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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, tyramide 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 candidate autoantigens that could potentially improve the diagnosis and help uncover the pathophysiology of T1D. The successful use of NAPPA for T1D AAb profiling will open the window for larger studies including more human antigen genes and other autoimmune diseases.

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 courses and improving our understanding of autoimmune islet cell 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]. However, until now, 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 AAbs 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 [18], lung [19], 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 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 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 for 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 the University of Florida College of Medicine. 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 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 of