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# Disease specificity of anti-tryptophan hydroxylase-1 and anti-AIE-75 autoantibodies in APECED and IPEX syndrome

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**Abstract** Autoantibodies to autoimmune enteropathy-related 75 kDa antigen (AIE-75) and villin are disease markers of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which is characterized by a peripheral tolerance defect. On the other hand, anti-tryptophan hydroxylase-1 (TPH-1) antibodies are detected in autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy (APECED), a central tolerance defect, especially when complicated with gastrointestinal dysfunction. However, to date, anti-AIE-75 and anti-villin antibodies or anti-TPH-1 antibodies have not been tested in APECED or IPEX syndrome, respectively. In the present study, we confirmed the disease specificity of both anti-AIE-75 and anti-TPH-1, although anti-villin antibodies were detected in some patients with APECED. Our observation suggests that immunotolerance to AIE-75 depends on the peripheral mechanism, whereas the tolerance to TPH-1 depends on the central mechanisms.


*Abbreviations:* AIE-75, autoimmune enteropathy-related 75 kDa antigen; AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy; CMC, chronic mucocutaneous candidiasis; FOXP3, forkhead box transcription factor 3; GST, glutathione-S-transferase; IPEX syndrome, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; mTEC, medullary thymic epithelial cells; TPH-1, tryptophan hydroxylase-1; Treg, regulatory T; TSA, tissue-specific antigen

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## 1. Introduction

Both T cells and B cells acquire their diversification by random recombination of T cell receptor (TCR) and B cell receptor (BCR) genes, respectively. This results in generation of a significant number of self-reactive T and B lymphocytes, but the majority of them are eliminated or suppressed by several mechanisms that contribute to immunological tolerance [1,2]. Autoimmune regulator, *AIRE*, is involved in the intrathymic expression of tissue-specific antigens (TSAs) and plays a critical role in the negative selection of self-reactive T cells, also known as central immunotolerance [1]. Although some self-reactive T cells escape negative selection and efflux to periphery, they are in anergic state or inactivated by regulatory T (Treg) cells [2,3]. Forkhead box transcription factor 3, *FOXP3*, is a master gene in the development of Treg cells and contributes to peripheral dominant immunotolerance [2,3]. Failure of the immunotolerance mechanisms causes multiple organ-specific autoimmune disorders. Mutations of *AIRE* gene result in autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy (APECED) which is characterized by autoimmunity to endocrine tissues such as parathyroid gland and adrenal gland, and to cytokines critical for antifungal immunity, interleukin-17 [1]. Mutations of *FOXP3* genes cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is characterized by autoimmune enteropathy and endocrinopathies such as type-1 diabetes mellitus and thyroiditis [4,5].

We have identified autoimmune enteropathy-related 75 kDa antigen (AIE-75) and an actin binding protein, villin, as target autoantigens of enteropathy in IPEX syndrome [6–8]. Recent studies have confirmed the high specificity and sensitivity of these two antibodies regardless of ethnicity [9,10]. On the other hand, gastrointestinal dysfunction is observed in about 10% of APECED patients. Autoantibodies against tryptophan hydroxylase (TPH)-1 are detected in 89% of the patients with APECED complicated by gastrointestinal dysfunction and 34% of the patients without gastrointestinal complications [11–14]. Recently, Sayar et al. have suggested that some cases of APECED with gastrointestinal dysfunction could mimic IPEX syndrome [15]. Nevertheless there have been no studies that tested anti-AIE-75 or anti-villin antibodies in APECED and anti-TPH-1 antibodies in IPEX syndrome. In the present study, we examined autoantibodies to TPH-1, AIE-75 and villin in APECED and IPEX syndrome.

## 2. Materials and methods

### 2.1. Patients and sera

We investigated 7 patients with IPEX syndrome (6 Japanese and 1 American) and 23 patients with APECED (20 Italian, 2 Japanese and 1 American) (Tables 1 and 2). This work was approved by the Institutional Review Board of Hokkaido University Hospital with written informed consent from the patients or guardians. Clinical and laboratory features and genetic mutations of some patients have previously been reported [8,14,16,17]. Among the twenty Italian patients with APECED, 10 were positive for anti-TPH-1 antibodies as judged from immunoprecipitation (IP-positive), whereas the other 10 were negative for the antibodies (IP-negative) [13,14]. Sera from 2 Japanese and 1 American (Irish/Spanish) patients have not been tested for the antibodies by IP (IP-NT). Sera were obtained from the patients and stored at −20 °C until use.

### 2.2. Production of recombinant fusion proteins

Recombinant human TPH-1 was expressed as a fusion protein with glutathione-S-transferase (GST). Briefly, the primer pair was designed to amplify whole coding region with *BamHI* restriction site at the 5′ end and *XhoI* site at the 3′ end as the following; 5′-GGATCCATGATTGAAGACAATAAGGAG-3′, and 5′-CTCGAGTTAGATACTCGGCTTCCTGCT-3′. Complimentary DNA encoding TPH-1 (NM_004179) was amplified by polymerase chain reaction (PCR) using λgt11 human duodenal cDNA library (BD Biosciences Clontech, Palo Alto, CA) as a template. The PCR product was inserted into pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA), digested with both *BamHI* and *XhoI* and then subcloned into a GST fusion protein expression vector, pGEX4T-2. *Escherichia coli*, *BL-21*, was transformed with the plasmid containing correct nucleotide sequence of TPH-1. Fusion protein, GST-TPH-1, was expressed in the presence of 0.5 mM isopropylthiogalactoside (IPTG) and purified with glutathione-sepharose beads (Amersham Biosciences, Piscataway, NJ). Recombinant AIE-75 and GST-villin were expressed and used for immunoblotting as previously reported [7,8].

### 2.3. Immunoblotting

A 60 ng of the recombinant antigens was subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel, and electrically transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% skim milk, the membranes were incubated with 1:200 diluted rabbit polyclonal anti-TPH-1 antibody (Sigma Aldrich), 1:1000 diluted goat anti-GST antibody (Amersham Biosciences), or 1:80–1:5120 diluted human sera. Human sera were diluted with Tris-buffered saline containing 0.1% Tween-20 (TBST) and crude lysate prepared from *E. coli* expressing GST to block potential cross-reactivity with GST or components of *E. coli* except for some experiments. After incubation with primary antibodies, membranes were washed with TBST three times and incubated with diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG at 1:5000 (Biosource, Camarillo, CA), HRP-conjugated goat anti-rabbit IgG at 1:2000 (Biosource) or HRP-conjugated rabbit anti-goat IgG (Biosource) at 1:2000 for 1 h at room temperature. All the secondary antibodies were diluted with TBST. After washing with 50 mM Tris–HCl pH 7.6, immunoreactive bands were detected by 3,3′-diaminobenzidine (Sigma, St. Louis, MO) and nickel ion (0.03% $NiCl_2$).

## 3. Results

### 3.1. Production of recombinant fusion protein

The fusion protein was immunoreactive on blots with either anti-GST or anti-TPH-1 antibody (Fig. 1). The apparent