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Brief Communication

Identification of autoantibodies using human proteome microarrays in patients with IPEX syndrome

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ABSTRACT

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is one of the inborn errors of immunity, characterized by impaired function of the regulatory T cells. Clinical manifestations of IPEX syndrome are characterized by various autoimmune diseases with autoantibodies. The comprehensive analysis for autoantibodies using human proteome microarrays in the four patients with IPEX syndrome was performed. The numbers of the highly expressed autoantibody showing relative log2 ratios greater than 1 were 1876, 513, 234 and 831 (mean: 864), respectively. Some novel autoantibodies which could explain the phenotypes of patients, adrenal dysfunction, muscular hypotonia, afibrinogenemia, enteropathy and pancytopenia were identified. Various kinds of autoantibodies targeting testis-specific antigens were also identified. Human proteome microarray is a powerful tool to understand the pathophysiology of IPEX syndrome. The larger cohort analysis using this method will provide further understanding of the impaired immune tolerance in humans.

1. Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is one of the inborn errors of immunity (IEI), caused by mutations in *FOXP3* gene, a master regulator of regulatory T cells (Treg). Functional and/or numerical impairment of Treg results in impaired suppression of effecter T cells (Teff). Hyperproliferation of Teff leads to various autoimmunity, infiltrating tissues, secreting cytokines and recruiting other inflammatory cells [1]. Impairment of Treg also results in defective peripheral B-cell tolerance and the production of autoantibodies [2]. In fact, various autoantibodies are detected in the serum of the patients with IPEX syndrome, which target the specific organs or tissues including the gut, pancreas, skin, thyroid and hematopoietic cells [1]. Interestingly, although their clinical significance is not clear, anti-cytokine antibodies were reported in IPEX patients [3]. B-cell depletion therapy using rituximab ameliorates the autoimmune symptoms in IPEX syndrome [4]. This observation obviously advocates the important roles of the autoantibodies in the etiology of IPEX syndrome. It has been reported that anti-AIE75 and villin antibodies are disease-specific and useful markers for the screening of IPEX syndrome [1]. The deep understanding of autoantibodies in IPEX patients would provide further insights into the pathophysiology of the disease. However, most previous studies have focused on the identification of autoantibodies against the single organ or tissue due to limitation of approaches. Herein, we describe the first comprehensive analysis for autoantibodies using human proteome microarrays in the patients with IPEX syndrome.

Abbreviations: AIHA, autoimmune hemolytic anemia; IEI, inborn errors of immunity; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; ITP, immune thrombocytopenia; MDS, myelodysplasia syndrome; Teff, effecter T cell; Treg, regulatory T cell

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2. Materials and methods

2.1. Study approval

This study was conducted in accordance with the Helsinki Declaration and approved by the ethics boards of Tokyo Medical and Dental University (No. 92).

2.2. Patients

We enrolled 4 patients with IPEX syndrome who were genetically diagnosed (patient 1, p.R347H; patient 2, p.A384T; patient 3, p.R337Q; patient 4, pA84Dfs*45). Patient 2 was described elsewhere [5,6]. All the patients but patient 4 received corticosteroids and/or immunosuppressive drugs, but not hematopoietic stem cell transplantation at the blood sampling. The age was 15 years, 18 years, 10 months and 2 months, respectively. The clinical presentations were as follows; patient 1 had enteropathy, eczema, asthma, type 1 diabetes mellitus, autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), and myelodysplasia syndrome (MDS), patient 2 had eczema, asthma, interstitial pneumonia and adrenal dysfunction, patient 3 had enteropathy, eczema, AIHA and muscular hypotonia, patient 4 had enteropathy, AIHA and bleeding tendency with afibrinogenemia. Autoantibodies identified before this study are as follows; patient 1 had coombs antibody, platelet-associated IgG and anti-AIE75 antibody, patient 3 had coombs antibody and anti-villin antibody, patient 4 had coombs antibody, anti-GAD antibody, anti- IA-2 antibody, anti-insulin antibody, thyroid stimulating antibody, thyroid-stimulating hormone receptor antibody and anti- thyroglobulin antibody.

2.3. Human proteome microarrays

Serum antibody screening was performed using human proteome microarrays (Fukushima Translational Research Project, Fukushima, Japan). The protein microarray contains 16,680 human full-length proteins, which encompass 15,406 genes, including 1190 malignancy-associated mutants and fusion proteins. In this study, to evaluate the serum antibodies, we excluded malignancy-associated mutants and fusion proteins, and used 15,490 human full-length proteins. Serum antibody screening were performed as described elsewhere [7]. In brief, microarrays were incubated with diluted sera and Goat Reference Antibody Mixture I (Fukushima Protein Factory, Inc., Fukushima, Japan) after blocking, and stained with Alexa Fluor 647-conjugated anti-human IgG and Cy3-conjugated anti-goat IgG antibodies, respectively. After staining, the microarrays were scanned with a GenePix 4000B scanner (Axon instruments) as well as those not being incubated with serum as negative controls. To make comparisons between microarrays, the fluorescence intensity ratios (Alexa Fluor 647/ Cy3) were normalized and then relative values against negative controls were calculated to remove the cross-reaction of secondary antibodies. Sera from 2 individuals without autoimmune disease, who were 1 and 17 years old respectively, were used as the controls. Gene ontology classification and enrichment analysis were performed using DAVID [8]. The Benjamini-Hochberg method was used to correct for multiple comparisons. The significance level of 0.05 was used.

2.4. Immunoblotting

Recombinant Proteins, KRT20 (ATgen) and FGB (Rayebio) were separated by using SDS-PAGE and transferred to membranes and stained using Fast Green dye. The membranes were probed using patient's sera (X500 dilution). The autoantibodies were detected with horseradish peroxidase–conjugated anti-human secondary antibody (Bethyl).

3. Results

3.1. Identification of autoantibodies

Human proteome microarrays revealed various autoantibodies, which were largely differently expressed in each patient (Fig. 1A). To understand the outline of autoantibody expression in patients with IPEX syndrome, we firstly evaluated the highly expressed autoantibodies which showed relative log2 ratios greater than 1 (Supplementary Table 1). In each patient, 1876, 513, 234 and 831 (mean: 864) autoantibodies were identified, respectively (Fig. 1B). Of those, 257, 36 and 3 autoantibodies were overlapped with those in any 2, 3 and 4 patients, respectively. Importantly, autoantibodies were also identified in 2 controls; 257 and 683 autoantibodies. Nevertheless, some patients, especially patient 1, had more numbers of autoantibodies. Of total 2839 autoantibodies identified in the patients, approximately one-half targeted the cytoplasm components, one-third targeted the nucleus components and the others targeted the membrane or extracellular components (Fig. 1C). The enrichment analysis showed that nucleus, nucleoplasm, nuclear chromatin, cytoplasm and cytosol components were significantly enriched (Fig. 1D). Similar findings were obtained in case of the autoantibodies with relative log2 ratios greater than 2 or 3 were selected, as well as those in the controls (Supplementary Fig. 1, 2). The functions of each antigen showed a diversity and no significant function was enriched. Autoantibodies detected by comprehensive protein array was evaluated using immunoblotting against recombinant protein. Due to the limitation of sample resource, the immunoblot was performed using sera from only in patient 1 and 4 and confirmed the presence of the autoantibodies (Fig. 1E).

3.2. Clinical implications of autoantibodies

We next focused on the autoantibodies especially associated with the clinical implications. Among many significantly uncertain autoantibodies, we searched a novel autoantibody which could explain the phenotype of patients. The candidates for phenotype associated autoantibody were determined when either of the following criteria is applicable. (1) The clinical features of the patient mimic the clinical features of the patient who have loss-of-function mutations in the corresponding genes coding the same antigen. (2) The patient has clinical features in the specific organs or tissues and the antigens targeted by the autoantibodies were selectively expressed in the same organs or tissues. The list of candidate autoantigens is listed in Table 1.

Patient 2 had adrenal dysfunction since 0-year-old. Autoantibody to glucocorticoid receptor (NR3C1) was detected by this assay. The loss of function in *NR3C1* results in an autosomal dominant disease, generalized glucocorticoid resistance (OMIM: 615962) [9].

Patient 3 had muscular hypotonia soon after birth. He was suspected to have congenital myopathy before the diagnosis of IPEX syndrome, but his hypotonia was partially improved after receiving prednisolone. Anti-troponin T1 (TNNT1) and anti-troponin C2 (TNNC2) autoantibody was identified in this patient. *TNNT1* is known as the causative gene of nemalin myopathy (OMIM: 605355) [10]. TNNC2 is expressed selectively on skeletal muscle, although its involvement in human disease has not been reported.

Patient 4 developed bleeding tendency with afibrinogenemia at the age of 2 months. Anti-fibrinogen antibody was suggested by the cross-mixing test. Human proteome microarrays revealed anti-fibrinogen beta chain (FGB) autoantibody. *FGB* can cause congenital afibrinogenemia or dysfibrinogenemia (OMIM: 202400, 616004) [11].

Patients 1 and 3 suffered from enteropathy. Anti-AIE75 (patient 1) or villin (patient 3) antibody was previously detected by western blotting analysis [12]. However, anti-villin antibody was not detected by this assay. On the contrary, anti-villin antibody was detected in patient 2 who did not exhibit enteropathy (Supplementary Table 2). Antibody to keratin 20 (KRT20) was identified in the both patients.

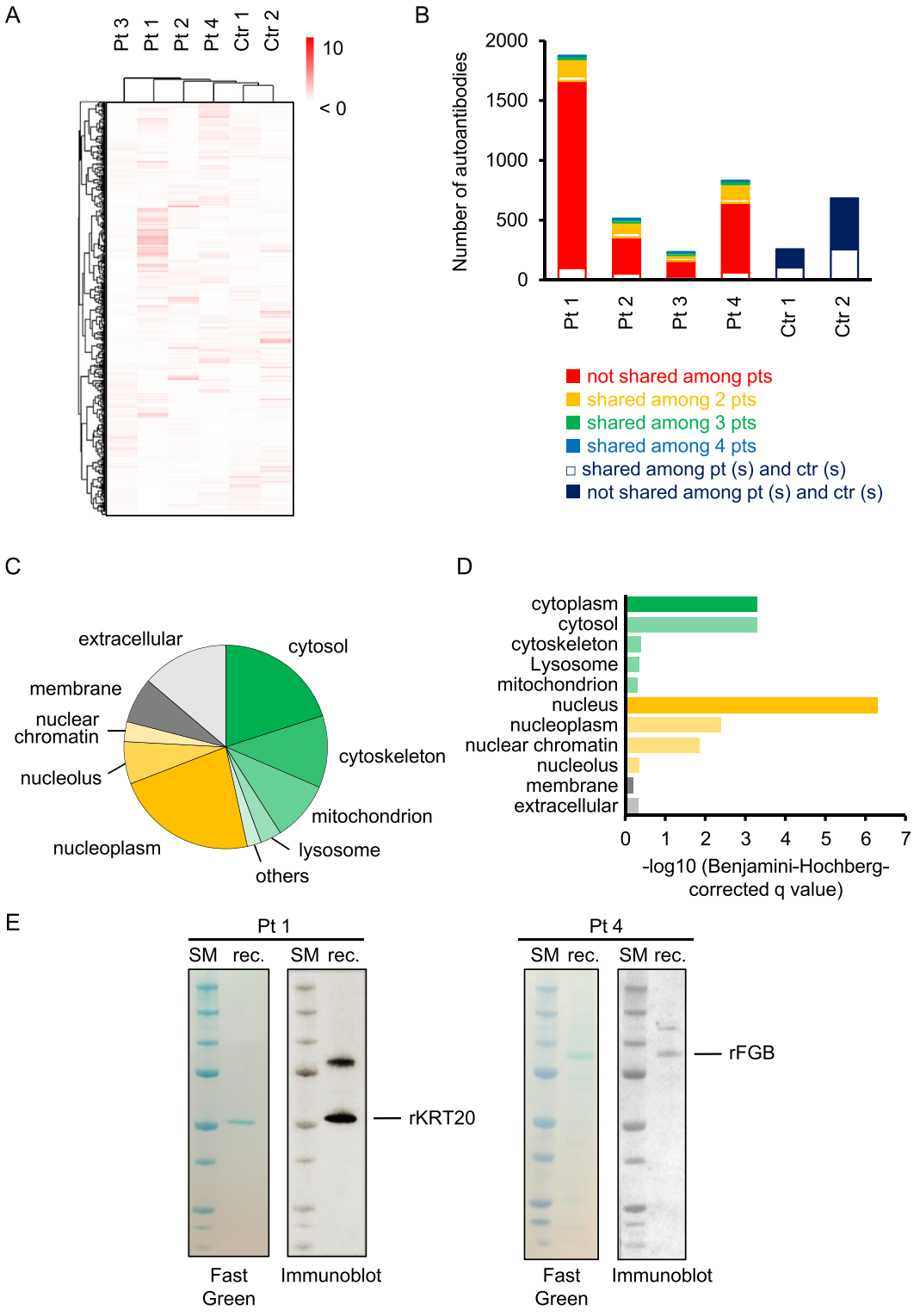

Fig. 1. Identification of autoantibodies.
(A) The heat map of the proteome microarrays. The numbers are shown in relative log2 ratios. (B) The distribution of the autoantibodies which showed relative log2 ratios greater than 1. (C) The distribution of the cellular components which were targeted by the autoantibodies. Some of the antigens are overlapped. (D) Benjamini-Hochberg-corrected q value of the cellular components which were targeted by the autoantibodies. (E) Left panel showing Fast Green staining of the membrane, right panel showing immunoblot using patient's sera. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although keratin 20-related human diseases have not been reported, its selective expression on the intestine and the observation of anti-keratin 14 autoantibodies in the enteropathy patients with IPEX syndrome support its involvement in the enteropathy [13].

Patient 1 developed pancytopenia at the age of 6 years. His laboratory test and bone marrow examinations revealed pancytopenia was developed by combination of AIHA, ITP and MDS (refractory cytopenia with multilineage dysplasia). Autoantibodies to Krueppel-like

Table 1
Candidates of autoantibody that determine clinical phenotypes.

patient	Phenotype	Symbol	ID	Name	Relative log2 ratio
Newly identified autoantibodies					
Patient 1	Enteropathy	KRT20	54,474	Keratin 20	6.98
	Anemia	KLF1	10,661	Kruppel-like factor 1	9.46
	Anemia	G6PD	2539	Glucose-6-phosphate 1-dehydrogenase	2.85
	Pancytopenia	ERCC4	2072	DNA repair endonuclease XPF	3.12
	Pancytopenia	CD34	947	Hematopoietic progenitor cell antigen CD34	2.11
Patient 2	Adrenal dysfunction	NR3C1	2908	Glucocorticoid receptor	3.37
Patient 3	Muscular hypotonia	TNNT1	7138	Troponin T1	4.12
	Muscular hypotonia	TNNC2	7125	Troponin C2	2.46
	Enteropathy	KRT20	54474	Keratin 20	2.59
Patient 4	Afibrinogenemia	FGB	2244	Fibrinogen beta chain	2.95
Previously described autoantibodies					
Patient 1	Enteropathy	AIE75	10083	Harmonin	1.12
Patient 2	Enteropathy	VIL1	7429	Villin-1	4.62

factor 1 (KLF1), DNA repair endonuclease XPF (ERCC4) and glucose-6-phosphate 1-dehydrogenase (G6PD) was identified in this patient. The mutations in these genes cause congenital dyserythropoietic anemia type 4 (OMIM: 613673), Fanconi anemia of complementation group Q (OMIM: 615272) and nonspherocytic hemolytic anemia due to G6PD deficiency (OMIM: 300908), respectively [14–16]. Although it has not been reported as the causative gene in human disease, autoantibody to hematopoietic progenitor cell antigen CD34 was also identified.

Although the clinical implications are not clear at this moment, many autoantibodies targeting testis-specific antigens were identified, especially in patient 1 (Supplementary Table 3).

4. Discussion

We performed the comprehensive analysis for autoantibodies in the patients with IPEX syndrome. Our study revealed that the patients with IPEX syndrome produced diverse autoantibodies and the pathogenic roles were proposed based on our criteria for only a small fraction of them while the majority of them were significantly uncertain. Several autoreactive B cells can escape from the central tolerance, but after that, they acquire tolerance in the periphery by Treg including FOXP3-positive follicular T cells [17]. In the peripheral tolerance, Treg can induce self-reactive B cell anergy and directly kill the autoreactive B cells by FAS/FAS ligand system or granzyme/perforin system [18–20]. As the apoptotic control system in central tolerance is incomplete, Treg cover this flaw as a peripheral tolerance. Therefore, autoimmunity is revealed in IPEX patients because imperfect central tolerance could not be compensated by attenuated peripheral tolerance caused by defective Treg. Precise determination of diversity of autoreactive B cell from patients with IPEX syndrome is an interesting topic, because it shows the incompleteness of the central tolerance in humans although it may be affected by other factors including the residual function of Treg or the regulation by regulatory B cells. However, it is technically challenging, therefore we evaluated diversity of autoantibodies instead. The wide diversity with least skewing of autoantibodies among the patients suggests that self-reactive B cells do not selectively escape from the central tolerance. Clinical heterogeneity of IPEX syndrome has been reported even in the same mutation, which might be contributed by this diversity and least skewing of autoantibodies [4]. On the other hand, several autoantibodies are often universally identified in patients with IPEX syndrome, including anti-GAD, insulin, AIE75 and villin antibodies, suggesting that the escapement from the central tolerance does not occur completely randomly [4]. The causes of this non-randomness are not clearly understood. However, recent studies demonstrated the nonrandom repertoire usage at the stage of gene rearrangement [21].

Our study revealed novel autoantibodies, which could cause the autoimmune diseases including adrenal dysfunction, muscular hypotonia, afibrinogenemia, enteropathy and pancytopenia. Identification of the testis antigen-specific autoantibody deserves more attention in the clinical setting as one of the main causes of male infertility [22]. Cell biological verification of the relationship between patient's specific autoimmune phenotypes and corresponding identified autoantibodies is not easy. Furthermore, many significantly uncertain autoantibodies were identified even in healthy controls, as previously described [23]. However, many candidates of autoantibody which may link autoimmune phenotypes were identified in our study. Although their clinical significance remains to be carefully confirmed, we believe the approach done in our study will be a breakthrough of understanding of autoimmune diseases. In this study, anti-villin antibody was not detected in patient 3, in whom anti-villin antibody by western blotting was previously detected. This discrepancy may be explained by the timing of the sampling or difference of antigen for capturing the autoantibody in proteome microarray.

In conclusion, comprehensive analysis for autoantibodies using human proteome microarrays was a powerful approach to investigate the pathophysiology of IPEX syndrome. The larger cohort analysis using this method will make the etiology of IPEX syndrome clearer. Further understanding of IPEX syndrome will lead to further understanding of the impaired immune tolerance in humans and development of adequate therapy development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.03.011.

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Declaration of interest

None.

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