ORIGINAL ARTICLE

# Proteomic identification of plasma signatures in type 2 diabetic nephropathy

Peir-Haur Hung[1,a], Ying-Chieh Lu[2,a], Yi-Wen Chen[2], Hsiu-Chuan Chou[3], Ping-Chiang Lyu[2], Ying-Ray Lee*[,1], Hong-Lin Chan*[2].

[1]Department of [1]Department of Medical Research, Chiayi Christian Hospital, Chiayi, Taiwan; [2] Institute of Bioinformatics and Structural Biology and Department of Medical Science, National Tsing Hua University, Hsinchu, Taiwan; [3] Department of Applied Science, National Hsinchu University of Education, Hsinchu, Taiwan; [a]These authors contribute equally to this work.

ABSTRACT

Recent advances in quantitative proteomics have offered opportunities to discover plasma proteins as biomarkers for tracking the progression and for understanding the molecular mechanisms of diabetes. We used quantitative proteomic analysis to identify novel biomarkers of nephropathy in plasma from type 2 diabetic patients. Plasma samples were analyzed by fluorescence two-dimensional differential gel electrophoresis (2D-DIGE), and differentially expressed proteins identification was performed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Proteomics analysis of the plasma proteome in type 2 diabetes mellitus with nephropathy identified 34 protein spots representing 31 unique proteins. These proteins mainly belonged to metabolic (such as 5'-AMP-activated protein kinase subunit beta-1) and growth regulatory (such as LIM homeobox protein 6) proteins. Additionally, our quantitative proteomic approach has identified numerous previous reported plasma markers of type 2 diabetes mellitus such as apolipoprotein A-I and ficolin-3. On the contrary, we have presented several putative type 2 diabetes mellitus biomarkers including calpain-7 and choline/ethanolamine kinase which have not been reported and may be associated with the progression and development of the disease. The potential of utilizing these markers for screening and treating type 2 diabetes mellitus warrants further investigation. Collectively, our results show that the proteins identified in this study may constitute potential biomarkers for the diagnosis of type 2 diabetics with nephropathy.

Keywords: Proteomics; Plasma biomarker; Type2 diabetic nephropathy.

## 1. Introduction

Diabetes mellitus is one of the most common metabolic diseases in the world, in which more than 90% diabetes mellitus patients are grouped to type 2 diabetes mellitus [1]. Type 2 diabetes mellitus is characterized by hyperglycemia due to defects of multiple organs which can not accurately measure the serum glucose level and uptake serum glucose. Additionally, type 2 diabetes mellitus is a chronic disease, which typically covers several decades from high serum glucose to significant diseases. The serum glucose level just reflects the outcome of multiple physiological disorders. Accordingly, numerous studies have been made to identify biomarkers to monitor the progression of type 2 diabetes mellitus [2-4]. However, most of these studies were concentrated on the genetic defects in gene expression level. Relative rare reports were given attention to protein level changes in response to

the progression of diabetes. For this, proteomic strategy might provide solutions for identification of large set of the proteins in tissues or in bio-fluid associated with type 2 diabetes mellitus.

Two-dimensional gel electrophoresis (2-DE) and MALDI-TOF MS has been widely used for profiling plasma proteins and some of the nonionic and zwitterionic detergents such as thiourea and CHAPS have been introduced to increase the solubility of the plasma proteins. In addition, a significant improvement of gel-based analysis of protein quantifications and detections is the introduction of 2D-DIGE. 2D-DIGE is able to co-detect numerous samples in the same 2-DE to minimize gel-to-gel variation and compare the protein features across different gels by means of an internal fluorescent standard. This innovative technology relies on the pre-

*Corresponding author : Ying-Ray Lee, Email Address: yingray.lee@gmail.com;  Hong-Lin Chan, Email Address: hlchan@life.nthu.edu.tw.

labeling of protein samples before electrophoresis with fluorescent dyes Cy2, Cy3 and Cy5 each exhibiting a distinct fluorescent wavelength to allow multiple experimental samples to include an internal standard. Thus, the samples can be simultaneously separated in one gel. The internal standard, which is a pool of an equal amount of the experimental protein samples, can facilitate the data accuracy in normalization and increase statistical confidence in relative quantitation across gels [5-10].

In order to examine differentially expressed levels of plasma proteins associated with type 2 diabetes mellitus, a proteomics-based approach was performed involving immunodepletion of high abundance proteins, 2D-DIGE analysis and subsequent MALDI-TOF MS analysis to obtain a panel of plasma proteins found to be differentially expressed between type 2 diabetes mellitus patients and healthy donors.

## 2. Material and methods

### 2.1 Chemicals and Reagents

Generic chemicals and albumin and IgG depletion kit were purchased from Sigma-Aldrich (St. Louis, USA), while reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). All the chemicals and biochemicals used in this study were of analytical grade.

### 2.2 Plasma sample collection and purification

From Jan 2009 to Dec 2009, eleven donors in a single center (Chiayi Christian Hospital, Chiayi, Taiwan) were enrolled in the study. Those included in the study were divided into type 2 diabetes mellitus patients (n=6) and healthy donors group (n=5). The criteria to assess the presence of type 2 diabetes mellitus were based on the guidelines proposed by the World Health Organization. All type 2 diabetic patients had typical diabetic symptoms along with a single fasting plasma glucose level of 7 mM or 2 hr postglucose or casual postprandial plasma glucose level of 11.1 mM. Healthy individuals with their fasting blood glucose below 5.5 mM were selected as controls. This study was approved by the Institutional Research Board and carried out according to the Helsinki Declaration Principles. Written informed consent was collected from all participating subjects.

To improve the performance of proteomic analysis of the plasma samples, the albumin and immunoglobulin G in the collected plasma samples were depleted using an albumin and IgG removal kit (Sigma, St. Louis, USA) in according with the manufacturer's instructions. The depleted plasma samples were precipitated by adding 1 volume of 100% (w/v) TCA to 4 volumes of sample and incubated for 10 min. at 4°C. The precipitated protein was then recovered by centrifugation at 13000 rpm for 10 min. (at -20°C), and the resulting pellet was washed twice with ice-cold acetone. Air-dried pellets were resuspended in 2-DE lysis containing 4% w/v CHAPS, 7M urea, 2M thiourea, 10mM Tris-HCl, pH8.3, 1mM EDTA.

### 2.3 Sample preparation for 2D-DIGE and gel image analysis

The plasma protein pellets were dissolved in 2-DE lysis buffer and protein concentrations were determined using coomassie protein assay reagent (BioRad). Before performing 2D-DIGE, protein samples were labeled with N-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5 following the protocol described previously [11,12]. Briefly, 150 µg of protein sample was minimally labeled with 375 pmol of either Cy3 or Cy5 for comparison on the same 2-DE. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per µg of protein as an internal standard for all gels. Thus, the triplicate samples and the internal standard could be run and quantify on multiple 2-DE. The labeling reactions were performed in the dark on ice for 30 min and then quenched with a 20-fold molar ratio excess of free L-lysine to dye for 10 min. The differentially Cy3- and Cy5-labeled samples were then mixed with the Cy2-labeled internal standard and reduced with dithiothreitol for 10 min. IPG buffer, pH3-10 nonlinear (2% (v/v), GE Healthcare) was added and the final volume was adjusted to 450 µl with 2D-lysis buffer for rehydration. The rehydration process was performed with immobilized nonlinear pH gradient (IPG) strips (pH3-10, 24 cm) which were later rehydrated by CyDye- labeled samples in the dark at room temperature overnight (at least 12 hours). Isoelectric focusing was then performed using a Multiphor II apparatus (GE Healthcare) for a total of 62.5 kV-h at 20°C. Strips were equilibrated in 6M urea, 30% (v/v) glycerol, 1% SDS (w/v), 100 mM Tris-HCl (pH8.8), 65 mM dithiothreitol for 15 min and then in the same buffer containing 240 mM iodoacetamide for another 15 min. The equilibrated IPG strips were transferred onto 26 x 20-cm 12.5% polyacrylamide gels casted between low fluorescent glass plates. The strips were overlaid with 0.5% (w/v) low melting point agarose in a running buffer containing bromophenol blue. The gels were run in an Ettan Twelve gel tank (GE Healthcare) at 4 Watt per gel at 10°C until the dye front had completely run off the bottom of the gels. Afterward, the fluorescence 2-DE were scanned directly between the low fluorescent glass plates using an Ettan DIGE Imager (GE Healthcare). This imager is a charge-coupled device-based instrument that enables scanning at different wavelengths for Cy2-, Cy3-, and Cy5-labeled samples. Gel analysis was performed using DeCyder 2-D Differential Analysis Software v7.0 (GE Healthcare) to co-detect, normalize and quantify the protein features in the images. Features detected from non-protein sources (e.g. dust particles and dirty backgrounds) were filtered out. Spots displaying a ≧ 1.3 average-fold increase or decrease in abundance with a p-value < 0.05 were selected for protein identification.

### 2.4 Protein staining

Colloidal coomassie blue G-250 staining was used to visualize CyDye-labeled protein features in 2-DE. Bonded gels were fixed in 30% v/v ethanol, 2% v/v phosphoric acid overnight, washed three times (30 min each) with ddH₂O and then incubated in 34% v/v methanol, 17% w/v ammonium sulphate, 3% v/v phosphoric acid for 1 hr., prior to adding 0.5g/liter

coomassie blue G-250. The gels were then left to stain for 5-7 days. No destaining step was required. The stained gels were then imaged on an ImageScanner III densitometer (GE Healthcare), which processed the gel images as .tif files.

### 2.5 In-gel digestion

Excised post-stained gel pieces were washed three times in 50% acetonitrile, dried in a SpeedVac for 20 min., reduced with 10 mM dithiothreitol in 5 mM ammonium bicarbonate pH 8.0 for 45 min at 50°C and then alkylated with 50 mM iodoacetamide in 5 mM ammonium bicarbonate for 1 hr. at room temperature in the dark. The gel pieces were then washed three times in 50% acetonitrile and vacuum-dried before reswelling with 50 ng of modified trypsin (Promega) in 5 mM ammonium bicarbonate. The pieces were then overlaid with 10 μl of 5 mM ammonium bicarbonate and trypsinized for 16 hr at 37°C. Supernatants were collected, peptides were further extracted twice with 5% trifluoroacetic acid in 50% acetonitrile and the supernatants were pooled. Peptide extracts were vacuum-dried, resuspended in 5 μl ddH$_2$O, and stored at -20°C prior to MS analysis.

### 2.6 Protein identification by MALDI-TOF MS

Extracted proteins were cleaved with a proteolytic enzyme to generate peptides, then a peptide mass fingerprinting (PMF) database search following MALDI-TOF MS analysis was employed for protein identification. Briefly, 0.5 μl of tryptic digested protein sample was first mixed with 0.5 μl of a matrix solution containing α-cyano-4-hydroxycinammic acid at a concentration of 1 mg in 1 ml of 50% acetonitrile (v/v) / 0.1% trifluoroacetic acid (v/v), spotted onto an anchorchip target plate (Bruker Daltonics) and dried. The peptide mass fingerprints were acquired using an Autoflex III mass spectrometer (Bruker Daltonics) in reflector mode. The algorithm used for spectrum annotation was SNAP (**S**ophisticated **N**umerical **A**nnotation **P**rocedure). This process used the following detailed metrics: Peak detection algorithm: SNAP; Signal to noise threshold: 25; Relative intensity threshold: 0%; Minimum intensity threshold: 0; Maximal number of peaks: 50; Quality factor threshold: 1000; SNAP average composition: Averaging; Baseline subtraction: Median; Flatness: 0.8; MedianLevel: 0.5. The spectrometer was also calibrated with a peptide calibration standard (Bruker Daltonics) and internal calibration was performed using trypsin autolysis peaks at $m/z$ 842.51 and $m/z$ 2211.10. Peaks in the mass range of $m/z$ 800-3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (release on 05-Oct-10) with 521016 entries using Mascot software v2.3.02 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*; tryptic digest with a maximum of 1 missed cleavage; carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation and partial modification of glutamine to pyroglutamate and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p<0.05$), spectrum

annotation and observed versus expected molecular weight and p$I$ on 2-DE.

## 3. Results and Discussion

### 3.1 Plasma sample preparation

Because albumin and immunoglobulin G account for around 70-80% proteins in human plasma, these high abundant proteins are the obstacle for 2-DE analysis. Accordingly, removing the high-abundance albumin and immunoglobulin G from plasma samples can increase the viability of the low- and middle-abundance proteins and enable for accurate analysis. In this study, the high abundance proteins were removed before performing 2D-DIGE analysis, and the effect of depletion was evaluated by 1D-SDS-PAGE (data not shown).

### 3.2 2D-DIGE and mass spectrometry analysis of the immuno-depleted plasma proteome

In order to study the alteration of plasma protein in the type 2 diabetic patients, comparative proteomics analysis was performed between type 2 diabetic patients and healthy donors. The 2-DE images of the samples of two groups were minimally labeled with Cy3 and Cy5 dyes and distributed to each gel. A pool of both samples was also prepared for labeling with Cy2 as an internal standard to run on all gels to facilitate image matching across gels. The plasma samples arrangement for a triplicate 2D-DIGE experiment is shown in Figure 1A. Thus, the triplicate samples resolved in different gels can be quantitatively analyzed by means of the internal standard on multiple 2-DE. After resolving protein samples with 2D-DIGE technique, the DeCyder image analysis software indicated that 65 protein features were showing greater than 1.3-fold change in expression level with student t-test ($p$-value) less than 0.05. MALDI-TOF MS identification revealed that 34 proteins were differentially expressed (Figure 1B, Figure 2 and Supplementary Table 1). With the basis of a Swiss-Prot search and KEGG pathway analysis, numerous potential biological functions and subcellular locations of the identified proteins were determined. Most of these identified proteins belong to cytoplasm proteins (29%), nuclear proteins (26%) and mitochondrial proteins (12%), and these proteins are found to be involved in metabolism (20%) and growth regulation (17%) (Figure 3).

Representative examples for the evaluation by DeCyder of alteration in spot intensities using the 2D-DIGE system are displayed in Figure 4. To display visually alterations in corresponding spot intensity proportions, selected identified spots are shown as 3-D images as well as the associated graph views of standardized abundances of the selected spots (Figure 4).

## 4. Concluding remarks

Proteomic analysis of the human diseases usually adopt a comparative method that is defined by the differential expression of the proteins under different disease states. Our 2D-DIGE / MALDI-TOF analysis revealed 34 altered expression

A



B

Green: Normal Red: DM2
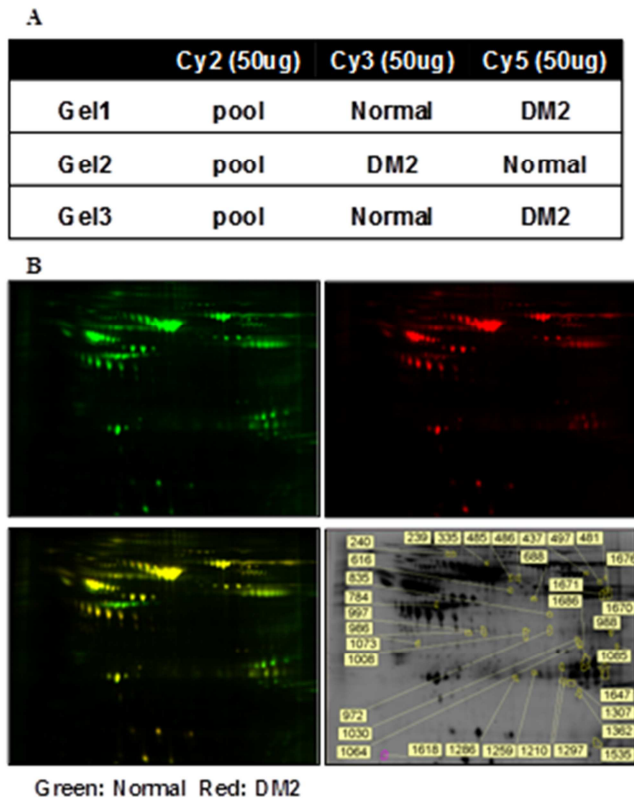
**Figure 1**. 2D-DIGE analysis of type 2 diabetes mellitus-induced differentially expressed proteins. *(A)* Plasma samples arrangement for a triplicate 2D-DIGE experiment. *(B)* Plasma samples (50 mg each) were labeled with Cy-dyes and separated using 24 cm, pH 3-10 non-linear IPG strips. 2D-DIGE images of the plasma samples from type 2 diabetic patients and healthy individuals at appropriate excitation and emission wavelengths were shown (Upper images) as well as overlaid pseudo-colored image processed with ImageQuant Tool (GE Healthcare) (left bottom). The differentially expressed identified protein features are annotated with spot numbers (right bottom).

of plasma proteins corresponding to 31 unique plasma proteins (Supplementary Table 1). A majority of altered proteins belong to three major functional groups, metabolism, growth regulation and transportation, while other affected categories included cytoskeleton, immune regulation and membrane trafficking (Figure 3). Of these, 20% participate in various metabolic pathways including 5'-AMP-activated protein kinase subunit beta-1 (regulation of energy production and consumption), 6-phosphofructokinase (glycolysis), carnitine O-palmitoyltransferase I (long-chain fatty acid transportation), choline/ethanolamine kinase (biosynthesis of phosphatidylcholine / phosphatidylethanolamine), steroid 21-hydroxylase (biosynthesis of the steroid hormones aldosterone and cortisol), glutamine-dependent NAD(+) synthetase (biosynthesis of NAD). 5'-AMP-activated protein kinase subunit beta-1 is one of the main subunits of AMPK proteins which plays as a key player in regulating energy metabolism and renders it at the center role in studies of the metabolic diseases including diabetes. AMPK is also reported as a critical molecule to maintain glucose homeostasis and its activation and expression is a positive regulator to reduce the con-

centration of blood glucose and facilitate the oxidation of fatty acids and glycolysis [13]. In current study, we have observed the down-regulated of AMPK-beta subunit, carnitine O-palmitoyltransferase I and 6-phosphofructokinase in type 2 diabetes mellitus plasma suggesting the up-stream regulators might inhibit AMPK expression subsequently by hampered the transportation of fatty acid into mitochondria for beta-oxidation via the inhibition of carnitine O-palmitoyltransferase I and down-regulated glycolysis via the inhibition of 6-phosphofructokinase in this disease.

Apolipoprotein A-I is a component of the high density lipoprotein responsible for the cholesterol transport into the liver [14]. Apolipoprotein A-I is also associated with diabetes and demonstrates a downregulation of apolipoprotein A-I in diabetic patients [15-18]. Our current 2D-DIGE analysis is consistent with the previous observations. Ficolins are a group of proteins containing collagen-like domains and fibrinogen-like domains. In addition, ficolins have a calcium-independent lectin activity and are able to activate the complement pathway to improve host defense activity [19]. In our current work and recent plasma proteomic studies demonstrated that ficolin-3 was significantly correlated with type 2 diabetes mellitus and supposed to play an important role in the chronic low-grade inflammation and stimulation of the innate immune system associated with type 2 diabetes mellitus [16,20]. Other identified proteins including LIM homeobox protein, 6-phosphofructokinase, steroid 21-hydroxylase and Rho GTPase-activating protein 25 have been well-studied in type 2 diabetes mellitus [21-23] suggesting the current 2D-DIGE playing a powerful complementary role in the assumed biomarker discovery and disease studies. On the other hand,
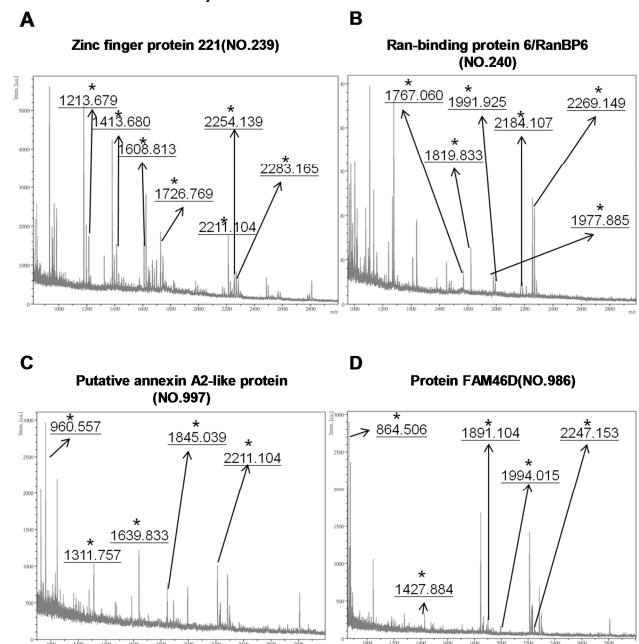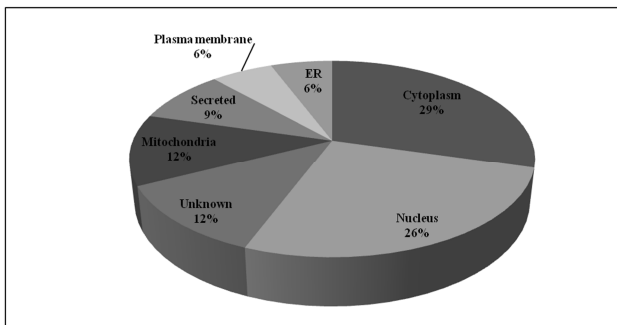


**Figure 2**. Peptide mass fingerprint of identified proteins *(A)* Zinc finger protein 221; *(B)* Ran-binding protein 6 / RanBP6; *(C)* Putative annexin A2-like protein; *(D)* Protein FAM46D. Peptides contributes to protein identifications are marked with asterisk (*).

## A    Functional Ontology
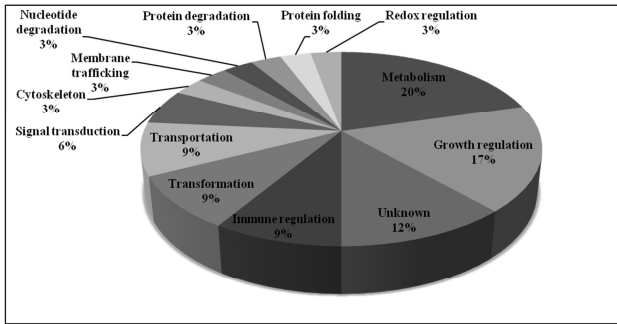


## B    Subcellular Location



**Figure 3.** Percentage of plasma proteins identified from albumin and imunnoglobulin G depleted plasma by 2D-DIGE / MALDI-TOF MS for type 2 diabetes mellitus according to their biological functions *(A)* and subcellular location *(B)*.

our current research also identifies alpha-(1,3)-fucosyltransferase, SCaMC-3, calpain-7, choline/ ethanolamine kinase, paraneoplastic antigen-like protein 6B, ranbinding protein 6/RanBP6, Sulfide:quinone oxidoreductase glutamine-dependent NAD(+) synthetase as putative plasma marker proteins of the type 2 diabetes mellitus. Further investigations would be necessary to validate their expression in plasma and elucidate their mechanistic role in type 2 diabetes mellitus.

The isolation of low-abundance proteins from plasma is frequently complicated because of the existed of high abundance proteins such as serum albumin and immunoglobulin. Serum albumin and immunoglobulin is the most (50-70%) and second (10-20%) abundant protein in plasma, respectively. These proteins will mask the low-abundant proteins and limit the amount of the total plasma proteins that can be applied and resolved by proteomic analysis. To removal of these two high-abundant proteins, we used albumin and IgG depletion kit from Sigma-Aldrich, which contains prepacked spin columns allows removal of the most of both serum albumin and immunoglobulin. At the same time, we used trichloroacetic acid / acetone to precipitate, desalt and enrich the plasma proteins to make them well resolved by 2D-DIGE. Our results demonstrated that this strategy did make the plasma proteins well prepared and separated.

In conclusion, the quantitative plasma proteomics analysis provided a valuable impact for type 2 diabetes mellitus research. Our quantitative proteomic approach has identified numerous previous reported plasma markers of type 2 diabetes mellitus such as apolipoprotein A-I and ficolin-3. Additionally, we have presented several putative type 2 diabetes mellitus biomarkers which may be associated with the progression and development of the disease and has a potential to serve as a useful tool for monitoring the course of the disease. The potential of utilizing these markers for screening and treating type 2 diabetes mellitus warrants further investigation.
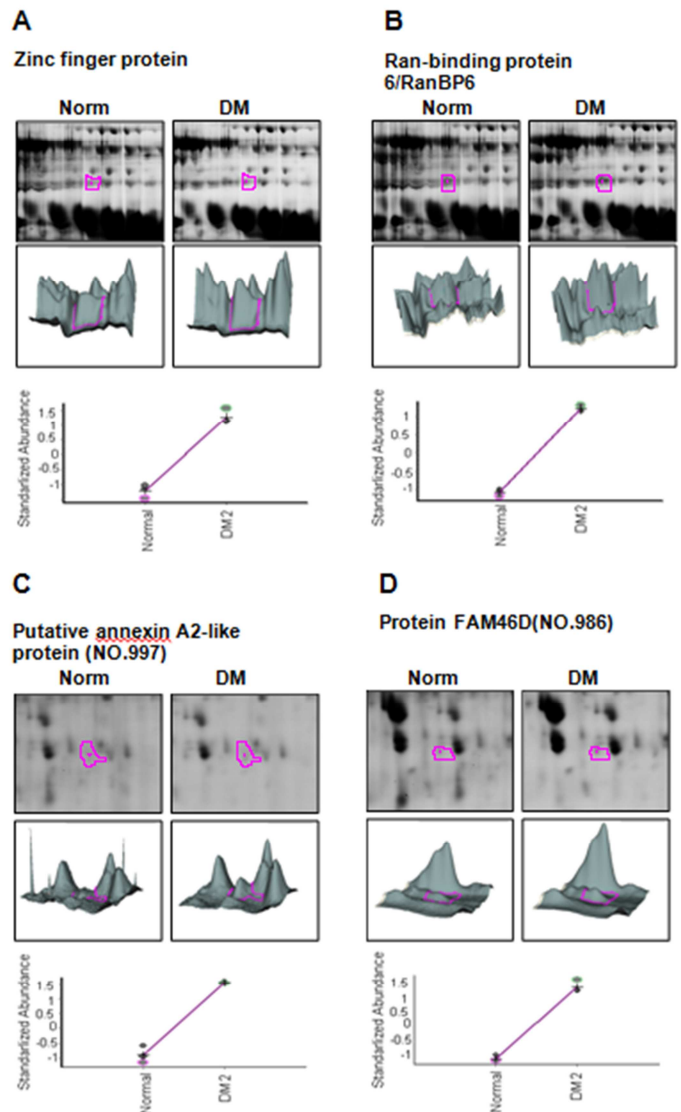


**Figure 4.** Representative images of the identified spots ((A) Zinc finger protein 221; (B) Ran-binding protein 6 / RanBP6; (C) Putative annexin A2-like protein; (D) Protein FAM46D) displaying type 2 diabetes mellitus–dependent protein expression changes. The levels of these proteins were visualized by 2-DE images (top panels), three-dimensional spot images (middle panels) and protein expression map (bottom panels).

## 5. Supplementary material

Supplementary material regarding this manuscript is online available in the web page of JIOMICS.

http://www.jiomics.com/index.php/jio/rt/suppFiles/44/0

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