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Autoantibodies to villin occur frequently in IPEX, a severe immune dysregulation, syndrome caused by mutation of *FOXP3*

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Abstract Intractable diarrhea is a major symptom of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and associated with autoantibodies against enterocytes. Although autoimmune enteropathy (AIE)-related 75 kDa antigen (AIE-75) is a prominent autoantigen involved in the enteropathy associated with IPEX syndrome, some patients with this syndrome demonstrated autoantibody recognizing a 95 kDa protein rather than AIE-75 in the small intestine. We, herewith, identified villin, an actin-binding protein, as the 95 kDa antigen. Four of five sera from patients with IPEX syndrome reacted with a fusion protein of glutathione-S-transferase and full length villin (GST–villin), whereas only three of 98 control sera weakly reacted with GST–villin. Anti-AIE-75 antibody was detected in all five IPEX sera but not in normal or control disease sera. We conclude that both AIE-75 and villin appear to be brush border autoantigens in IPEX syndrome and could be used for the diagnosis of AIE in patients with presumptive IPEX syndrome.

Abbreviations: IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; AIE-75, autoimmune enteropathy-related 75 kDa antigen; FOXP3, forkhead box protein 3; GST, glutathione-S-transferase; DAB, 3,3′-diaminobenzidine; PCR, polymerase chain reaction; Treg, regulatory T cell.

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1. Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare disorder characterized by multi-organ system failure particularly of the gastrointestinal tract and endocrine glands especially the thyroid and pancreatic β cells (OMIM 304790) [1–3]. Mutations responsible for IPEX syndrome have been mapped to the *FOXP3* gene (NM_014009) on chromosome Xp11.23. This gene encodes a forkhead/winged helix transcription factor which plays a critical role in the development of $CD4^+CD25^+$ regulatory T (Treg) cells [3–9]. Immune dysregulation typically results in protracted diarrhea that develops in infancy and requires early intervention with aggressive immunosuppression or stem cell transplantation [10–19]. Because diarrhea in IPEX is commonly associated with anti-enterocyte autoantibodies, the IPEX syndrome overlaps with autoimmune enteropathy (AIE) defined as intractable diarrhea refractory to diet therapy or total parenteral hyperalimentation and the presence of circulating autoantibody against enterocytes [3,10,20–22]. We have identified the autoimmune enteropathy-related 75 kDa antigen (AIE-75) as a target of the anti-enterocyte autoantibodies present in sera from three patients with AIE including two with IPEX syndrome [23]. AIE-75 is predominantly expressed in brush border of the small intestine and proximal tubules of the kidney. Although anti-AIE-75 antibody can be detected in most patients with IPEX syndrome [24,25], some patients have low levels of autoantibody to this antigen despite apparent immunoreactivity with the brush border. In the present study, we identified an actin-binding protein, villin, as another brush border antigen that is aberrantly targeted in IPEX syndrome.

2. Materials and methods

2.1. Sera

Serum samples were obtained from five Japanese patients with IPEX syndrome confirmed to have germline mutations in the *FOXP3* gene before the commencement of immunosuppressive drugs or immunoglobulin (Cases 1–5). Clinical features of these patients are summarized in Table 1 [5,23,26,27]. Control sera were obtained from normal volunteers (n=18) or patients with bronchial asthma (n=15). Autoimmune sera other than AIE were obtained from patients with systemic lupus erythematosus (n=8), mixed connective tissue disease (MCTD) (n=3), primary Sjögren's syndrome (n=10), juvenile idiopathic arthritis (n=7), dermatomyositis (n=8), and autoimmune hepatitis (n=2) and used as autoimmune patient controls. Enterocolitis control sera were obtained from patients with ulcerative colitis (n=7), Crohn's disease (n=2), Behçet disease involving small intestine (n=2), common variable immunodeficiency with diarrhea (n=1), and other undefined enteritides including atypical sprue (n=15). Eight sera from colon cancer patients were also included as controls.

2.2. Immunohistochemistry

With informed consent, samples of apparently normal jejunum were obtained as excess tissue from surgical specimens resected from cancer patients. Immediately post resection, small slices of specimens were fixed in periodic-lysine-paraformaldehyde at 4 °C for 6–12 h. After washing in 10–20% sucrose in phosphate buffered saline (PBS), the specimens were embedded in OCT medium and rapidly frozen in cooled isopentane. Frozen sections were cut on a cryostat at 5 μm thickness. They were mounted on silane-coated glass slides and air-dried. After immersion in 3% bovine serum albumin in PBS, dilutions of human sera were applied and incubated overnight at 4 °C. Immunodetection was achieved using HRP-labeled anti-human IgG antibody (Amersham Biosciences, Piscataway, NJ), and 3,3′-diaminobenzidine (DAB, Sigma, St. Louis, MO) as a chromogen. Endogenous peroxidase was inactivated in 0.3% H_2O_2 in methanol for 10 min after incubation with the primary antisera, and by adding sodium azide to the DAB development solution (65 mg/100 ml).

2.3. Screening of a cDNA library

A λgt11 human duodenal cDNA library (BD Biosciences Clontech, Palo Alto, CA) was immuno-screened with serum containing autoantibody to a 95 kDa protein in jejunal tissue but low levels of anti-AIE-75 antibody, according to the

Table 1 Summary of the IPEX cases examined.

Case no.	*FOXP3* mutations	Clinical manifestations	Treatment	Outcome	Ref
1	c.1117T>G (F373V)	Diarrhea	CSA, CS, IVIG, Tacrolimus, BMT	Alive	27
2	c.−23+1G>T*	Diarrhea, nephrotic syndrome, T1DM	CSA, CS	Alive	
3	c.227delT (L76fsx53)	Diarrhea, thyroiditis, interstitial nephritis	Tacrolimus, CS	Alive	26
4	c.1087A>G (I363V)	Diarrhea, thyroiditis, interstitial nephritis		Died (Sepsis)	26
5	c.1293_1294 del (P431fsX457)	Diarrhea	CBSCT	Died (GVHD, VOD, CMV)	5

*c.−23+1 G>T mutation, a substitution of G by T at the splicing donor site of intron 1, resulting in splicing defect and makes an incorrect initiation codon in the intron which causes a frame shift before the proper translation initiation site in exon 2 of the *FoxP3* gene. Abbreviations; CSA, cyclosporine A; CS, corticosteroid; IVIG, high-dose intravenous immunoglobulin therapy, BMT, bone marrow transplantation; CBSCT, cord blood stem cell transplantation; T1DM, type I diabetes mellitus; GVHD, graft-versus-host disease;VOD, veno-occlusive disease; CMV, cytomegalovirus infection.