Identification of Novel Autoantibodies in Type 1 Diabetic Patients Using a High-Density Protein Microarray

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Autoantibodies can facilitate diagnostic and therapeutic means for type 1 diabetes (T1DM). We profiled autoantibodies from serum samples of 16 T1DM patients, 16 type 2 diabetic (T2DM) patients, and 27 healthy control subjects with normal glucose tolerance (NGT) by using protein microarrays containing 9,480 proteins. Two novel autoantibodies, anti-EEF1A1 and anti-UBE2L3, were selected from microarrays followed by immunofluorescence staining of pancreas. We then tested the validity of the candidates by ELISA in two independent test cohorts: 1) 95 adults with T1DM, 49 with T2DM, 11 with latent autoimmune diabetes in adults (LADA), 20 with Graves disease, and 66 with NGT and 2) 33 children with T1DM and 34 healthy children. Concentrations of these autoantibodies were significantly higher in T1DM patients than in NGT and T2DM subjects ($P < 0.01$), which was also confirmed in the test cohort of children ($P < 0.05$). Prevalence of anti-EEF1A1 and anti-UBE2L3 antibodies was 29.5% and 35.8% in T1DM, respectively. Of note, 40.9% of T1DM patients who lack anti-GAD antibodies (GADA) had anti-EEF1A1 and/or anti-UBE2L3 antibodies. These were also detected in patients with fulminant T1DM but not LADA. Our approach identified autoantibodies that can provide a new dimension of information indicative of T1DM independent of GADA and new insights into diagnosis and classification of T1DM.

Type 1 diabetes (T1DM) results from immune-mediated pancreatic β-cell destruction. Several autoantibodies, such as GAD65 antibody (GADA) (1), islet cell antibody (ICA) (2), protein tyrosine phosphatase antibody (IA-2 antibodies [IA-2A]) (3), and zinc transporter antibody (ZnT8A) (4), were identified and used for diagnosis and prediction of T1DM. However, the nature and mechanism of β-cell destruction in humans seem to be variable, which results in a broad spectrum of T1DM spanning from fulminant T1DM (5) to latent autoimmune diabetes in adults (LADA) (6). Fulminant T1DM is a rapidly progressing form of T1DM without evidence of autoimmunity, whereas LADA is autoimmune diabetes not requiring insulin at diagnosis. In most cases of LADA, which account for 10% of incident cases of diabetes in adults, β-cell function is severely impaired within 6 years, leading to insulin dependency (6). Fulminant T1DM is an important subtype of T1DM in Asian adults (7), accounting for 15%–20% of T1DM with ketosis or ketoacidosis in Japan (5). Moreover, T1DM in adults often lacks evidence of autoimmunity. The prevalence of autoantibodies associated with T1DM declines as the onset age increases, especially in the case of IA-2A (8) and ZnT8A (4,9). ZnT8A was observed in 80% of individuals in late adolescence (4,9), 12% of adults <50 years of age, and very rarely for adults

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>60 years of age (9). Consequently, there has been a need for novel markers that can reflect β-cell destruction in such a broad spectrum of T1DM, which in turn can be used to differentiate T1DM from other classes of diabetes.

Thus, we systematically explored autoantibodies associated with T1DM in a large-scale screening study of autoantibody repertoires in subjects with T1DM by using a high-density, fluorescence-based protein microarray containing duplicate spots of 9,480 human proteins derived from the Ultimate ORF Clone Collection (Life Technologies) to immunologically characterize a broad spectrum of T1DM. Among 9,480 proteins, we selected two candidate autoantibodies—antieukaryote translation elongation factor 1a (EEF1A1) autoantibody (EEF1A1-AAb) and antitubulin-conjugating enzyme 2L3 (UBE2L3) autoantibody (UBE2L3-AAb)—based on multivariate partial least squares–discriminant analysis (PLS-DA) and immunofluorescence staining and validated them in two independent cohorts.

RESEARCH DESIGN AND METHODS

Sample Preparation

For autoantibody profiling, we carefully selected the first cohort of 16 Korean patients with recent-onset T1DM, 16 patients with type 2 diabetes (T2DM), and 27 healthy control subjects with normal glucose tolerance (NGT). This cohort was used to screen candidate autoantibodies using protein microarrays. Serum samples were drawn from T1DM patients with 1) a fasting C-peptide level <0.3 nmol/L or serum C-peptide <0.6 nmol/L after glucagon loading (10), 2) an initiation of insulin treatment within 6 months after diagnosis, and 3) a duration of diabetes ≤12 months. Mean age of patients with T1DM in the first cohort was 42 ± 16 years (Table 1). The control serum samples were obtained from T2DM patients who were treated only with oral antidiabetic drugs for at least 5 years and from subjects with NGT who had no history of diabetes, no first-degree relatives with diabetes, a fasting plasma glucose concentration of <6.1 mmol/L, and an HbA1c value of <5.8% (40 mmol/mol) (Table 1).

The second cohort to test the validity of the autoantibody candidates comprised 95 T1DM, 49 T2DM, 11 LADA, and 66 NGT subjects, where T1DM patients met the first and second selection criteria of the first cohort. Among them, 11 patients with fulminant T1DM, as defined according to a previous report (11), were included. Mean age of patients with T1DM in the second cohort was 32 ± 11 years, and median duration of diabetes was 3 (range 0–14) years (Table 1). The same criteria for T2DM and NGT in the first cohort were used. Patients with LADA defined as previously reported (6) (n = 11) and patients with Graves disease who had no history of diabetes as the autoimmune disease control (12) (n = 20) were also included in the second cohort. The third cohort, comprising 33 children with T1DM and 34 healthy children, was used as an

Table 1—Clinical characteristics of subjects

<table>
<thead>
<tr>
<th>T1DM</th>
<th>T2DM</th>
<th>LADA</th>
<th>NGT</th>
<th>Graves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>49</td>
<td>11</td>
<td>66</td>
</tr>
<tr>
<td>Ages (years)</td>
<td>42 (16–69)</td>
<td>50 (22–72)</td>
<td>48 (11–61)</td>
<td>63 (20–90)</td>
</tr>
<tr>
<td>Female sex</td>
<td>6 (37.5)</td>
<td>14 (29.2)</td>
<td>7 (63.6)</td>
<td>20 (30.3)</td>
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<tr>
<td>Duration (years)</td>
<td>0 (0–1)</td>
<td>1 (0–5)</td>
<td>3 (0–9)</td>
<td>0 (0–6)</td>
</tr>
<tr>
<td>BM (kg/m²)</td>
<td>23.7 (21.3–25.7)</td>
<td>23.3 (21.3–25.7)</td>
<td>23.3 (21.3–25.7)</td>
<td>23.3 (21.3–25.7)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.1 (6.9–9.2)</td>
<td>7.9 (6.9–9.2)</td>
<td>7.9 (6.9–9.2)</td>
<td>7.9 (6.9–9.2)</td>
</tr>
<tr>
<td>Fasting C-peptide (ng/mL)</td>
<td>0.4 (0.2–0.6)</td>
<td>0.4 (0.2–0.6)</td>
<td>0.4 (0.2–0.6)</td>
<td>0.4 (0.2–0.6)</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n (%), or median (range) unless otherwise indicated. T1DM excluding LADA.
independent test set. The same criteria for T1DM in the second cohort were used for the third cohort. Age of patients with T1DM in the third cohort was 8 ± 4 years, and median duration of diabetes was 0 (range 0–7) years. Mean age of healthy children was 10 ± 1 years (Table 1).

For patients with T1DM, serum GADA concentration was measured by a radioimmunoassay (RSR Ltd.). GADA values >1.45 International Units/mL were considered positive.

This study was conducted in accordance with the provisions of the Declaration of Helsinki for participation of subjects in human research. The institutional review board of the Clinical Research Institute at Seoul National University Hospital approved the study protocol, and written informed consent was obtained from each subject.

**Protein Microarray Experiments**

Protein microarray platform version 5.0 (ProtoArray; Invitrogen) contains duplicate spots of 9,480 N-terminal glutathione S-transferase–tagged human proteins printed on a nitrocellulose slide. In this array, the antigens have their native cellular enzymatic activities and conformations. Assays were performed according to the instructions of the manufacturer (13) as follows: Serum diluted 1:500 in PBS with Tween was incubated on microarray slides for 90 min, and Alexa Fluor 647–conjugated anti-human IgG was used to detect the reaction. The array slides were scanned with a GenePix 4000B fluorescent scanner (Molecular Devices).

**Analysis of ProtoArray Data**

Signal intensities were acquired using ProtoArray Prospector, a software tool developed for ProtoArray data by Invitrogen (14). We converted fluorescent intensities for the spot proteins (or autoantigens) to log2 intensities and then normalized across the total 59 data sets by using the quantile normalization method (15). Protein microarray data used in this study are available from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/gds) under submission number GSE 50866. To determine the presence of autoantibodies for each probe, we calculated a z score defined as follows: (spot intensity – the averaged intensity of all probes) / SD of all probes. The autoantibody with z score >1.64 (95th percentile of all probe intensities) was considered present. To identify autoantibodies showing a significant difference between two groups (T1DM vs. NGT, T1DM vs. T2DM, or T1DM vs. T2DM + NGT), the M-statistic test in ProtoArray Prospector was applied to the normalized intensities as reported previously (16–20). For each probe, M test 1 produced the number of samples (M-statistic counts) with the intensity greater than or equal to the M-statistic threshold in two groups and 2) computed the P value (the significance of the difference between two groups) from the sample sizes and the M-statistic counts in the two groups. As an initial set of autoantibody candidates, we selected the proteins with P < 0.05 and z score >1.64 in at least one of T1DM samples.

We then applied PLS-DA to compute the variable importance in projection (VIP) representing the relative contribution of each autoantibody to the separation among T1DM, T2DM, and NGT samples (21). To determine a cutoff for significant VIPs, we estimated an empirical distribution of VIP for the null hypothesis (i.e., an autoantibody has no contribution to the separation) by applying PLS-DA followed by gaussian kernel density estimation method (22) to randomly permuted experiments. We then determined a VIP cutoff of 1.59 as the 90th percentile of the empirical VIP distribution. Thus, the autoantibodies with VIP >1.59 were selected as those significant contributing to the separation among T1DM, T2DM, and NGT samples.

**Immunofluorescence Staining and Immunohistochemistry**

Primary anti-EEF1A1 (Merck Millipore), anti-UBE2L3 (Santa Cruz), and anti-chromosome 7 open reading frame 53 (c7orf53) (Santa Cruz) antibodies were loaded onto slides of pancreas tissue of a subject who underwent partial pancreatectomy due to serous adenoma. Alexa Fluor 647– (Merck Millipore), 594– (Invitrogen), or 488– (Invitrogen) conjugated secondary antibodies were used to visualize insulin, glucagon, and candidate autoantigens, respectively. The cell nuclei were counterstained with DAPI (Invitrogen). An Olympus FluoView FV1000 confocal laser scanning microscope was used to acquire data. To investigate the expression of candidate autoantigens in other tissues, including liver, kidney, stomach, intestine, muscle, brain, and thyroid gland, immunohistochemistry using tissue array (SuperBioChips) was performed with EnVision System-HRP–labeled polymer (Dako) according to the manufacturer’s instructions.

**ELISA and Immunoprecipitation**

The differential expression of autoantibodies identified by ProtoArray in T1DM patients was confirmed by ELISA using corresponding recombinant proteins. Plates coated with EEF1A1 or UBE2L3 (Origene) were incubated with diluted serum samples (1:1,000–1:10,000) in PBS. All experiments were performed in triplicate for each patient and control sample. The recombinant proteins and their corresponding antibody, anti-EEF1A1, and anti-UBE2L3 were used as the positive control for the assay. Autoantibody bound to the protein was detected with goat anti-human IgG-HRP (Jackson ImmunoResearch). The cutoff point for the presence or absence of each autoantibody was determined according to the mean absorbance + 3 × SD of NGT, which corresponded to ~99% of normal control. Because titers of autoantibodies may differ between the solid and liquid phases as a result of the difference in exposure of binding epitopes, the relative levels of EEF1A1-AAb and UBE2L3-AAb were analyzed using an immunoprecipitation–Western blot method. T1DM patient samples categorized as high, medium, and low were selected from the second cohort based on ELISA absorbance values at 450 nm. Autoantibodies present in
these groups of T1DM and NGT samples were immuno-precipitated using EEF1A1-flag or UBE2L3-flag. Autoantibody:antigen immune complex captured with antiflag M2-antibody–conjugated magnetic beads was eluted from the beads following the manufacturer’s protocol (Sigma) and analyzed by Western blot using anti-human IgG-HRP. Antigens coeluted from the beads were detected using antibodies for EEF1A1 and UBE2L3. An irrelevant human IgG antibody (ForteBio) was used as a control for the immunoprecipitation assay.

**Statistical Analysis**

Statistical analysis for the microarray studies was as just described. Results of ELISA experiments were evaluated by means of independent t test. The χ² test was used to evaluate differences in the proportion of antibody-positive subjects per group. The number of test sets for ELISA was determined from the results of ELISA in the second cohort. That is, to reliably identify a significant group difference by the two-sample t test with a type I error rate of 0.05 and statistical power of 0.8, 32 subjects in disease and control groups were needed for the test sets.

**RESULTS**

**Serum Autoantibody Profiling**

We profiled autoantibodies in the sera from 16 recent-onset T1DM, 16 T2DM, and 27 NGT subjects by using the ProtoArray human protein microarray version 5 containing 9,480 different proteins spotted in duplicate. These 9,480 proteins correspond to 6,867 of the 19,050 annotated proteins (36.0%) (Swiss-Prot 2013_05), which includes 1,632 of 4,673 genes (34.9%) encoding proteins localized in plasma membrane or extracellular regions based on their gene ontology cellular component annotations and 809 of 1,929 proteins (41.9%) previously detected in human serum and plasma samples according to the human plasma proteome project (23). The autoantibodies against 3,060 among the 9,480 proteins were found to be present in at least one of the samples. To assess the data quality, we examined whether the signal intensity of a known autoantigen, GAD65, obtained from ProtoArray reflects a serum concentration of GADA that was independently measured by a conventional radioimmunoassay and confirmed a significant correlation between them (Spearman r = 0.863, P < 0.001). On the other hand, none of IA-2A, ICA, or ZnT8 is spotted in other hand, none of IA-2A, ICA, or ZnT8 is spotted in these groups of T1DM and NGT samples were immuno-precipitated using EEF1A1-flag or UBE2L3-flag. Autoantibody:antigen immune complex captured with antiflag M2-antibody–conjugated magnetic beads was eluted from the beads following the manufacturer’s protocol (Sigma) and analyzed by Western blot using anti-human IgG-HRP. Antigens coeluted from the beads were detected using antibodies for EEF1A1 and UBE2L3. An irrelevant human IgG antibody (ForteBio) was used as a control for the immunoprecipitation assay.

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**Selection of Autoantibodies for Validation in a Large-Scale Cohort**

To identify a set of biomarkers predictive of T1DM, it is important to evaluate the relative contribution of the 69 proteins to the separation between T1DM and T2DM/NGT samples. To this end, we applied multivariate PLS-DA (21) to the abundance of the 69 autoantibodies to evaluate their collective contribution to the separation (Fig. 2A). The PLS-DA result showed that a clear separation between T1DM and T2DM/NGT samples can be achieved by the differential expression of the 69 proteins (Fig. 2B and Supplementary Table 3). The relative collective contribution of individual autoantibodies to the candidates whose abundance increased in T1DM. The Venn diagram shows the numbers of these candidates identified from the three comparisons: 1) T1DM vs. NGT, 2) T1DM vs. T2DM, and 3) T1DM vs. NGT + T2DM. B: Increased abundance of the 69 autoantibodies in T1DM revealed by hierarchical clustering analysis. The columns and rows in the heat map represent samples and autoantibodies, respectively. In each row, the intensities were divided by the SD after subtracting the mean intensity from them (i.e., autoscaling). Yellow and blue represent the increase and decrease in abundance, respectively. The color bar represents the gradient of the auto-scaled log₂ intensities.

**Identification of Autoantibodies Predominantly Expressed in T1DM**

To identify autoantibodies predominantly expressed in T1DM samples, the relative abundance of 3,060 autoantibodies detected in the profiling were compared among 1) T1DM versus NGT, 2) T1DM versus T2DM, and 3) T1DM versus NGT + T2DM by using M test. From these comparisons, we identified 103, 27, and 79 autoantibodies, respectively, whose abundance increased in T1DM with M-test P < 0.05 and z > 1.64 in at least one of the T1DM samples (Fig. 1A). Of these, we focused on the 69 autoantibodies (Fig. 1B and Supplementary Table 2) identified from T1DM versus NGT + T2DM, which overlapped with those in either T1DM versus NGT or T1DM versus T2DM.

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separation achieved by PLS-DA was estimated as their VIP values (21) (Supplementary Table 4). A large VIP value indicates a high contribution to the separation. Among the 69 autoantibodies, we selected 50 with VIP values >1.59 (Fig. 2C) (see RESEARCH DESIGN AND METHODS). We also evaluated the contribution of individual samples to the separation captured by the PLS-DA model by using the sample contribution analysis. This analysis showed no significant correlation between disease duration and the distances to the PLS-DA model (r = -0.465 and P = 0.070).

Figure 2—Selection of three autoantibodies associated with T1DM. A: The overall scheme for selection of two novel autoantibodies tested in large-scale cohorts. See RESEARCH DESIGN AND METHODS for detailed procedures. B: Discrimination of T1DM from T2DM and NGT using PLS-DA. The two-dimensional PLS-DA score plot shows a separation among T1DM, T2DM, and NGT samples. The line indicates a decision function between T1DM and T2DM + NGT samples. C: Selection of a second list of autoantibodies with significant collective contribution to the separation-based VIP values. The cutoff of 1.59 (line) was determined as described in RESEARCH DESIGN AND METHODS. Among the 50 autoantibodies, four (GADA, EEF1A1-AAb, c7orf53-AAb, and UBE2L3-AAb) were selected (see RESEARCH DESIGN AND METHODS). D: A heat map showing the increased abundance of GADA, EEF1A1-AAb, c7orf53-AAb, and UBE2L3-AAb in T1DM patients compared with T2DM patients and NGT subjects. See Fig. 1B legend for the descriptions of the heat map.
We further selected the following three autoantibodies that were detected in none of NGT samples (i.e., z < 1.64 in all NGT samples) (Fig. 2D): 1) EEF1A1-AAb, 2) UBE2L3-AAb, and 3) c7orf53-AAb. For the selection of the autoantibodies targeting β-cells, we performed immunofluorescence staining of the pancreas using antibodies with specificity for EEF1A1, UBE2L3, and c7orf53. EEF1A1 and UBE2L3 were predominantly expressed in β-cells, whereas c7orf53 was exclusively found in α-cells (Fig. 3). On the basis of these results, EEF1A1-AAb and UBE2L3-AAb were selected as the candidates for the T1DM-associated autoantibodies for the subsequent studies. Both EEF1A1 and UBE2L3 were expressed in skin, stomach, colon, kidney, lung, and adrenal gland tissues (Supplementary Fig. 2), which was consistent with previous reports showing extrapancreatic expression of EEF1A1 and UBE2L3 (24–27).

### Validation of Novel Autoantibodies

We measured the amount of EEF1A1-AAb and UBE2L3-AAb in serum samples by ELISA in a large cohort, which comprised 95 T1DM, 49 T2DM, 11 LADA, and 66 NGT subjects as well as 20 patients with Graves disease. EEF1A1-AAb and UBE2L3-AAb were significantly higher in their abundance in T1DM samples than in NGT (P < 0.001 in both) and T2DM (P < 0.001 in both) samples (Fig. 4A). Of note, EEF1A1-AAb showed a significantly higher abundance in T1DM compared with Graves disease (P = 0.003). On the other hand, UBE2L3-AAb was not significantly different between T1DM and Graves disease. The prevalence of EEF1A1-AAb and UBE2L3-AAb was 29.5% and 35.8% in T1DM, respectively, which was significantly higher than T2DM and NGT (P < 0.001 in both). These antibodies were detected in only 2.0% of T2DM and 1.5% of NGT subjects (Table 2). To quantitatively assess the accuracy for prediction of T1DM, we generated receiver operating characteristic curves for the autoantibodies and computed the areas under the curve (AUCs). The AUCs (0.73 for EEF1A1-AAb, 0.78 for UBE2L3-AAb) revealed high accuracy of the autoantibodies in predicting T1DM (Fig. 4B). The comparison of EEF1A1-AAb and UBE2L3-AAb in the third cohort of children between T1DM and NGT showed a significantly higher abundance of these autoantibodies in T1DM than in NGT subjects (P = 0.007 and 0.020, respectively) (Fig. 4C), which is consistent with the results in the adult T1DM cohort.

Western blot analysis of the pull-down autoantibodies showed that the relative abundance of autoantibodies in the T1DM samples measured by the immunoprecipitation method reasonably agrees with the titers of autoantibodies measured by ELISA (Supplementary Fig. 3), and they were well correlated with each other (r = 0.779,
Clinical Implications of Novel Autoantibodies

Clinical characteristics of the novel autoantibodies were evaluated in the second cohort. The prevalence of EEF1A1-AAb and UBE2L3-AAb in T1DM was 29.5% and 35.8%, respectively, which was comparable to that of ZnT8A in adult T1DM (28). The most prevalent autoantibody, GADA in T1DM (8), was detected in 76.3% of T1DM patients (71 of 93). To further examine the clinical importance of this finding, the prevalence of the novel autoantibodies in the T1DM patients without GADA was analyzed. Of the 22 GADA-negative T1DM patients, an equal distribution (31.8%) of autoantibody between EEF1A1-AAb and UBE2L3-AAb was observed, and a total

| Table 2—Prevalence of anti-EEF1A1 and anti-UBE2L3 antibodies from ELISA |
|-------------------------------------------------|--------------------|--------------------|-------------------|--------------------|-------------------|--------------------|
| Prevalence of positivity*                       | T1DM (n = 95)      | T2DM (n = 49)      | LADA (n = 11)     | Graves disease (n = 20) | NGT (n = 66)      |                  |
| EEF1A1                                          | 28 (29.5)          | 1 (2.0)            | 0                 | 2 (10.0)             | 1 (1.5)           | <0.001            |
| UBE2L3                                          | 34 (35.8)          | 1 (2.0)            | 0                 | 4 (20.0)             | 1 (1.5)           | <0.001            |

Data are n (%). *The signal above (mean + 3 × SD) healthy controls was considered as positive. †P values from χ² test.
of 40.9% (9 of 22) had either EEF1A1-AAb or UBE2L3-AAb (Fig. 5A), which was higher than that of ZnT8A in an Asian population (29). Additionally, it is worth noting that the proportion of patients with evidence of autoimmunity in T1DM was increased from 76.3% to 86.0% when two newly identified novel autoantibodies were added to GADA.

We also observed the trend of negative correlation between the age of onset and the prevalence of EEF1A1-AAb. The age of disease onset was significantly younger in T1DM patients with EEF1A1-AAb than in those without EEF1A1-AAb (\(P < 0.001\)) (Table 3). Categorical analysis showed that the prevalence of EEF1A1-AAb in T1DM increased when the age of disease onset was young, which was not observed for GADA (\(P < 0.001\) for trend) (Fig. 5B), which was maintained even in the stratified analysis according to duration of diabetes (Fig. 5C). Duration of diabetes did not affect the prevalence of EEF1A1-AAb (Table 3), and EEF1A1-AAb titer was not associated with disease duration (\(r = 0.153, P = 0.139\)), whereas ZnT8A has been shown to decline rapidly in the first years after disease onset and is less persistent than IA-2A or GADA in the longer term (30). Of note, the prevalence of EEF1A1-AAb increased as the C-peptide level decreased (\(P = 0.03\) for trend), which was not observed for GADA (Fig. 5E). In addition, EEF1A1-AAb was detected in 27.3% of subjects with fulminant T1DM (3 of 11), whereas GADA was not detected in fulminant T1DM patients (Fig. 5F and Table 3). There was no difference in the prevalence of other autoimmune diseases or history of diabetic ketoacidosis between T1DM patients with or without EEF1A1-AAb. However, EEF1A1-AAb notably was not detected among the patients with LADA (Table 2). Furthermore, the characteristics found in patients with EEF1A1-AAb were similar to those with UBE2L3-AAb (Table 3 and Fig. 5B, D, and G). UBE2L3-AAb was detected in 18.2% of patients with fulminant T1DM, whereas UBE2L3-AAb was not detected in patients with LADA.

**DISCUSSION**

Novel autoantibodies indicative of the autoimmune destruction in the serum of T1DM patients can be important in developing new diagnostic and therapeutic means for T1DM patients. In this study, we extensively profiled autoantibodies by using a protein-antigen microarray in the sera of 16 T1DM, 16 T2DM, and 27 NGT subjects. Among 9,480 different proteins in the array, we initially selected three novel autoantibody candidates (EEF1A1-AAb, c7orf53-AAb, and UBE2L3-AAb) by M test coupled with PLS-DA. Considering potential autoantigens to be expressed in pancreatic \(\beta\)-cells, EEF1A1-AAb
and UBE2L3-AAb were selected as autoantibody candidates based on immunofluorescence staining for further validation studies. The increase in abundance of the selected novel autoantibodies in T1DM were confirmed by ELISA in a second cohort and a third test set of children.

The prevalence of EEF1A1-AAb and UBE2L3-AAb was ~30% for T1DM patients and 1.5% for NGT subjects in the present study, which is comparable to that of ZnT8A in adult T1DM patients (9). The two identified autoantibodies EEF1A1-AAb and UBE2L3-AAb have several clinical advantages compared with ZnT8A. First, the disease duration did not affect the prevalence of these autoantibodies. By contrast, ZnT8A was shown to decline rapidly in the first years after disease onset and to be less persistent than IA-2A or GADA in the longer term (30). This may imply that both EEF1A1-AAb and UBE2L3-AAb are more suitable for the young patients without GADA. Third, the prevalence of EEF1A1-AAb in GADA-negative T1DM was 31.8%, which is higher than that of ZnT8A in an Asian population (29). The prevalence further increased to 40.9% when adding UBE2L3-AAb. Considering that the prevalence of these autoantibodies increased for young T1DM patients (45.6% in T1DM patients with an age of onset <30 years), the use of both autoantibodies should be more efficient for the young patients without GADA. Third, T1DM patients with and without GADA showed no significant difference in the prevalence of EEF1A1-AAb and UBE2L3-AAb (38.0 and 28.2% in GADA-positive T1DM and 31.8 and 27.3% in GADA-negative T1DM for EEF1A1-AAb and UBE2L3-AAb, respectively). In addition, they were detected in 27% of patients with fulminant T1DM without GADA but were not detected in LADA patients. Thus, the two autoantibodies can provide novel diagnostic information and new insights into the classification of and therapy for T1DM as well as into T1DM pathogenesis.

EEF1A1 and UBE2L3 are involved in protein translation (31) and degradation (24), respectively. We previously reported aminocyl-transfer RNA synthetase as a novel marker of T1DM (32), which is also involved in protein translation. EEF1A1 is associated with Felty syndrome, an autoimmune disease (33), and UBE2L3 is associated with the risk of such autoimmune diseases as rheumatoid arthritis (34), Crohn disease (35), and systemic lupus erythematosus (36). These data indicate that protein homeostasis represented by these autoantibodies may reflect the autoimmunity in T1DM. Of note, the two autoantibodies correlated well in their levels (Supplementary Fig. 5). Additionally, the immunofluorescence staining of the corresponding two autoantigens in the pancreas using antibodies specific for EEF1A1 and UBE2L3 showed that both autoantigens were expressed higher in islets of Langerhans in the pancreas, more specifically in β-cells, than in the acinar cells and α-cells. The tissue array data showed that they are expressed in many tissues, suggesting that autoantibodies targeting EEF1A1 and UBE2L3 may come from epitope spreading, which is a typical phenomenon occurring during the preclinical and clinical disease process in T1DM (37). Nonetheless, all these data suggest that the protein homeostasis represented by these proteins can be used to show the

| Table 3—Clinical characteristics of T1DM patients with anti-EEF1A1 antibody, anti-UBE2L3 antibody, and anti-GADA |
|---------------------------------|---------------------------------|---------------------------------|
|                                | Anti-GADA                       | Anti-UBE2L3 antibody            |
|                                | Positive (n = 71)               | Positive (n = 34)               |
|                                | Negative (n = 22)               | Negative (n = 61)               |
| Female sex                     | 36 (50.7)                      | 12 (35.3)                      |
|                                | 9 (40.9)                       | 35 (57.4)                      |
| Age (years)                    | 29 (17–69)                     | 25 (17–33)†                    |
|                                | 32 (19–58)                     | 34 (17–69)                     |
| Age of onset (years)           | 25 (13–66)                     | 21 (13–33)†                    |
|                                | 29 (10–58)                     | 30 (10–66)                     |
| Disease duration (years)       | 3.5 ± 3.0                      | 3.3 ± 2.7                      |
|                                | 2.6 ± 3.2                      | 3.2 ± 3.7                      |
|                                |                                | 2.9 ± 2.7                      |
|                                |                                | 3.4 ± 3.7                      |
| Insulin requirement            | 0.68 ± 0.30                    | 0.73 ± 0.28                    |
| (International Units/day/kg)   | 0.75 ± 0.21                    | 0.69 ± 0.28                    |
| History of DKA                 | 20 (28.2)‡                     | 11 (35.3)                      |
|                                | 16 (72.7)                      | 27 (40.3)                      |
| Fulminant T1DM                 | 0‡                            | 3 (10.7)                       |
|                                | 11 (50.0)                      | 8 (11.9)                       |
| Other autoimmune disease       | 15 (21.4)                      | 5 (18.5)                       |
|                                | 3 (14.3)                       | 14 (21.2)                      |
| BMI (kg/m²)                    | 21.1 ± 3.1                     | 20.9 ± 3.2                     |
|                                | 21.6 ± 3.3                     | 21.4 ± 3.2                     |
| Fasting C-peptide (ng/mL)      | 0.33 ± 0.32                    | 0.22 ± 0.23                    |
|                                | 0.22 ± 0.24                    | 0.33 ± 0.33                    |
| HbA1c (%)                      | 9.7 ± 3.1                      | 9.7 ± 3.6                      |
|                                | 8.5 ± 2.0                      | 9.4 ± 2.9                      |
| Prevalence of GADA             | —                             | 21 (75.0)                      |
|                                | —                             | 50 (76.9)                      |
| Prevalence of anti-EEF1A1      | 27 (38.0)‡                     | 26 (92.9)‡                     |
|                                | 7 (31.8)                       | 8 (11.9)                       |
| Prevalence of anti-UBE2L3      | 21 (29.6)                      | —                             |
|                                | 7 (31.8)                       | —                             |
| Prevalence of anti-GADA        | —                             | 26 (76.5)‡                     |
|                                | —                             | 2 (3.3)                       |

Data are n (%), median (range), or mean ± SD. Anti-EEF1A1 and anti-UBE2L3 antibodies were measured by ELISA and anti-GADA by radioimmunoassay. DKA, diabetic ketoacidosis. †P < 0.05 from independent t test comparing the subjects without respective autoantibody. ‡P < 0.05 from χ² test comparing the subjects without respective autoantibody.
autoimmune destruction of pancreatic islet β-cells. Detailed functional studies can be designed to elucidate the mechanisms underlying the links of the protein homeostasis to the T1DM-related autoimmunity in the pancreas.

Furthermore, our approach involving autoantibody profiling provided a comprehensive list of autoantibody candidates that extends extensively the current list of autoantibodies identified by previous approaches, such as protein expression profiling of pancreas (4) and measurements of molecular targets associated with insulin secretion in β-cells (3,38) or pathologically proven autoantigens (2,39). Other than the two autoantibodies tested in study, an additional 66 potential novel autoantibodies, including fms-like tyrosine kinase receptor 3 ligand (FLT3LG), were previously shown to be associated with diabetes (40) and identified in the current study (Fig. 1B and Supplementary Table 2). FLT3LG treatment was reported to delay diabetes onset in the NOD mouse model of autoimmune diabetes (41). Protein kinase C (PRKCA), also shown in our list, is associated with multiple sclerosis (42), which is known to be initiated by a misdirected immune response against myelin autoantigens (43). In addition, our list includes the molecules associated with T1DM-related pathophysiology in pancreatic β-cells (Supplementary Fig. 6), such as insulin secretion [PRCKA (44), ACVR2B (45), GLUL (46), and PSMD9 (47)] and glucose metabolism [GPI (48)], indicating their potential roles in the autoimmune destruction of the pancreatic β-cells in T1DM. Thus, the list of autoantibody candidates we identified in this study can be a comprehensive basis for understanding the links to autoantibodies to T1DM diagnosis and pathogenesis.

The clinical implications of the two novel autoantibodies can be further tested with a larger number of GADA-negative and fulminant T1DM patients. In addition, longitudinal studies can be designed to further demonstrate the nature of dynamic changes of these autoantibodies during the course of disease progression, and new subtypes of T1DM patients might be further characterized based on the dimension of protein homeostasis represented by these autoantibodies when larger numbers of adult T1DM patients are used. Nevertheless, our approach successfully demonstrated the possibility of the use of the two autoantibodies as diagnostic markers and/or therapeutic targets. Finally, our autoantibody profiling approach can be applied to other autoimmune diseases for which autoantibody markers are unknown, and the newly identified autoantibodies can contribute to the understanding of the mechanisms underlying autoimmune destruction. In summary, our approach successfully identified two novel auto-antibodies, EEF1A1-AAB and UBE2L3-AAb, that can add a new dimension to the diagnosis, classification, therapy, and pathogenesis of T1DM.

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References


