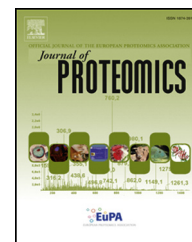


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Serological autoantibody profiling of type 1 diabetes by protein arrays

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ABSTRACT

The need for biomarkers that illuminate the pathophysiology of type 1 diabetes (T1D), enhance early diagnosis and provide additional avenues for therapeutic intervention is well recognized in the scientific community. We conducted a proteome-scale, two-stage serological AAb screening followed by an independent validation study. In the first stage, the immunoreactivity was compared between T1D cases and healthy controls against ~6000 human proteins using the nucleic acid programmable protein array (NAPPA). Genes identified with higher signal intensities in patients were challenged with a larger sample set during the second stage. Statistical analysis revealed 26 novel autoantigens and a known T1D associated autoantigen. During validation, we verified the presence of AAbs to dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) using the Luciferase ImmunoPrecipitation System (LIPS) assay (36% sensitivity, 98% specificity). The AUC for a combination of DYRK2A and the classical T1D AAb IA-2A was 0.90 compared to 0.72 for DYRK2A and 0.64 for IA-2A alone. This is the first systematic screening for seroreactivity against a large number of human proteins in T1D patients. We demonstrated the application of protein microarrays to identify novel autoantigens in T1D, expanded the current T1D “autoantigenome” and help fulfill the goal of searching for novel biomarker candidates for T1D.

Biological significance

Protein microarrays provide a high-throughput platform that enables the profiling of serum antibodies to a large number of protein antigens. The value of AAb biomarkers in diagnosis, prognosis and treatment is well recognized in autoimmune diseases including T1D. We performed a systematic screening for new T1D-associated autoantigens by adapting the

Abbreviations: T1D, type 1 diabetes; AAb, autoantibody; NAPPA, nucleic acid programmable protein array; TSA, thymid signal amplification; FDR, false discovery rate; CV, coefficient of variance; ROC, receiver operator characteristic.

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innovative protein array platform NAPPA. We believe that the discovery in this study will add information on the candidate autoantigens that could potentially improve the diagnosis and help uncover the pathophysiology of T1D. The successful use of NAPPA in T1D AAb profiling will open the window for larger studies including more human antigen genes and other autoimmune diseases.

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1. Introduction

Early studies of diabetes demonstrated the seroreactivity of individuals with T1D to islet cells in pancreatic cryosections [1], suggesting an AAb-mediated autoimmune component in this disease. The identification and study of AAbs associated with T1D [2–6] have confirmed their roles as biomarkers with value in diagnosis [7], prognosis, patient treatment stratification [8], tolerizing therapies as well as providing insights into the pathophysiology of disease [9]. AAbs against insulin, glutamic acid decarboxylase 65 (GAD65), protein tyrosine phosphatase receptor N (IA-2) and zinc transporter member 8 (ZnT8) antigen proteins in T1D have a combined clinical performance enabling the detection of >90% of T1D [4]. The known T1D-associated AAbs are frequently used for research studies and increasingly used in clinical management for purposes such as identifying individuals with T1D risk, stratifying patients with different disease course and improving our understanding of autoimmune destruction [10–12]. Despite this, known T1D associated AAbs against antigens like insulin and GAD65 are not diabetes specific. Insulin autoantibody (IAA) appears in insulin autoimmune syndrome while GAD65 autoantibody (GADA) appears in Stiff-Man Syndrome [13]. In addition, many diabetic individuals positive for islet cell antibody (ICA) staining are negative for all known anti-islet AAbs [14], suggesting the existence of additional, yet-to-be-identified autoantigens [14]. Comprehensive identification of autoantigens targeted in T1D will help fully characterize the heterogeneity of disease represented in the “autoantigenome” that may enable enhanced diagnostics, personalized therapies and a fundamental understanding of diabetes pathology.

Autoantigen discovery in T1D has been slow with the identification of four major autoantigens over the past four decades. Previous AAb biomarkers were discovered based on either the known understanding of T1D pathogenesis (e.g., IAA) using radioimmunoassay (RIA) or immunoprecipitation of autoantigens from cell lysates by patient serum. With the development of genomics and bioinformatics, ZnT8 was identified as a major T1D autoantigen by analyzing pancreatic gene expression profiles followed by RIA [4]. No large-scale screening of individual proteins at the proteome level has been conducted to search for new T1D AAb biomarkers perhaps due to the lack of appropriate high-throughput proteomic techniques.

The advent of proteomic technologies like protein microarrays has provided attractive opportunities to profile AAb against a large number of human proteins in T1D. Protein microarrays in particular are an invaluable tool for simultaneous interrogation of thousands of proteins and possess immense potential as a non-biased discovery tool to identify AAbs targeting self-antigens. They have been used in a variety of systematic and organ-specific autoimmune diseases including autoimmune hepatitis [15], rheumatoid arthritis [16], multiple

sclerosis [17] as well as in various cancers including breast cancer [18], lung cancer [19], colon cancer and colorectal cancer [20,21].

Conventional protein microarrays printed from purified proteins, however, suffer from practical limitations including the substantial cost and time associated with purifying proteins from sizable libraries that can make large scale screening cost-prohibitive. The wide range of protein concentrations that deposited and limited shelf lives due to the instability of purified proteins, can further compromise the array utility. Recent conceptual advances achieved with the nucleic acid programmable protein array (NAPPA) platform have circumvented these primary challenges associated with conventional protein microarrays by printing cDNA encoding plasmids instead of purified proteins [22] (Fig. 1). Plasmids encoding genes with a C-terminal affinity tag are co-printed on the array along with anti-tag antibody allowing for the cell-free expression and immediate capture of thousands of functional proteins with a wide range distribution of protein sizes on glass slides [22]. Slides are stable at room temperature for at least one year and can be expressed at the time-of-use. NAPPA has been applied in a variety of AAb discovery studies such as breast cancer and arthritis [18,23].

In the present work, we report the first large scale use of protein microarrays in profiling of AAbs in T1D. We conducted a two-stage, sequential serological immunoreactivity screening and an independent validation study. The two stages of screening resulted in the identification of 26 candidate autoantigens including ZnT8 ($p < 0.005$, FDR < 10%). Candidate genes were selected for further validation via LIPS assay in an independent serum set [24]. Using this assay, we confirmed a sensitivity of 36% at the specificity of 98% for the dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2), thus demonstrating the use of protein microarrays in the search for novel T1D associated AAbs.

2. Methods and material

2.1. Serum samples

T1D serum samples included in the study were obtained from individuals diagnosed with T1D, according to American Diabetes Association (ADA) criteria, at College of Medicine, the University of Florida. Peripheral blood samples were drawn from the antecubital vein and serum was prepared according to a standardized protocol before freezing serum aliquots at -80°C . Control samples were drawn and prepared in the identical fashion to T1D patients and selected to be age-matched to the patient set. Control samples were considered to be at low risk of T1D with the absence of known T1D-associated AAbs and T1D family history. Independent sample sets were used in each stage

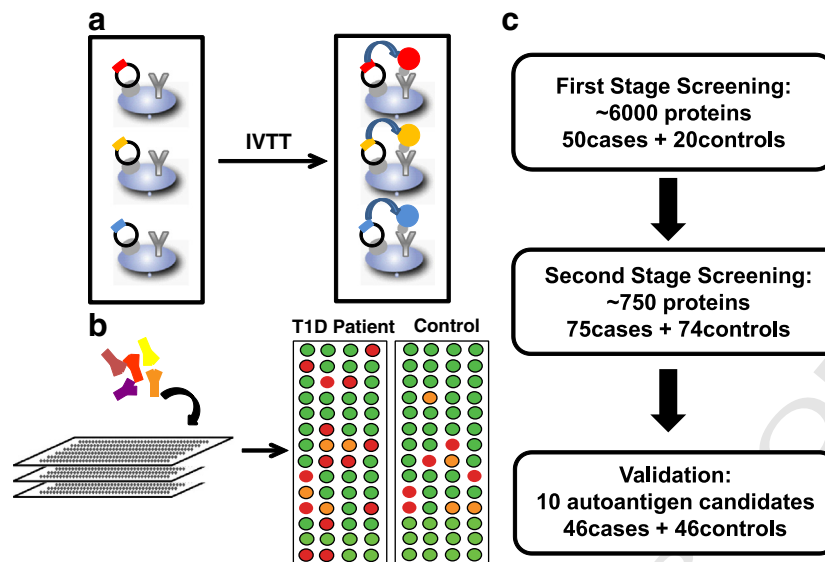


Fig. 1 – Scheme of NAPPA and the strategy of T1D AAB screening. (a) Nucleic acid programmable protein array (NAPPA) — spotted plasmids are printed along with a capture antibody, expressed in situ using a cell free expression system, and the protein of interest is captured by an anti-tag antibody; (b) protein displaying-microarray slides are challenged with serum from either patient or control individuals. The difference in immunoreactivity is determined by tyramide-amplified signals from the HRP-labeled anti-human IgG secondary antibody. Antigens that repeatedly show a higher intensity in patients than controls are considered candidate T1D autoantigens. Red dots represent high reactivity, yellow dots represent medium reactivity; green dots represent low reactivity; (c) three stages of AAb discovery strategy: First stage screening: A large number of expressed antigen genes were challenged with a smaller number of sera samples to eliminate uninformative genes; second stage screening: Antigens that exhibited higher responses among patient serum were challenged with a larger number of patient and control serum samples to scale down the number of candidates for validation; Validation stage: Top candidates from microarray analysis were validated by an independent luminescent immunoprecipitation (LIPS) method.

of study. Neither patients nor control samples were known to have any other underlying autoimmune disease. All samples were collected with written informed consent, under the guidelines of the Institutional Review Boards (IRB) at the University of Florida, the Harvard Medical School and Arizona State University through the entire study. The detailed sample information is indicated in Table 1.

2.2. Array production and quality assessment

Human genes used in this study were obtained from DNASU (<http://dnasu.asu.edu/DNASU/>). NAPPA was produced and

the quality of DNA printing and protein expression was controlled as previously described [25–27]. The correlation of protein expression within one printing run or two printing runs was compared to determine the reproducibility of slide production.

2.3. Profiling of serum AABs

Serum AAb profiling on NAPPA was performed as previously described [18]. Briefly, once slide quality had been confirmed, expressed slides were challenged with serum samples, HRP-linked anti-human IgG secondary antibodies and developed by

Table 1 – Demographic data of the patient and control samples used in this study.

		Total	Gender male (%)	Age mean ± SD (median)	Years from diagnosis mean ± SD (median)
First-stage screening	T1D	50	41%	17.6 ± 10.4 (14.4)	6.7 ± 8.4 (4.0)
	Healthy	20	55%	24.3 ± 9.6 (23.4)	N/A
Second-stage screening	T1D	74	46%	21.1 ± 11.3 (17.0)	5.5 ± 7.7 (2.3)
	Healthy	75	43%	18.5 ± 12.1 (15.5)	N/A
Validation stage	T1D	46	25%	17.2 ± 11.0 (13.0)	6.8 ± 8.5 (3.5)
	Healthy	46	17%	17.7 ± 11.5 (15)	N/A

Tyramide Signal Amplification [28]. All slides were scanned by a ScanArray ProScanArray HT scanner (Perkin Elmer) at the same settings. Individual spot signal intensities were quantified using Microvigen Image Analysis Software V2.9.9.2. Slide images were vetted to remove visually defective features (i.e. with scrapes, smudges, dust particles, or obvious contribution of signal from adjacent features).

2.4. Study design and data analysis

Screening efforts were structured in two stages (Fig. 1). In the first stage of screening, we used 50 cases and 20 control serum to screen >6000 unique proteins across three arrays. Signal intensities were normalized by first subtracting the background signal estimated by the 25th percentile of the negative control (printing mix alone without plasmid DNA) on each slide and then scaling data of each slide to the same overall median intensity and median absolute deviation. Normalized signal intensities were analyzed by comparing the 90th percentiles of case and control samples using three statistical tests: Quantile regression [29], Fisher's exact test and a binomial proportion test. The antigens that did not exhibit statistically significant ($p < 0.05$) differential reactivity in cases versus controls according to any of the three tests were eliminated. The remaining antigens were ranked by the difference between the summed signals of cases versus controls above the 90th percentile of the controls. ~750 antigens from three arrays were advanced to the second stage screening (Table S1).

In the second stage, 74 case and 75 control sera were screened using ~750 antigen genes identified from the first stage. After background subtraction, Wilcoxon Rank-Sum Test and the partial Area Under the Curve at 95% specificity (pAUC95) were applied in the analysis of normalized data to rank genes that have differential reactivity between patient and control group. The sensitivity and p-values of each candidate were generated. The rationale to include two statistical methods to analyze the second-stage screening data was to be more inclusive with autoantibody biomarker candidates for validation.

During the validation stage, we investigated the sensitivity and specificity of the combination of IA-2A and DYRK2A by constructing a classification rule using logistic regression [30–32]. We conducted leave one out cross validation to assess the performance of the logistic regression classifier. Specifically, disease status for each sample was predicted using a logistic regression classifier that was trained using the remaining samples. These cross validation predictions were then used to conduct receiver operator characteristic (ROC) analysis and calculate the AUC for the combination of IA-2A and DYRK2A.

2.5. Validation of candidates by LIPS assay

The liquid-phase, luminescent immunoprecipitation assay system (LIPS) was used to verify a selection of candidate autoantigens from the second stage screening [33]. C-terminal renilla luciferase fusion products (Ag-cRuc) for each antigen were generated using a modified pRL-CMV vector (Promega), engineered to contain a gateway-compatible death cassette (available at DNASU) and T7 promoter. Gateway compatibility enabled the rapid and accurate transfer of candidate genes from

entry vectors into the expression vector. The incorporation of T7 promoter to pRL-CMV parental vector allows the choice of expression in either mammalian cells via the CMV promoter or in-vitro by cell free expression via T7 promoter, thus increasing ease, speed and versatility. Where available, entry clones encoding the autoantigen candidate genes were used to generate the expression clones by a LR recombination reaction. In all other instances, inserts were cloned into a pDON221 entry vector by PCR amplification and homologous recombination using BP Clonase II (Invitrogen) and then into the pRL-CMV vector using LR Clonase II (Invitrogen). Plasmid DNA encoding the antigen-cRuc was purified and sequence verified from single colonies and stored as glycerol stocks.

For expression, plasmid DNA encoding antigen-cRuc was mixed at a ratio of 2 μ g/20 μ L in rabbit reticulocyte lysate (RRL) supplemented with 10 μ g/mL tRNA for protein expression at 30 °C for 90 min. Expression of each clone was verified by 1) luciferase activity of RRL-expressed antigen (3×10^5 to 1×10^6 ALU/ μ L lysate) versus the same volume of no DNA control retic; and 2) western blot using anti-renilla antibody or antigen specific antibody. For assay of serum responses, serum samples were diluted at a 1:10 ratio in buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) and prepared in a 96 well plate. 50 μ L diluted antigen-cRuc protein was incubated with 10 μ L diluted serum sample and 40 μ L buffer A at 4 °C overnight with gently shaking at 500 rpm on a plate shaker (Eppendorf). The antigen and serum mixture was transferred to a Millipore HTS filter plate (Millipore, Bedford, MA) with 5 μ L of 30% slurry protein A/G beads (Pierce Biotechnology, Rockford, IL) and incubated at RT for 2 h. After intense wash with 8 $\times$ of 100 μ L buffer A each time and 2 $\times$ of 200 μ L PBS, the luminescent intensity of each sample was quantified in duplicate by reading on a Glomax Luminometer (Promega) with a 5 second integration time following automated injection of 50 μ L of coelenterazine substrate. Jitter plots were then generated from log transformed raw luminescent signal intensities using R software to enable the comparison of patient and control population immunoreactivity to candidate autoantigens. Sensitivity for each autoantigen was calculated at the 98% specificity.

3. Results

3.1. Array production and quality assessment

High quality NAPPA arrays that comprised proteins displayed at high level are keys to a successful serum screening study. Prior to serum challenge, protein display on NAPPA was quality assessed by anti-tag antibodies, confirming the robustness of protein expression (Fig. 2). Signal intensities for protein levels on the array were similar to those previously estimated to be in the average magnitude of 9 fmol per feature [34]. Importantly, scatter plots of signal intensities of anti-tag staining from replicate arrays revealed correlation coefficients of >0.84 from two randomly selected slides within a print run or between two print runs attesting the reproducibility of slide printing and protein display, further confirming the suitability for serum screening (Fig. 2).

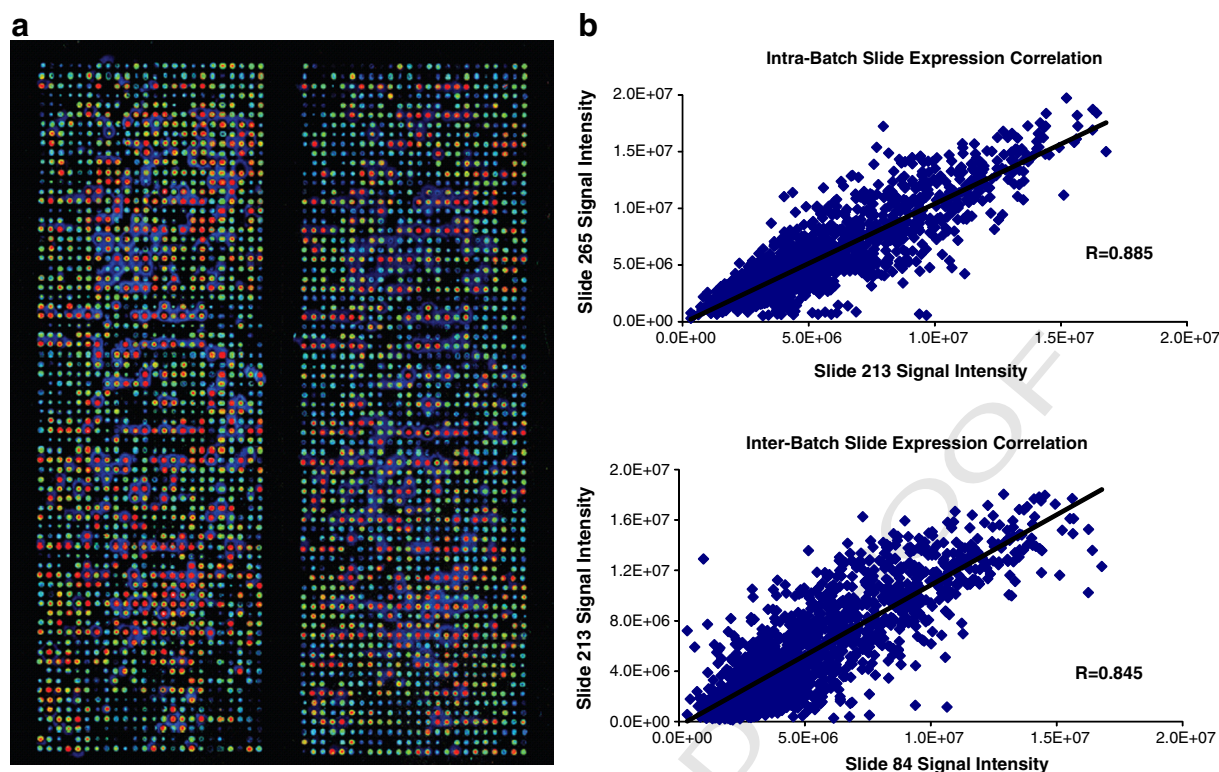


Fig. 2 – Reproducibility of protein expression on NAPPA. (a) Pseudo-colored image of anti-GST staining on two expressed slides to assess the level of protein display: slides were developed with a mouse monoclonal anti-GST to an epitope distinct from that used for antigen capture enabling assessment of protein expression, capture and display and confirming reproducibility; **(b)** plots of signal intensities from all spots for two arrays within or between printing batches showed $R > 0.84$.

3.2. Screening strategy

Screening efforts were structured in two stages (Fig. 1). During the first stage, >6000 unique proteins, printed across three arrays were challenged with serum from 50 T1D patients and 20 healthy controls. The goal was to facilitate the second stage by eliminating uninformative antigens. Thus any antigens that showed no immune response in either group (i.e., most antigens) or no appreciable difference in response were eliminated. During the second stage, ~750 candidate genes (Table S1) identified from the first stage were printed in duplicate and challenged with an independent sample set of 74 patients and 75 healthy controls to confirm the reproducibility of enhanced immunoreactivity and to further scale down the number of candidate autoantigens for independent validation. The advantages of this two-stage screening experimental design are three-fold: 1) It minimized the cost of screening against such a large initial group of antigens; 2) it ultimately reduced the logistical burden of handling large numbers of slides during screening and 3) it limited the potential for overfitting of the data.

In the first stage screening, statistical analysis revealed approximately 12% of these ~6000 proteins displayed enhanced reactivity when challenged with patient sera as opposed to that from healthy controls. During the second stage, both the case and control samples were independent of the samples used in the first stage. Raw screening data were produced and normalized after removing the within block and pin effects. Analysis of the normalized signal intensity by the Wilcoxon Rank-Sum Test

identified 26 proteins whose median antigen reactivity was higher in patients than in controls ($p < 0.005$) (Table 2). Similarly, analysis of the normalized antibody-dependent signal intensity resulted in the identification of 25 proteins that displayed differential immunoreactivity when the statistical significance of the partial area under the curve at 95% specificity (pAUC95) was tested to be higher in T1D patients than controls ($p < 0.005$) (Table S2).

To enable the visual comparison of immunoreactivity obtained from the second-stage screening, image scans of serum challenged slides were adjusted to be pseudo-colored in identical black and full color threshold scale. Slides with duplicate features designated as seropositive in different patients by statistical analysis were compared to a slide challenged with control serum with similar median signal intensities (Fig. 3). Visually discernible differences for the antigens calpain-11 (CAPN11), CDK3, astrocytic phosphoprotein (PEA15) and sphingosine-1-phosphate receptor 3 (SIPR3) suggested that the normalization scheme employed did not distort the data in a manner that either 1) creates signal differentials that do not exist or 2) destroys true signal differentials. In order to provide a visual representation of signal intensities of candidate antigens across all samples, jitter plots were generated from normalized and log-transformed signal intensities to illustrate the comparison of immunoreactivity of known T1D associated autoantigens as well as the novel ones in patient and control groups (Fig. 4).

Table 2 – List of 26 candidate autoantigens from the second screening stage analyzed by Wilcoxon Rank-Sum Test ($p < 0.005$), individual sensitivities were determined at 95% specificity.

Second stage screening candidates ranked by Wilcoxon Rank-Sum Test

		PAUC-95	PAUC-95	PAUC-95	Median of	Median of	Wilcoxon	AUC	Sensitivity	Specificity
			p-value	q-value	patients	controls	Rank-Sum p-value			
t2.6	TBCA	0.0130	0.0000	0.0010	1.29	1.19	0.0000	0.72	32.00%	94.59%
t2.7	DOM3Z	0.0064	0.0453	0.1920	0.27	0.21	0.0003	0.66	27.03%	94.59%
t2.8	TTC4	0.0038	0.0382	0.1905	1.07	1.02	0.0004	0.66	9.33%	94.59%
t2.9	Slc30A8-NC	0.0037	0.1229	0.2411	2.14	1.88	0.0004	0.66	16.00%	94.59%
t2.10	LIMK2	0.0063	0.0139	0.1266	1.23	1.12	0.0005	0.66	20.00%	94.59%
t2.11	CAPN11	0.0149	0.0000	0.0007	1.71	1.61	0.0005	0.66	36.49%	94.37%
t2.12	TSPAN31	0.0043	0.0389	0.1905	0.96	0.91	0.0006	0.65	9.33%	94.52%
t2.13	PEA15	0.0112	0.0000	0.0032	0.89	0.84	0.0006	0.65	25.33%	94.59%
t2.14	CDK6	0.0046	0.0542	0.1967	1.02	1.00	0.0007	0.65	14.67%	94.59%
t2.15	DYRK2	0.0046	0.0233	0.1647	0.81	0.73	0.0008	0.65	12.00%	94.52%
t2.16	GPR120	0.0038	0.1187	0.2411	1.27	1.18	0.0011	0.64	17.33%	94.59%
t2.17	POU2F1	0.0048	0.0808	0.2188	1.36	1.27	0.0014	0.64	18.67%	94.59%
t2.18	CDK4	0.0062	0.0452	0.1920	1.75	1.61	0.0018	0.64	20.27%	94.44%
t2.19	KCNK13	0.0077	0.0134	0.1266	1.11	1.06	0.0019	0.64	24.00%	94.52%
t2.20	FOXO1	0.0025	0.2340	0.2901	0.81	0.77	0.0021	0.64	10.67%	94.59%
t2.21	CDKN1A	0.0045	0.1307	0.2411	1.71	1.59	0.0022	0.64	23.29%	94.59%
t2.22	HOXB7	0.0085	0.0022	0.0603	1.27	1.21	0.0025	0.63	22.97%	94.59%
t2.23	ARF6	0.0006	0.5963	0.4706	1.24	1.11	0.0026	0.63	2.67%	94.59%
t2.24	GTF2A1	0.0133	0.0000	0.0007	0.97	0.93	0.0033	0.63	26.67%	94.59%
t2.25	HTR1E	0.0089	0.0033	0.0716	1.35	1.28	0.0034	0.63	28.00%	94.44%
t2.26	IL13RA2	0.0081	0.0024	0.0603	1.01	0.99	0.0040	0.63	24.00%	94.44%
t2.27	CDC2	0.0019	0.2352	0.2901	1.03	0.99	0.0040	0.63	5.33%	94.59%
t2.28	SERPINH1	0.0060	0.0542	0.1967	1.10	1.08	0.0045	0.62	21.33%	94.52%
t2.29	KLK3	0.0024	0.2166	0.2812	1.56	1.45	0.0046	0.62	10.67%	94.44%
t2.30	TBRG4	0.0061	0.0082	0.1168	1.57	1.51	0.0048	0.62	15.07%	94.59%
t2.31	ZFP64	0.0067	0.0137	0.1266	1.30	1.23	0.0048	0.62	18.92%	94.59%

3.3. Autoantigen candidates identified from the second-stage screening

Known T1D autoantigens, such as ZnT8-NC (Slc30A8), GAD65 and IA-2, were included as positive controls on the second stage screening array. The display levels of these antigens were confirmed by anti-GST staining of arrays (data not shown). In this protein microarray assay, the median signal of known autoantigen-ZnT8-NC (Slc30A8) in patient group was significantly higher than the control group ($p < 0.0004$) from the Wilcoxon Rank-Sum Test (Table 2); however, none of the other three known autoantigens, insulin, IA-2, or GAD65 exhibited statistically significant increase reactivity toward patient sera. The absence of immunoreactivity to those three known T1D associated autoantigens may reflect a differential performance of solid-phase immune-profiling techniques in the detection of some epitopes. Nevertheless, we were able to identify 25 additional candidate autoantigens besides ZnT8-NC (Slc30A8) using our unbiased screening strategy (Table 2). Some of these autoantigens are known to be expressed in the pancreas or enriched in islet cells such as PEA-15 and DYRK2 [35,36].

3.4. Validation of candidate autoantigens

Candidate autoantigens were selected based on the significance of differential reactivity (as indicated by p-value) and their potential roles in beta-cell physiology for validation in an independent serum set (46 patients and 46 controls) by LIPS assay [24]. We decided to employ LIPS as our validation platform

because it was a solution based assay platform orthogonal to protein array and the performance of LIPS on the known T1D associated autoantigens (GAD65 and IA-2) was similar to the widely accepted RIA in clinical T1D studies. We modified the published LIPS assay expression vector pRL-CMV so that it was compatible with Gateway® Cloning, allowing us to transfer our candidate genes into the modified vector easily and to produce candidate antigens by the rabbit reticulocyte lysate (RRL) IVTT system (Fig. 5) consistent with the antigen production used throughout this study and in most reported RIA assays [37]. We first tested the modified pRL-CMV vector on IA-2, a known T1D-associated autoantigen. LIPS assay confirmed IA-2 with a sensitivity of 54% at 98% specificity, comparable to the performance of LIPS assay and commonly used RIA assay reported by Burbelo, P.D. et al. [33].

Besides IA-2, 9 sequence-verified candidate genes (PEA15, CAPN11, DYRK2, CDK1, CDK3, CDK4, CDK6, TBCA, GSK) including candidates from supplement statistical analysis (Table S2) and previous published T1D associated autoantigens were cloned into the modified pRL-CMV expression vector with c-terminal luciferase fusion and immunoreactivity to these antigens was measured by LIPS. Luminescent data for each sample collected were from the average of the replicate measurements. Average patient signals that exceed the 98th percentile of control signals were considered to exhibit positive reactivity.

One candidate antigen, DYRK2 showed 36% sensitivity at 98% specificity (Fig. 5); whereas, the other nine candidates showed lower sensitivities (data not shown). We suspected the failure to validate these latter candidates may be due to the platform difference. The performance of DYRK2 was

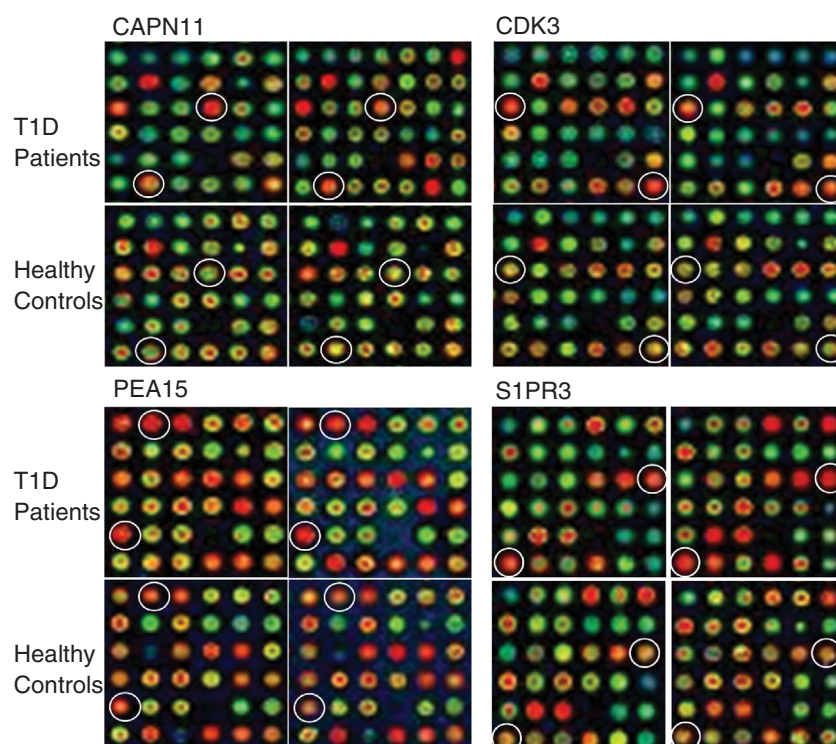


Fig. 3 – Visual comparison of candidate autoantigens identified by statistical analysis of feature intensity data. Array images challenged with serum sample from different patients that deemed positive by statistical analysis were adjusted to identical black and color thresholds to enable visual comparison. Differences were readily perceived upon visual examination of immunoreactivity of candidate features between patient and control serum-challenged slides. Intensity scale = red > orange > yellow > green.

confirmed by several repeated assays (data not shown). The addition of DYRK2A improved the AUC for IA-2A from 0.64 to 0.90. DYRK2A has an AUC of 0.72 on its own. The combination of DYRK2A and IA-2A has a sensitivity of 72% at 98% specificity.

4. Discussion

The rapid rise in diabetes incidence imposes a substantial economic burden on health care systems worldwide [38]. Primarily affecting children and young adults, T1D is characterized by the progressive destruction of insulin producing pancreatic beta cells and imposes a lifelong responsibility to maintain glucose homeostasis through regular insulin injections. It is believed the autoimmune reactions mediated by B cells and T cells play an important role in disease pathogenesis and autoantibodies to self-proteins are good predictors for disease diagnosis and progression. Historically, four autoantigens (Insulin, GAD65, IA-2, ZnT8) have been reported as major targets of AAb with individual sensitivity more than 55%, the combination of these four autoantigens detects >90% of T1D patients [39]. Nevertheless, the prevalence of individuals that are ICA positive but negative for all four known AAbs is not rare at all ages and thus provides impetus for the discovery of novel autoantigens that are not yet identified [14,40]. Minor T1D-associated autoantigens with low sensitivity and specificity such as carboxypeptidase-H [41], ICA69 [42], GM2-1 [43], microsomal

hepatic protein [44], SOX13 [45], HSP70 [46], topoisomerase II [47], VAMP2 and NPY [48] were reported continuously and provide deeper understanding of T1D pathogenesis in addition to the four major ones. The above AAb biomarker discovery studies relied upon a targeted strategy and restricted inquiry to the landscape of the pancreas using low-throughput experimental techniques such as phage display, ELISA and RIA [48].

The value of AAbs in prediction and diagnosis of T1D is well-established; however, their pathogenic role is contentious [49]. AAbs, generated during the influx of immune cells into the peri-islet space, are most often considered byproducts of a pre-existing underlying pathology, rather than a causative agent themselves. A comprehensive knowledge of the 'autoantigenome' in T1D will undoubtedly assist in generating and testing hypotheses aimed at answering questions regarding mechanisms of beta cell damage and the temporal expression patterns of autoantibodies that aid in disease characterization. We believe that a systematic profiling of serological AAbs will expand the current 'autoantigenome' in T1D and illuminate T1D pathogenesis.

To date, no unbiased, proteome scale and high-throughput AAb discovery studies have been published for T1D. The development and successful use of new discovery technologies such as protein microarrays offer a valuable opportunity to design non-biased, data-driven studies to profile serum AAbs to a large number of human proteins in a high-throughput manner. Our innovative protein array platform, NAPPA, has been used successfully in identifying circulating antibodies

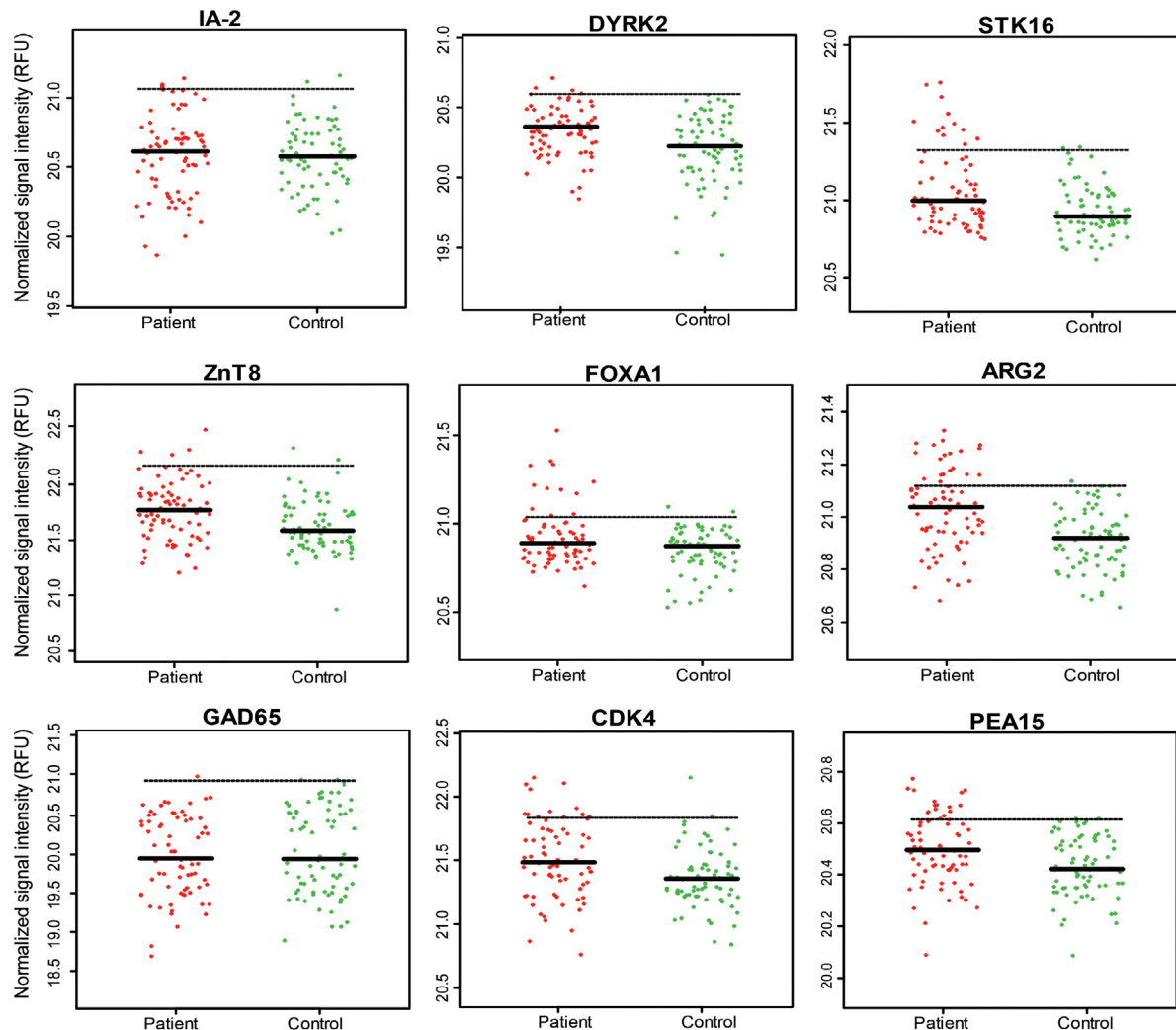


Fig. 4 – Jitter plots of serological immunoreactivity for indicated antigens from the second stage screening data. Each dot represents the signal from one patient or control serum to the indicated antigens; the solid lines indicate median signal intensities of patient (n = 74) or control group (n = 75). The upper line across both patient and control groups, indicates the 95th percentile of control signal.

in infectious diseases, autoimmune diseases and cancers [18,23,50]. In this study, we reported that the establishment of a NAPPA protein array based high-throughput pipeline of AAb discovery in T1D and demonstrated an initial success of identifying DYRK2 as a minor autoantigen using this pipeline.

Our high-throughput discovery pipeline involved two large screening processes followed by a targeted solution-based validation study. Although a known T1D-associated autoantigen-ZnT8 was among the final candidate list, neither GAD65 nor IA-2 was identified presumably due to the differences in the manner in which responses to those antigens are typically measured. Nevertheless, several candidate autoantigens we identified are known to play critical roles in beta cell physiology and are associated with exocytotic machinery. PEA15 was implicated in the regulation of glucose induced insulin secretion through the inhibition of potassium channel expression and [35], STX6 has been shown to function in the process of endosomal maturity and its delivery to lysosomes and may further influence the secretory pathway in live pancreatic beta-cells [36]. Several ubiquitously expressed cyclin-dependent kinases (CDKs)

were identified in our list including CDK3, CDK4 and CDK6. Studies in human and mice have revealed that beta cell replication and proliferation can be stimulated by the over-expression of either CDK4 or CDK6 without evidence of cell death, loss of function or aberrant differentiation [51,52], but how they would be targeted in the pancreas as autoantigens is unclear. In addition, candidates that show no strong connection with diabetes such as S1PR3 and endoplasmic reticulum to nucleus signaling 2 (ERN2) were also identified. The diversity of functions of the identified autoantigen candidates demonstrated the power of our unbiased screening strategy.

To confirm our findings, we took an initial step in validating the above autoantigen candidates in an independent serum sample set using an orthogonal liquid-phase assay originally developed by Burbelo, P.D. et al. [37]. To adapt this assay platform for compatibility with the IVTT system, used in our study, we modified the expression vector by engineering it with a Gateway™-compatible cassette to simplify the process of gene transfer and T7 promoter to enable antigen production in an RRL IVTT system. We selected the candidate antigens for

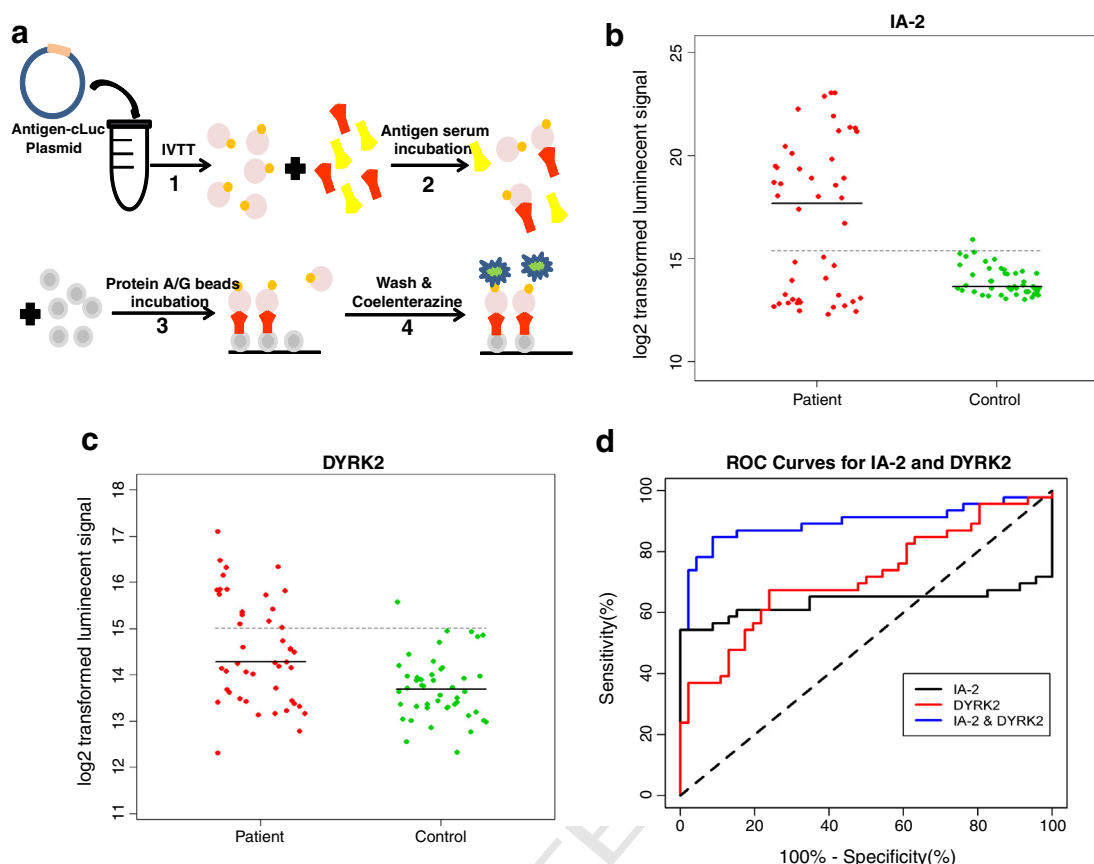


Fig. 5 – Validation of candidate autoantigens using LIPS assay. (a) Schematic representation of LIPS assay; (b,c) LIPS assay confirmed the performance of IA-2 and DYRK2 with 54% and 36% sensitivity at 98% specificity respectively. The solid lines represent the median signal of patient (n = 46) and control group (n = 46), the dash line indicates the 98% percentile of signal intensities from control group. (d) ROC curves for IA-2 and DYRK2 alone and the combination of the two.

validation based on their classification performance and putative roles in beta-cell biology.

DYRK2 was validated with a 36% sensitivity at 98% specificity, further analysis indicates that DYRK2 complements IA-2 by a moderate increase in sensitivity, whereas it did not complement GAD65. IA-2A has a sensitivity of 54% at 98% specificity on its own. We used the widely accepted linear regression model to combine the two markers and generate the ROC curve. The addition of DYRK2A improved the AUC for IA-2A from 0.64 to 0.90. An important next step will be to validate the performance of the DYRK2A and IA-2A combination in future studies on independent sample sets.

The identification of minor autoantigens like DYRK2 and future characterization of the correlation of their seropositivity with other clinical features may benefit other aspects of clinical patient management (e.g. stratification) and advance our understanding of disease etiology and heterogeneity. DYRK2 is a member from novel dual specificity protein kinase family and has been implicated in the glucose metabolic pathway as responsible for the phosphorylation and inactivation of glycogen synthase [53]. DYRK2 is highly expressed in the pancreas based on protein atlas data and has been implicated in the apoptosis pathway in response to severe DNA damage through the activation of the p53 signaling [54]. Moreover, a Genome wide association study (GWAS) found that DYRK2 is significantly

associated with type 2 diabetes among Mexican Americans [55]. Its role in T1D pathogenesis requires further investigation.

Other candidates from our screening did not validate in the LIPS assay, which may reflect differences in the detection methods. Antigen conformation and/or binding characteristics of autoantibody markers to their target antigens that can be identified in a solid-phase assay may be very different from those identified in a liquid-phase assay. The original rationale for choosing a liquid phase validation platform included using a method that had previously been shown to work for two classical T1D AABs and to ensure the markers we report can perform on two orthogonal validation platforms. It is expected that some markers will only perform well on one platform but not both. This may explain why some of the markers discovered on the arrays did not validate in the liquid phase assay. Some classical autoantibodies in T1D have been reported to behave differently on different platforms. For example, it has been reported that insulin antibody (IAA), a known T1D autoantibody, showed lower sensitivity, specificity and AUC in liquid phase assays compared with a solid phase ELISA assay [56,57]. On the other hand, IA-2A performs better when measured by the liquid phase Radio Immuno Assay (RIA) than on the solid phase ELISA platform [58]. It is possible that some conformational epitopes of antigen proteins might be blocked by the capture antibody in our array platform. Whereas in the liquid LIPS assay

platform, these epitopes were exposed and recognized by serum autoantibodies. On the other hand, the array platform might display some epitopes that are not exposed in a liquid phase assay. Assessment of sero-reactivity to these candidates on different platforms such as the multiplexed self-assembling bead arrays on the Luminex platform [59], plate-based programmable ELISAs [23] or targeted small content NAPPA arrays [18] may shed light on the behavior of these antigens presented in different formats.

In conclusion, we have developed a discovery pipeline that enables the first large scale proteome scale searching for AAb biomarkers in T1D on an innovative protein array platform using a two-stage screening strategy, resulting in the discovery of minor autoantigen-DYRK2 (36% sensitivity at 98% specificity), thus, demonstrating the applicability of NAPPA to AAb discovery in T1D and providing impetus to expand this technology to the whole human proteome with the established discovery pipeline.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.10.018>.

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