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Temporal expression profiling of plasma proteins reveals oxidative stress in early stages of Type 1 Diabetes progression

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ABSTRACT

Blood markers other than islet autoantibodies are greatly needed to indicate the pancreatic beta cell destruction process as early as possible, and more accurately reflect the progression of Type 1 Diabetes Mellitus (T1D). To this end, a longitudinal proteomic profiling of human plasma using TMT-10plex-based LC-MS/MS analysis was performed to track temporal proteomic changes of T1D patients (n = 11) across 9 serial time points, spanning the period of T1D natural progression, in comparison with those of the matching healthy controls (n = 10). To our knowledge, the current study represents the largest (> 2000 proteins measured) longitudinal expression profiles of human plasma proteome in T1D research. By applying statistical trend analysis on the temporal expression patterns between T1D and controls, and Benjamini-Hochberg procedure for multiple-testing correction, 13 protein groups were regarded as having statistically significant differences during the entire follow-up period. Moreover, 16 protein groups, which play pivotal roles in response to oxidative stress, have consistently abnormal expression trend before seroconversion to islet autoimmunity. Importantly, the expression trends of two key reactive oxygen species-decomposing enzymes, Catalase and Superoxide dismutase were verified independently by ELISA.

Biological significance: The temporal changes of > 2000 plasma proteins (at least quantified in two subjects), spanning the entire period of T1D natural progression were provided to the research community. Oxidative stress related proteins have consistently different dysregulated patterns in T1D group than in age-sex matched healthy controls, even prior to appearance of islet autoantibodies – the earliest sign of islet autoimmunity and pancreatic beta cell stress.

1. Introduction

T1D is an autoimmune chronic disorder resulted from the progressive destruction and dysfunction of insulin-producing β-cells in the pancreatic islets, which further results in severe insulin deficiency, hyperglycemia, and secondary complications [1–3]. Clinically, seroconversion to islet cell autoantibodies, including autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GAD65A), protein tyrosine phosphates IA2 (IA2A) and zinc cation efflux transporter 8 (ZnT8A), is used to predict the risk of developing this disease. In particular, the presence of multiple autoantibodies reveals high risk of T1D [4–6]. However, not all islet autoantibody-positive subjects progress to T1D and independent biomarkers are needed to elucidate the etiology of this disease [7], monitor β-cell destruction and accurately stage the progression of pre-clinical T1D for timely therapeutic intervention.

Cross-sectional characterization of serum proteome at diagnosis of T1D has suggested associations with proteins involved in inflammation and immune response [8–10]. However, samples used in these studies were collected at or shortly after the clinical diagnosis of T1D, reflecting late stages of β-cell destruction with hyperglycemia. In contrast, prospective cohorts allow profiling of serum/plasma proteome as children develop islet autoantibodies and progress to T1D [11].

We investigated whether the temporal expression trends of plasma proteins are able to differentiate T1D patients from healthy controls in the early stage of disease, multiple time point samples collected prior to T1D onset are necessary to accurately track the temporal changes in proteome. To this end, longitudinally collected plasma from subjects enrolled in the Diabetes Autoimmunity Study in the Young (DAISY)

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prospective cohort [12,13] were analyzed by quantitative proteomics, 9 time points per subject were selected, with the time points covering from birth to development of islet autoimmunity and overt T1D. Tandem mass tag (TMT)-based isobaric labeling quantitation approach [14–17] was applied to the serial 9 time point samples that can be quantified in parallel, and increased time points in a longitudinal study helped improve the statistical power needed to identify the expression patterns/trends significantly variant between the T1D progressors and healthy controls. To our knowledge, this study presented the most comprehensive profiling in temporal proteome changes in T1D research, and pattern recognition was being applied to differentiate the temporal changes of proteins correlated to disease progression from those in age-sex matched healthy controls. Importantly, the temporal expression trends of proteins observed by mass spectrometry were consistently verified by antibody-based ELISA platform. Our results provide a promising list of protein markers that temporally dysregulate before appearance of islet autoimmunity, and those proteins have a role in response to oxidative stress.

2. Experimental section

2.1. Study population and design

This is a nested case-control study. Participants were selected from the DAISY cohort, and they were identified with T1D susceptible HLA-DR/DQ alleles through genotyping at birth and followed prospectively. The details of screening [12] and follow-up [13] have been published previously. Informed consent was obtained from the parents of each study subject. The Colorado Multiple Institutional Review Board approved all study protocols.

Islet autoantibodies (to IAA, GADA, IA-2A, and ZnT8A) were measured in the laboratory of Dr. George Eisenbarth at the Barbara Davis Center in Denver at 9, 15 and 24 months and yearly thereafter [13]. Children positive for any autoantibody were followed in 3–6 month intervals for autoantibodies, hemoglobin $A1_C$, and random blood glucose until diagnosis of diabetes. Islet autoimmunity (IA) is a pre-T1D phenotype defined here as the presence of one or more of the autoantibodies on at least two consecutive visits 3–6 months apart or development of T1D within 6 months after a positive autoantibody test. Diabetes was diagnosed using the American Diabetes Association criteria [18]. Venous non-fasting blood samples were collected at each study visit and plasma was separated and stored at − 80 °C. All positive autoantibody values and 5% of negative ones were confirmed by blind duplicate re-testing.

Archived plasma samples from 11 T1D and 10 healthy subjects enrolled in DAISY were selected for this study. A series of 9 plasma samples were selected from each subject in the T1D group to represent different phases of disease progression, i.e. from autoantibody negativity to seroconversion and the ultimate T1D diagnosis. Healthy individual samples were matched on age, sex and sampling frequency to the T1D group except having permanent autoantibody negativity during the entire follow-up period and designated as NP group in this study. Frozen plasma samples were transferred to proteomics measurement laboratories for sample processing and measurement. In total, 99 and 90 plasma samples from the T1D and control groups, respectively, were used in this MS-based proteomics profiling study and they were processed according to the experimental strategy (Fig. 1) [17].

2.2. Proteomics sample preparation

Unless otherwise specified, all reagents and chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO). The kit for BCA protein assay was obtained from ThermoFisher Scientific (Rockford, IL). Sequencing-grade trypsin was purchased from Promega (Madison WI). All solvents used were HPLC-grade.

All samples were prepared in the same batch by the same researcher to ensure minimal variations in sample preparation. The detailed experiments for sample preparation have been published previously [17]. Briefly, 5 mg plasma proteins from each sample were depleted to remove the top 14 most abundant proteins using an MARS Human-14 column (Agilent Technologies), and the flow-through fractions (depleted plasma) were collected and concentrated. The depleted plasma proteins were denatured, reduced, alkylated, and digested as previously described [10], and the C_{18} SPE desalted peptide solution was completely dried before isobaric labeling. Aliquots of each individual sample from all healthy subjects were pooled to create the common reference sample used for healthy control group, to be used in subsequent TMT labeling as reference channel. The 9 serial samples from each subject and the common reference sample were included in one TMT10 labeling experiment set as shown in Fig. 1. This labeling strategy avoids the potential missing value issues resulted from data-dependent MS/MS acquisitions [19] because all samples from the same subject were included in the same labeling experiment. The same pooling and labeling strategy was applied to T1D patient samples (sample labels in Supplementary material Tables S1 and S2). After labeling, the 10 labeled samples in each TMT labeling experiment set were pooled, concentrated and fractionated using high-pH RPLC on an XBridge C_{18} analytical column (particle size of 5 µm, 250 × 4.6 mm, Waters). Mobile phases A and B consisted of 10 mM ammonium formate in water (pH 10) and 90% CH_3CN (pH 10), respectively. In total, 24 fractions were generated [16,20], dried, stored at − 80 °C, and reconstituted in 0.1% FA until LC-MS/MS analysis.

2.3. Quantitative LC-MS/MS analysis

LC-MS/MS analysis was conducted using an UltiMate 3000 RSLCnano system coupled to a Q Exactive HF mass spectrometer through an EASY-Spray ion source (ThermoFisher Scientific). Peptides were separated on a PepMap C_{18} analytical column (2 µm particle, 50 cm × 75 µm i.d.). A binary solvent system consisting of 0.1% FA in ddH_2O (solvent A) and 0.1% FA in CH_3CN (solvent B) was used to separate peptides at a flow rate of 250 nL min^{-1}. LC separation was performed using the following gradient setting: held at 4% B for 3 min, from 4% to 8% B in 0.1 min, 8% to 40% B in 90 min, 40% to 90% B in 0.1% min, held at 90% B for 10 min, 90% to 4% B in 0.1 min, and held at 4% B for 17 min for re-equilibrating column.

MS data was acquired in profile mode using a data-dependent top 15 method and resolution for full scans (m/z 400–1950) was set to 120,000 at m/z 200 with maximum fill time of 50 ms. Precursors were isolated with a window of 1.4 m/z [21] and fragmented with HCD fragmentation with normalized collision energy of 32. Resolution for MS/MS spectrum was set to 60,000 at m/z 200 with maximum fill time of 100 ms. AGC target for full scan and MS/MS scan was 3e6 and 1e5, respectively. Precursor ions with unassigned, single, seven, and higher charge states were excluded, and dynamic exclusion time was set to 20 s.

2.4. Database search

All raw files obtained from LC-MS/MS analyses were analyzed using MaxQuant software version 1.5.3.30, and searched against Swiss-Prot human protein database (91,960 protein entries, 02/17/2016 release) using the built-in Andromeda search algorithm. Semispecific Trypsin/P was selected as the enzyme. Cysteine carbamidomethylation and TMT-10plex labeled N-terminus and lysine were set as fixed modifications. Methionine oxidation was set as variable modifications. FDR was set at 1% for proteins and peptides level identification using decoy database, and precursor intensity fraction was set to > 0.75 [22]. Other parameters were used as default settings for Orbitrap-type data. The search results in proteinGroups.txt generated by MaxQuant were processed in Perseus software version 1.5.1.6 [23]. Identified protein is represented as protein group in the database search output when the search