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博士論文

Disease specificity of anti-tryptophan hydroxylase-1 and
anti-AIE-75 autoantibodies in APECED and IPEX syndrome
(APECED と IPEX における抗 TPH-1 抗体と抗 AIE-75 抗体の疾患特異性)

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Highlights

1. Anti-AIE-75 antibodies are detected in IPEX syndrome but not in APECED, whereas anti-TPH-1 antibodies are detected only in APECED
2. Autoantibodies could differentiate intestinal dysfunction associated with IPEX syndrome or APECED

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 cells (Treg) [2, 3]. Forkhead box transcription factor 3, *FOXP3*, is a master gene in the development of Treg 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 a 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]. Although some cases of APECED with gastrointestinal dysfunction could mimic IPEX syndrome [15], there have been no studies, which tested anti-AIE75 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.

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 *Bam*HI restriction site at the 5' end and *Xho*I site at the 3' end as following; 5'-GGATCCATGATTGAAGACAATAAGGAG-3', and 5'-CTCGAGTTAGATACTCGGCTTCCTGCT-3'. Complimentary DNA encoding TPH-1 (NM_004179) was amplified by polymerase chain reaction (PCR) using λ gt11 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 *Bam*HI and *Xho*I and then subcloned into a GST fusion protein expression vector, pGEX4T-2. *E. 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 were 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 1:5,000 (Biosource, Camarillo, CA), HRP-conjugated goat anti-rabbit IgG at 1:2,000 (Biosource) or HRP-conjugated rabbit anti-goat IgG (Biosource) at 1:2,000 for 1 hour 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% NiCl₂).

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 molecular size of the fusion protein was approximately 75 kDa, consistent with the sum of GST (26 kDa) and TPH-1 (51 kDa).

3.2. Autoantibodies to TPH-1

As shown in Fig. 2A, an IP-NT serum (APECED-2) reacted with GST-TPH-1 but not with GST alone. GST-TPH-1 reacted with 8 of 10 IP-positive APECED patients, but none of the sera from 10 IP-negative or 2 IP-NT patients (Fig. 2B and data not shown). However, there was no correlation between the titers of antibodies measured by IP and immunoblot (Fig. 3). On the other hand, none of the sera from 7 patients with IPEX reacted with GST-TPH-1 (Fig. 4).

3.3. Reactivity of the sera to AIE-75 and villin

Anti-AIE-75 antibodies were detected in 5 of 7 patients with IPEX syndrome but not in any patients with APECED (Fig. 5 and Table 2). Anti-villin antibodies were detected in 4 of 7 patients with IPEX syndrome and 3 of 23 patients with APECED (Fig. 6 and Table 2).

4. Discussion

In the present study, we demonstrate that anti-AIE-75 antibodies and anti-TPH-1 antibodies are specific to IPEX syndrome and APECED, respectively. These confirm the specificity and diagnostic value of anti-TPH-1 and anti-AIE-75 autoantibodies in APECED and IPEX, respectively [6-14]. On the other hand, anti-villin antibodies were detected in sera from 3 of 23 patients with APECED (1 with and 2 without GI dysfunction). Low levels (1:160 or lower) of anti-villin antibodies are also detected in some patients with other collagen vascular diseases such as SLE and mixed connective tissue disease [8]. Thus, villin could be highly immunogenic, and anti-villin autoantibodies are produced in several autoimmune diseases. Anti-TPH-1 antibodies were detected in 8 of 10 IP-positive patients with APECED. Interestingly, there were no correlations between the titers determined by immunoblotting and the binding index by the IP assay. IP assays using deletion mutants of TPH-1 have revealed three major epitopes of the antigen [13]. Although the conformation of antigens is preserved in IP, immunoblot detects antibodies against linear peptide sequences of denatured antigens. Our result suggests that the autoantibodies to conformation of TPH-1 are dominant in some patients with APECED, whereas those to linear peptides are dominant in the others. However, our study could not clarify whether autoantibodies against the conformational or linear epitopes are associated with clinical GI tract dysfunction.

Most of APECED patients enrolled in our study were Italian, whereas most IPEX patients were Japanese. Both anti-AIE-75 and anti-villin antibodies are prevalent in patients with IPEX regardless of their ethnicities [7-10]. On the other hand, although the most frequent mutation in *AIRE* gene differs between races, genotype-phenotype correlations have not been clarified except for the association between large truncations of *AIRE* and candidiasis [18]. Thus, the influence of the bias in the ethnicities on our results remains unclear.

APECED and IPEX syndrome share several common features such as type-1 diabetes mellitus and thyroiditis, however, Addison's disease and hypoparathyroidism are prevalent in APECED but not in IPEX syndrome [1, 3, 19]. AIE-75 and villin are expressed in the duodenal epithelial cells, whereas expression of TPH-1 is limited to enterochromaffin cells in the intestine [7, 8, 14]. These are consistent with histopathological findings; severe inflammation is associated with villous atrophy of the duodenal tissue in IPEX syndrome, whereas only minimal inflammation and loss of enterochromaffin cells without apparent villous atrophy are observed in the intestine of anti-TPH-1 antibody-positive APECED [3, 14, 20]. Coincidence of the target cells and distribution of the autoantigens suggest a pathological role of antigen-specific autoimmunity in the development of enteropathy in both diseases. Because all of the

three antigens are cytoplasmic proteins, circulating autoantibodies may not directly bind them. Indeed, interstitial nephritis is not necessarily observed in IPEX syndrome positive for autoantibodies to these antigens despite the expression of both AIE-75 and villin in the renal tubules [7, 8]. Furthermore, anti-TPH-1 antibodies are positive in one-third of the cases of APECED without gastrointestinal dysfunction [11-14]. Given that autoreactive T cells share the same antigen-specificity with autoantibodies in autoimmune diseases [21-23], autoreactive T cells likely play a critical role in the tissue- or cell type-specific destruction in both diseases. Autoantibodies or autoreactive BCR may bind cytoplasmic antigens that had leaked from apoptotic or necrotic cells and facilitate the activation of T cells by antigen presenting cells [24]. Indeed, depletion of B cells in *Scurfy* mice, counterparts of human IPEX syndrome, reduces tissue damages [25]. Furthermore, follicular Treg cells which are derived from thymic naturally occurring Treg cells directly suppress autoreactive B cells in the peripheral lymphoid tissues [26-29]. As a result, Treg dysfunction allows the accumulation of autoreactive B cells in IPEX syndrome [30].

AIRE is expressed mainly in the medullary thymic epithelial cells (mTEC), acts as a transcription factor responsible for the thymic expression of TSAs, and contributes to the elimination of self-reactive T cells [1]. In addition, *AIRE* is expressed in the

peripheral lymphoid tissues, and accordingly, may play a role in the peripheral tolerance. On the other hand, *FOXP3* is a master gene of Treg cells which play a central role in peripheral immunotolerance [2, 3]. Thus, immunotolerance mechanisms may differ between autoantigens; tolerance to TPH-1 depends on intrathymic negative selection or AIRE-dependent peripheral tolerance, whereas tolerance to AIE-75 depends on Treg cells. Both TPH-1 and villin are expressed in mTEC of *AIRE*-deficient mice, although the expression of AIE-75 has not been studied [31]. Some target autoantigens associated with APECED are also expressed in mTEC in *AIRE*-independent manners [32-34]. Thus, AIRE must have roles in negative selection through a mechanism distinct from intrathymic transcription of TSA, e.g., antigen presentation by mTEC or interdigitating reticular cells in the peripheral lymphoid organs [35]. Otherwise, expression of TSAs in human mTEC may be different from those in mice. To date, whether AIRE contribute to the selection of Treg cells in the thymus is still controversial [34, 36]. However, given the severe clinical features of IPEX syndrome compared with APECED, at least some of Treg cells may develop independent of AIRE.

5. Conclusions.

Autoantibodies to AIE-75 and TPH-1 could be used for the differential diagnosis of IPEX syndrome and APECED. Coincidence of the distribution of autoantigens and target cell types suggests the involvement of antigen-specific mechanisms in the intestinal dysfunctions in both diseases. Immunotolerance to AIE-75 and TPH-1 may depend on the peripheral and central mechanisms, respectively.

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Figure legends

Figure 1: Expression of recombinant GST-TPH. GST or GST-TPH-1 is immunoblotted with anti-GST (A) or anti-TPH-1 (B) antibodies. In this experiment, the serum was diluted with TBST without *E. coli* extract. Both GST and GST-TPH-1 reacted with anti-GST antibodies, whereas only GST-TPH-1 reacted with anti-TPH-1 antibodies. Arrow and arrowhead indicate recombinant GST-TPH-1 and GST alone, respectively.

Figure 2: Anti-GST-TPH-1 antibodies in sera from APECED patients. (A) The serum of APECED-2 reacted with GST-villin but not GST alone. In this experiment, the serum was diluted by TBST without *E. coli* extract. (B) GST-TPH-1 reacted with 10 APECED sera (cases 3-12) in which the autoantibodies have been detected by immunoprecipitation. Anti-TPH-1 antibodies were not detected in case 10 and 11 by immunoblotting. Arrows indicate GST-TPH-1.

Figure 3: Anti-TPH-1 measured by immunoprecipitation and immunoblotting.

The tiers of anti-TPH-1 antibodies in APECED patients were compared with the binding unit of the antibodies measured by immunoprecipitation. There is no significant correlation between the binding unit by immunoprecipitation and titers by immunoblotting (Spearman's rank correlation coefficient; $r_s = -0.56$, $p = 0.088$). Closed

circles indicate patients with gastrointestinal manifestations.

Figure 4: Autoantibodies to TPH-1 are specific to APECED. GST-TPH-1 reacted with the serum of APECED-8 but none of IPEX syndrome. All the sera were diluted with TBST containing extracts of *E. coli* expressing GST.

Figure 5: Autoantibodies to AIE-75 are specific to IPEX syndrome. (A) AIE-75 reacted with the serum from IPEX-1 but not with sera from APECED-1 and 2. (B) Anti-AIE-75 antibodies were detected in 5 of 7 sera from IPEX syndrome patients but none of 23 APECED sera. An arrow indicates recombinant AIE-75. Closed circles indicate patients with gastrointestinal manifestations.

Figure 6: Autoantibodies to GST-villin. (A) GST-villin reacted with the serum from IPEX-1 but not with sera from APECED-1 and 2. The molecular weight of GST-villin is approximately 120 kDa which is consistent with that calculated from GST (26kDa) and villin (91 kDa). (B) Anti-villin antibodies were detected in 4 of 7 sera from IPEX syndrome patients and 3 of 23 sera from APECED patients. All of the sera were diluted with TBST containing extracts of *E. coli* expressing GST. An arrow indicates GST-villin. Closed circles indicate patients with gastrointestinal manifestations.

Tables

Table 1: Clinical and laboratory features of patients with APECED

Case No.	Clinical manifestation	Intestinal dysfunction	Autoantibodies	Anti-TPH-1 Abs by IP [binding index]	Ethnicity
1	AD, HP, CMC, M	yes	Not tested	Not tested	Japanese
2	HP, CMC, AIH	no	Not tested	Not tested	Japanese
3	AD, HP, CMC, POF, K	yes	IFN ω Abs	84.13	Italian
4	AD, HP, CMC, EH, demyelinating polyneuropathy	no	IFN ω Abs, AADC	167.6	Italian
5	AD, HP, AIT, POF, Ch, CTD, AG, PA, AIH, M, K, ND, A	yes	IFN ω Abs, AADC	90.1	Italian
6	AD, HP, CMC, AIT, POF, AG, PA, Co	no	IFN ω Abs, AADC	200	Italian
7	AD, HP, CMC, POF, CTD, AG, PA, K, Va	no	IFN ω Abs, AADC	56.7	Italian
8	AD, HP, CMC, AG, M, K, V, A	yes	IFN ω Abs, AADC	137	Italian
9	AD, HP, CMC, AIT, GHD, AG, M, Co, V	yes	IFN ω Abs, AADC	114.9	Italian
10	AD, HP, CMC, POF, AG, AIH, A	no	IFN ω Abs, AADC	74.4	Italian
11	AD, HP, CMC, ND	no	IFN ω Abs	7.7	Italian
12	AD, HP, CMC, ND, A	no	IFN ω Abs, AADC	201.22	Italian
13	AD, HP, CMC, POF, Co, CTD, AG	no	AADC	Negative	Italian
14	HP, POF	no	IFN ω Abs	Negative	Italian
15	AD, CMC, POF, ND, A, EH	no	IFN ω Abs	Negative	Italian

16	AD, HP, CMC, POF	no	IFN ω Abs, AADC	Negative	Romanian
17	AD, HP, CMC, POF, GHD, ND, EH	yes	IFN ω Abs	Negative	Italian
18	AD, HP, CMC,	no	IFN ω Abs	Negative	Italian
19	AD, HP, AG	no	IFN ω Abs	Negative	Italian
20	AD, HP, CMC, Ch, CTD, AG, A, TMC	no	IFN ω Abs, AADC	Negative	Italian
21	AD, HP	no	IFN ω Abs	Negative	Italian
22	AD, HP, CMC, AIH	no	IFN ω Abs, AADC	Negative	Italian
23	AIH, AIT, GN, IDDM	yes	Not tested	Not tested	Irish/Spanish

Abbreviations; AADC, antibodies to aromatic L-amino acid decarboxylase; IFN ω Abs, antibodies to Interferon- ω ;

Abs, antibodies; A, alopecia; AD, Addison disease; AG, atrophic gastritis; AIH, Autoimmune hepatitis; AHA,

autoimmune hemolytic anemia; AIT, Autoimmune thyroiditis; As, asplenia; CD, Celiac Disease; Ch, cholelithiasis;

Co, constipation; CTD, connective tissue disease; EH, enamel hypoplasia; GHD, growth hormone deficiency; GN:

glomerulonephritis; HP, hypoparathyroidism; IDDM: insulin-dependent diabetes mellitus; IP, immunoprecipitation; K,

keratoconjunctivitis; M, malabsorption; ND, nail dystrophy; PA, pernicious anemia; POF, premature ovarian

failure; Ps, psoriasis; TMC, tympanic membrane calcification; TIN, tubulointerstitial nephritis; V, vitiligo; Va,

vasculitis.

Table 2: Clinical and laboratory features of IPEX patients

Case No.	Clinical manifestation	Anti-AIE-75 Abs (IB)	Anti-villin Abs (IB)	Ethnicity	Outcome
1	Diarrhea	1:80	1:2,560	Japanese	Alive
2	Diarrhea, NS, T1DM	1:640	Negative	Japanese	Alive
3	AHA, Diarrhea, AIT, TIN	1:320	1:320	Japanese	Died
4	Diarrhea, AIT, TIN	1:160	1:320	Japanese	Died
5	Diarrhea	negative	1:160	Japanese	Died
6	Alopecia, psoriasiform dermatitis, Diarrhea	negative	Negative	Caucasian	Alive
7	Diarrhea, food allergy, eczema, NS, AIH	1:2,560	Negative	Japanese	Alive

Abbreviations; AIE-75, autoimmune enteropathy-related 75 kDa antigen; Abs, antibodies; AHA, autoimmune

hemolytic anemia; AIT, autoimmune thyroiditis; AIH, autoimmune hepatitis; NS, nephrotic syndrome; TIN,

tubulointerstitial nephritis.

Figures

Fig.1

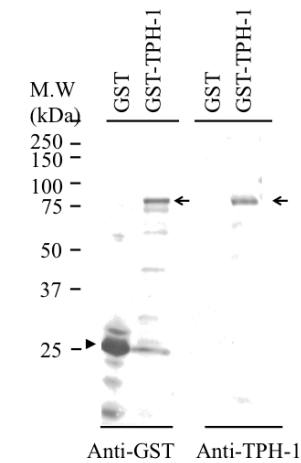


Fig.2

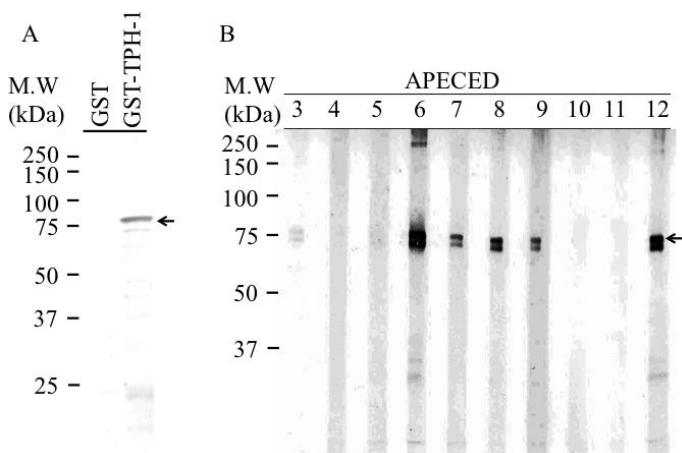


Fig.3

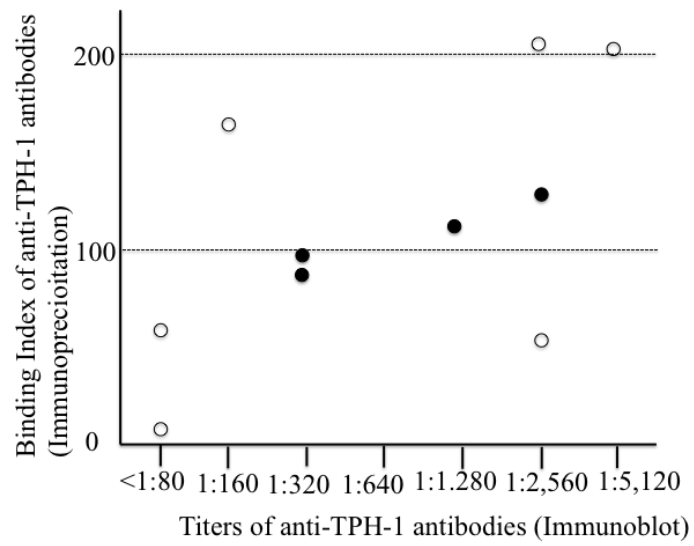
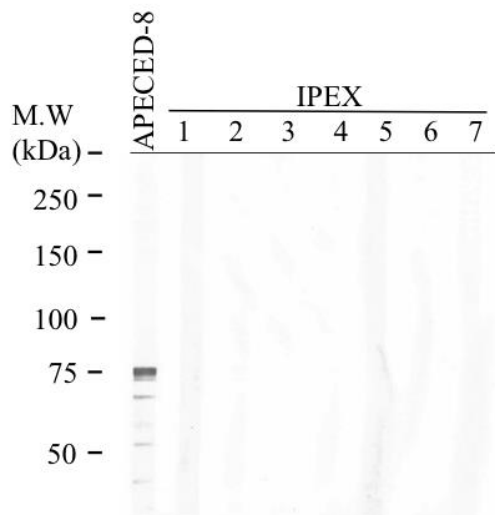


Fig.4



Fog.5

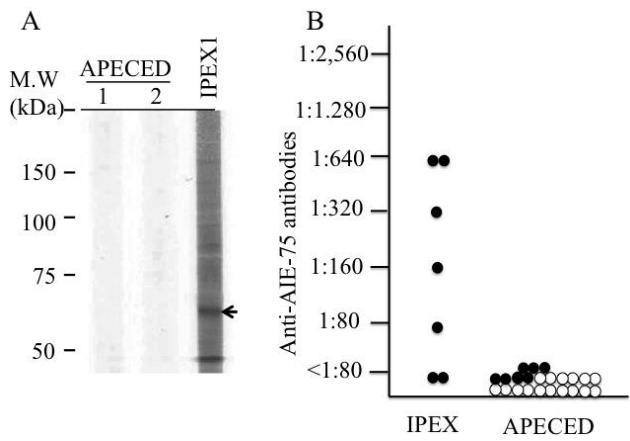


Fig.6

