 (14). However, there have been no reports of Japanese APECED patients with R257X or K83E mutations. An analysis of the heterozygous mutations that have been discovered in Japanese APECED patients should be included

in future studies of genetic factors in childhood-onset type 1 DM and Graves' disease in Japanese patients.

In conclusion, no K83E and R257X heterozygous mutations in the *AIRE-1* gene were found in Japanese children with isolated type 1 DM or Graves' disease. Further study is needed to conclude whether there is an association between heterozygous *AIRE-1* gene mutations or polymorphisms and isolated type 1 DM or Graves' disease in Japanese patients.

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