[Frontiers in Bioscience 16, 2671-2681, June 1, 2011]

Serum biomarker of diabetic peripheral neuropathy indentified by differential proteomics

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1. ABSTRACT

At least one in four diabetic patients is affected by peripheral neuropathy. In this study, the MALDI-TOF-MS mass spectra of peptides and proteins were generated following WCX CLINPROT bead fractionation of 39 diabetic peripheral neuropathy (DPN), 39 diabetes mellitus (DM), and 35 control (CON) serum samples. The spectra were analyzed statistically using flexAnalysisTM and Clin-ProtTM bioinformatics software. Identification of the selected markers was performed and affinity bead-purified plasma protein was subjected to LTQ Orbitrap XL MS/MS analysis followed by Mascot identification of the peptide sequences. 89 differentially expressed peaks of serum proteins were identified. 17, 10 and 4 most significant peaks between CON vs. DM, CON vs. DPN, DM vs. DPN, respectively, were selected out using the ClinProTool software package and used to train a Supervised Neural Network. A veracity rate of 100% was obtained for all sets. Following this analysis, a 6631-Da marker was identified as a fragment of the Apolipoprotein C-I precursor. The peptides identified may have clinical utility as surrogate markers for detection and classification of DM and DPN.

2. INTRODUCTION

Diabetic neuropathy has been defined as a demonstrable disorder, evident either clinically or subclinical, that occurs in the setting of diabetes in the absence of other causes for peripheral neuropathy (1). Distal symmetric sensory or distal sensorimotor polyneuropathy (DSP) represents the most relevant clinical manifestation, affecting 30% of the hospital-based population and 25% of community-based samples of diabetic patients (2). Diabetic peripheral neuropathy (DPN) is the most common chronic complication of diabetes (3), and there are many methods utilized in DPN screening, such as the Toronto clinical neuropathy score (TCSS), Michigan Neuropathy Screening Instrument (MNSI), diabetic neuropathy symptom (DNS) score, and the 128Hz tuning fork examination, single-wire inspection and others. Though there is validation through clinical data which supports these methods, the actual efficiency of diagnoses varies due to each method's different emphasis. Diagnosis of PDN requires the presence of a neuropathy consistent with diabetes, as well as the exclusion of other possible etiologies of neuropathy. The differential diagnosis is vast, including alcoholic, idiopathic, nutritional, and many other

types of neuropathy. Nerve conduction studies and electromyography can assist in the description and objective confirmation of the source of neuropathy. Nerve conduction studies are, however, best suited to rule out the other causes of neuropathy or to identify additional neuropathies. MNSI and DNS scores ignore a portion of patients with no obvious symptoms of DPN. The tuning fork and 10g monofilament examinations are simple, but the early screening positive rate is low. A nerve biopsy, although usually not required, can reveal the involvement of unmyelinated fibers, which are not routinely evaluated by electrophysiological tests. More recently, neuropathy associated with changes in intraepidermal nerve fiber density and dendritic nerve fiber length has been demonstrated in patients with impaired glucose tolerance and dysmetabolic syndrome.

Human blood plasma is the most complex human derived proteome, as well as the most informative proteome from a medical point of view. The attractiveness of plasma for disease diagnosis lies in two characteristics: the ease with which it can be obtained and the fact that it comprehensively samples the human phenotype (the bodily state at a particular point in time). The proteins in plasma can be categorized into several functional groups. These include the proteins secreted by solid tissues, immunoglobulin (about 10 million different sequences) that circulate in plasma, “long distance” receptor ligands which include the classical peptides and protein hormones, “local” receptor ligands which include cytokines and other short distance mediators of cellular response, temporary passengers, tissue leakage products, aberrant secretions and foreign proteins which are proteins from infectious organisms or parasites that are released into the circulation. Given this variety of classes of protein components, plasma is thus the most comprehensive and the largest version of the human proteome. On the other hand, more than half of the total protein mass in plasma is comprised of one protein (albumin), while the top ten proteins together make up 90% of the total. This enormous dynamic range (nearly 12 orders of magnitude between the high abundance and very low abundance) of proteins currently falls outside the range of available technologies in proteomics. To address this complexity, plasma samples can be fractionated using multidimensional separation techniques, for example fractionation at the peptide level where the proteome is first digested with a protease such as trypsin, then the peptides are separated using reverse phase and cation exchange liquid chromatography. Alternatively, separations at the protein level, using pre-fractionation methods such as ion exchange, size exclusion, hydrophobic interaction, and various affinity methods have been attempted. One such pre-fractionation method is the removal of high abundance proteins, which can dramatically improve the number of proteins identified by reducing the dynamic range of protein levels in biological fluids to better match the analytical platform. A variety of depletion methods for specific removal of high abundance proteins from bodily fluids have been developed. One example is matrix-assisted laser desorption/ionization time-of-flight known as MALDI-TOF (4-9).

Proteomic expression profiles generated with mass spectrometry have been suggested as potential tools for the early diagnosis of diseases. Different protein profiles can be associated with varying responses to therapeutics. It has been postulated that on the basis of the presence/absence of multiple low-molecular-weight serum proteins using time-of-flight (TOF) mass spectrometry technologies, such as SELDI-TOF and MALDI-TOF, biomarkers can be identified (10-14). Although the data from these studies are encouraging, critical notes have been made on both study design and experimental procedures for proteomic profiling (15, 16). In addition, the importance of avoiding confounding biological variables, as well as technological factors that may bias the results, have previously been stressed by several authors. Among the huge amount of biomarkers discovered by other technologies, only a few of them have been identified due to the technique limitations concerning direct identification on a chip using ProteinChip Array. In fact, identification of these candidates will not only assist in exploring the mechanism of disease occurrence, but also facilitate the development of a more traditional multiprotein antibody array for the early detection of DPN.

3. MATERIALS AND METHODS

3.1. Patients and blood sample

The diagnostic criteria for diabetes mellitus (DM): fasting blood glucose (FPG) $\geq$ 7.0 mmol/L and (or) 2 hours after glucose load blood glucose (2hPG) $\geq$ 11.1 mmol/L. Diabetic peripheral neuropathy (DPN) diagnostic criteria: meeting the diagnostic criteria for diabetes mellitus and with abnormal NCV examination (excluding unrelated reasons for peripheral neuropathy). Healthy controls (CON) populations were collected from the health examination in the Changzheng Hospital from November 2005 through January 2007, including 238 inpatient cases and 158 cases of healthy volunteers. The study groups included a normal control (CON) group of 35 patients, diabetes not accompanied by any chronic complication (DM) group of 39 patients, and diabetes only associated with peripheral neuropathy (DPN) group of 39 patients. The final study groups excluded cases of dyslipidemia and hypertension, therefore 25 CON cases, 25 DM cases, and 25 DPN cases were used to construct the model; and 10 CON cases, 14 DM cases, and 14 DPN cases were used for the blind test and general information such as included in Table 1.

Neural electrophysiological examination for DM patients: A Nihon Kohden Neuropaek-2 was used to evoke potential/EMG, and the instrument was placed in a separate shielded room. Three parameters of motor conduction velocity (MNCV) and sensory conduction velocity (SNCV) were determined, including distal latency, distal amplitude of median nerve, and superficial peroneal nerve. For the assessment of neuropathological symptoms of peripheral neuropathy, the total symptoms score (TSS) was used for assessment of the drug treatment of DPN. Scoring included lower limb and foot numbness, paresthesia, burning sensation, gill pain and the severity of four symptoms (none, mild, moderate, severe) and frequency (occasional, frequent, almost continuous), respectively, were scored and

Table 1. Serum samples characteristics

	Modeling group			**Testing group**			
	characteristics	CON(25)	DM(25)	DPN(25)	CON(10)	DM(14)	DPN(14)
Gender	Male/n	15	13	13	6	8	10
	Female/n	10	12	12	4	6	4
Age	<45/n	12	5	6	5	2	1
	≥45/n	13	20	19	5	12	13
	means+/-SD	46.4+/-14.0	55.1+/-10.1	58.2+/-13.1	40.7+/-20	62.9+/-13.1	58.6+/-9.8
Stage	<5Y/n	—	14	14	—	4	6
	≥5Y/n	—	11	11	—	10	8
	means+/-SD	—	3.6+/-4.9	5.3+/-5.7	—	8.6+/-7.7	7.1+/-6.0
Family history	Yes/n	12	12	13	3	7	5
	No/n	13	13	12	7	7	9
HbA1c	<6.5%/n	—	5	3	—	8	1
	≥6.5%/n	—	20	22	—	6	13
	means+/-SD	—	7.7+/-1.4	8.1+/-1.7	—	6.7+/-1.1	8.6+/-1.7

counted as 0 ~ 3.66 to record the rate of TSS before and after treatment (data not shown).

3.2. Blood sample preparation

Blood samples were drawn upon study entry (Day 0) and in the 6th month via an indwelling arterial catheter. Serum samples were collected in glass tubes without additive (BD Vacutainer™ Franklin Lakes, NJ) and was allowed to clot at room temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 15 min, immediately split into 200μl aliquots and frozen at -80 °C until time of analysis. The time from collection to frozen storage was no more than 60 min for all samples. The processing, collection and storage protocols for all individuals were identical. For serum protein fractionation, the sera were left at 4~6□ for 2h, centrifuged at 10000 rpm, 4□ for 10 min. 10Ml MB-WCX binding solution and 5Ml serum sample were added to the beads and mixed completely. The samples were then placed on the magnetic bead separation device (MPC-auto96, Dynal, Oslo, Norway) where the beads were pulled to the side by magnetic force, allowing for the supernatant to be removed and discarded. The magnetic beads were washed three times with MB-WCX washing solution by shaking the beads up and down as needed. The supernatant was removed and the beads remained in place. 5Ml elution solvent was added to the bead pellet and mixed by pipeting up and down, then the beads were pulled to the side and a fraction of the eluate was transferred to another tube. 10Ml α-cyano-4-hydroxycinnamic acid (0.3 g/L in ethanol: acetone 2:1) was added to the 1Ml elution in a 348-well microtiter plate and mixed carefully. 1Ml mixture was spotted in quadruplicate onto a MALDI AnchorChip™ (Bruker Daltonics, Bremen, Germany).

3.3. Quality control and standards detection for MALDI-TOF-MS

Each standard was calibrated around a range of four molecular weight protein samples. Each point was acquired multiple times to obtain a standard map, and the molecular weight bias correction did not exceed 0.01%. A single standard serum was used as a quality control for the entire experimental process. Every 7 samples were matched with the standard serum. When a standard serum pattern map was obtained, it was compared with maps already present in the database, which had been constructed using the same standard serum maps treated with the identical WCX magnetic bead method. From this data, the coefficient of variation was calculated.

3.4. Mass spectrometry analysis to profiling serum proteome

For MALDI-TOF-MS analysis, 1 Ml of the above diluted purified serum was mixed with 0.5 μl of matrix solution (2 g/L α-cyano-4-hydroxycinnamic acid, and 1% formic acid in 50% acetonitrile) and allowed to dry onto a MALDI sample plate (600 μm AnchorChip™, Bruker Daltonics Company). Two peptides were also included in the matrix solution for internal calibration: 10 pmol/Ml angiotensin II and 10 pmol/Ml ACTH18-39 (Bruker Daltonics). Laser desorption was targeted randomly on the sample plate and samples were measured using an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics) operated in positive ion linear (reflection) mode. Ionization was achieved by irradiation with a 50 Hz nitrogen laser. Spectra are the mean of 100 ionizations with a fixed laser power in linear geometry mode and mass maps were obtained in reflectron mode. The spectra were calibrated externally with a mixture of protein/peptide standards in the range of 1000 to 12000 Da (Bruker Daltonics). For databank analysis, all spectra were processed using automatic baseline subtraction, peak detection, recalibration, and peak area calculation according to predefined parameter settings. The criteria for peak detection were: Signal/Noise (S/N) ratio > 5, 2 Da peak width filter, and maximum peak number of 200. The intensities of the peaks of interest were normalized against the peak intensity of the ACTH internal standard. A +/-2 Da mass accuracy for each spectrum was observed which may be a result of the geometry of varied sample positions on the AnchorChip. These mass shifts were corrected by the flexAnalysis™ software after alignment with the 2 internal standards.

3.5. Statistical methods, evaluation of diagnostic efficacy

All MALDI-TOF-MS spectra were analyzed with flexAnalysis™ to detect the peak intensities of interest and CLINPROT™ software to compile the peaks across the spectra obtained from all samples (Bruker Daltonics Company). This analysis allowed for discrimination between the patient and control samples. We used a Supervised Neural Network (SNN) in CLINPROT 2.1 with the detected peaks from the discovery set to generate cross-

Table 2. The M/Z of the reference selected peaks in standards

Substance	mass(M+H)+	Within-run CV (%)
AngiotensinII	1047.18	0.29
AngiotensinI	1297.48	0.05
Substance P	1348.64	0.18
Bombesin	1620.86	0.22
ACTH clip 1-17	2094.42	0.12
ACTH clip 18-39	2466.68	0.19
Somatostatin 28	3149.57	0.12
Ubiquitin	4283.45	0.09
Insulin	5734.56	0.16
Cytochrome c	6181.05	0.23
Ubiquitin	8565.89	0.25

validated classification models. These were used to measure the reliability of the calculated model and to predict how a model would behave in the future. A random 20% of data points (taken over all classes) was selected and omitted from the model generation procedure. The model was constructed using the remaining 80% of data points and the previously omitted 20% set of data points was classified against the model. The obtained classification results were stored (10 CON cases, 14 DM cases, 14 DPN cases) and used for the blind test.

3.6. Identification of protein markers

Selected peptides were further purified using Nano Aquity UPLC C18 beads (Waters Corporation, Milford,USA) and serially eluted with 5% and 95% acetonitrile. These peptides were identified directly via LTQ Orbitrap XL (Michrom Bioresources, Auburn, USA) analysis in order to obtain the peptide sequences. For the Nano Ion Source, spray voltage was 1.8kV, MS scan time was 60min, and the scanning range was 400-2000m/z. Obitrap was used for the first scan (MS), with resolution of 100000 and LTQ was used for CID and the second scan (MS/MS). The 10 strongest ion intensities in the MS spectra were selected as the parent ion for the MS/MS (single charge exclusion, not as a parent ion). Peptide mass fingerprinting was performed with the International Protein Index (IPI human v3.45 fasta with 71983 entries) and a search of the National Center for Biotechnology Information (NCBI) protein-protein BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).

4. RESULTS

4.1. System stability and experimental reproducibility were ensured through the use of standards and standard serum

In this study, standard products and standard serum were used for quality control. The external standard calibration standards contained 11 peptides (Table 2), and almost all the CV were below 30% within each run. Average molecular weight deviation was less than 100ppm, and for every eight samples of data collection an external standard calibration was performed. Four standard serum samples were used to ensure quality control. Resultant mass spectrometry test results are shown in Figure 1.

4.2. Differentiation of peptides selected out between DM, DPN and CON groups

All 113 patients from the DM, DPN and CON groups' sera peptide profiles were analyzed using a new high-resolution MALDI-TOF MS coupled with bead fractionation. Samples were randomly distributed during processing and analysis. A total of 89 distinct m/z values were resolved in the 800–12000Da range. Differences in peak positions and intensities were observed and later used to statistically analyze the spectra. ClinprotTools ver 2.1 (Bruker Daltonic) was used for peak detection. Those peptides which displayed significant statistical significance (P<0.05) according to a Mann-Whitney U-test between CON and DM, CON and DPN, DM and DPN groups respectively. These data are shown in Table 3. Typical WCX spectra for three groups are shown in Figure 2, and indicate very good reproducibility for each group. MALDI-TOF mass spectral overlays of selected peaks were derived from serum peptide profiling of each pair of groups. Spectra were obtained, aligned, and normalized as described in the Methods and were displayed using a mass spectra viewer. The top two significantly differently expressed proteins are shown in Figure 3. Each pair of peaks appears to have significant discriminatory potential.

4.3. Establishment of Predicting Model

A Supervised Neural Network (SNN) in CLINPROT was trained with the detected peaks from the discovery set to generate cross-validated classification models. The recognition capability between each pair of groups for the best predicting model, and MALDI-TOF peaks from the best classification model are shown in Table 4, with 20% of randomly selected data points omitted in the cross validation step. The accuracy of the models was verified with the validation set data, consisting of the 20% omitted samples. All the samples were correctly classified by the SNN model.

4.4. Identification of markers

With this bead-based proteomic technology, identification of potential markers at 6631Da could distinguish both the DM and DPN groups from the CON group, and with a relative high peak intensity which is beneficial for further purification and identification. With this in mind, this peptide could be a potential marker for further immunoassay trials. After fractionation by Nano Aquity UPLC (Waters Corporation, Milford, USA), the eluted plasma samples were further purified by C18 beads with 5μm and 3.5μm, then serially eluted with 5% and 95% acetonitrile. Samples were then subjected to LTQ Orbitrap XL MS/MS (Michrom Bioresources, Auburn, USA) analysis. Marker 6631Da was significantly enriched (Figure 4A). This acetonitrile eluate was further subjected to TOF MS/MS analysis. The MS fingerprint was subjected to International Protein Index (IPI human v3.45 fasta with 71983 entries) searching for peptide sequence and further to NCBI database for protein identification. The sequence was identified as fragment of Apolipoprotein C-I precursor (Figure 4B and 4C).

5. DISCUSSION

In the search for clinically relevant biomarkers, the low mass range of the serum proteome, particularly peptides with a molecular mass below 3,000 Da, has not received the same attention as higher molecular weight

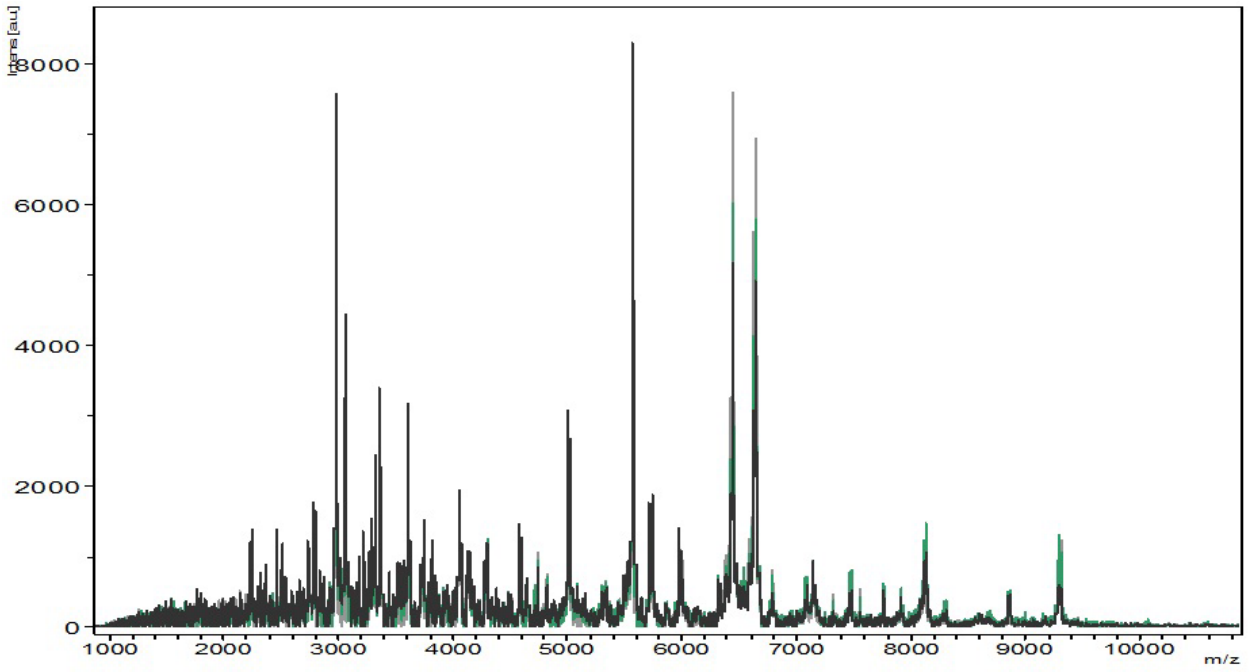

Figure 1. Self-Test for standard serum.

peptides and proteins. Meanwhile, a crucial point of discussion in the evolving field of clinical proteomics is validation of classification (17, 18). Given the sample size achievable within the experiment, the use of a separate (possibly set-aside) validation set was precluded. The other problem is ''predictive optimization''. The controversy with regard to the use of protein profiles as a pattern diagnostic without analysis of the diagnostic biomarkers remains to be solved for clinical applications. Further, identification and functional analysis of these discriminating proteins/peptides might render new insights on disease development and environmental responsiveness, which could eventually be translated into new diagnostic and prognostic insights for the clinician. Unfortunately, little success has been obtained so far in the assignment of reproducible discriminating biomarkers (19, 20). At the same time, the identification of the individual differentially expressed proteins that comprise the diagnostic expression profile will essentially facilitate significant progress in the development of a robust accurate diagnostic platform. In addition, if the proteins are identified and specific high affinity antibodies are generated for them, more direct but less expensive methods for analysis can be developed.

Results of this study demonstrate that, by using MALDI-TOF, the complexity of the human plasma proteome could be significantly reduced and allow for identification of potential protein biomarkers for disease studies by proteomic analysis. This approach is a moderately high throughput method suitable for biomarker discovery in clinical studies. By using spectral counting as a surrogate for peak area measurements, data can be quickly assessed to evaluate which proteins are differentially abundant and to determine which peak areas to measure manually across the different groups.

Recently performed experimental studies suggest a multifactorial pathogenesis of diabetic neuropathy. Most data have been generated in the diabetic rat model. Two approaches have contributed to the elucidation of the pathogenesis of diabetic neuropathy. The most common type of diabetic neuropathy is distal symmetric polyneuropathy, which may affect large and/or small fibers and may be either sensory or motor. The major neurotransmitter in small unmyelinated C fibers is substance P, and those of A fibers (such as glutamate) act on Na channels. Thus, capsaicin, which depletes substance P is usually effective for C fiber pain, whereas agents that correct Na channelopathy improve large fiber function (4). PDN is caused by the involvement of small nerve fibers, which may be affected without objective clinical findings, such as decreased peripheral reflexes or abnormalities on routine electrophysiological studies. Small fiber neuropathies may manifest as a number of different clinical symptoms, including allodynia, burning pain, defective warm thermal sensation, and defective autonomic function, e.g. decreased sweating, dry skin, and impaired vasomotor control.

We found 77 distinguishable peaks in the 800 to 12,000 m/z range, with 17 and 10 peaks having statistically significant differential expression between DM vs CON, DPN vs CON, respectively($P<0.005$). Using the SNN analysis package in the CLINPROT software, 10 and 4 particularly significant peaks were selected from the discovery set data and were used to generate a diagnostic model which was applied to data from the validation set. The veracity rate was 100% for all groups. Cross Validation was 74.47% for CON, 79.25% for DM and 100, for CON, and 100, for the DPN set. Further, we found the peptide fragments with respective m/z value 6631 with high

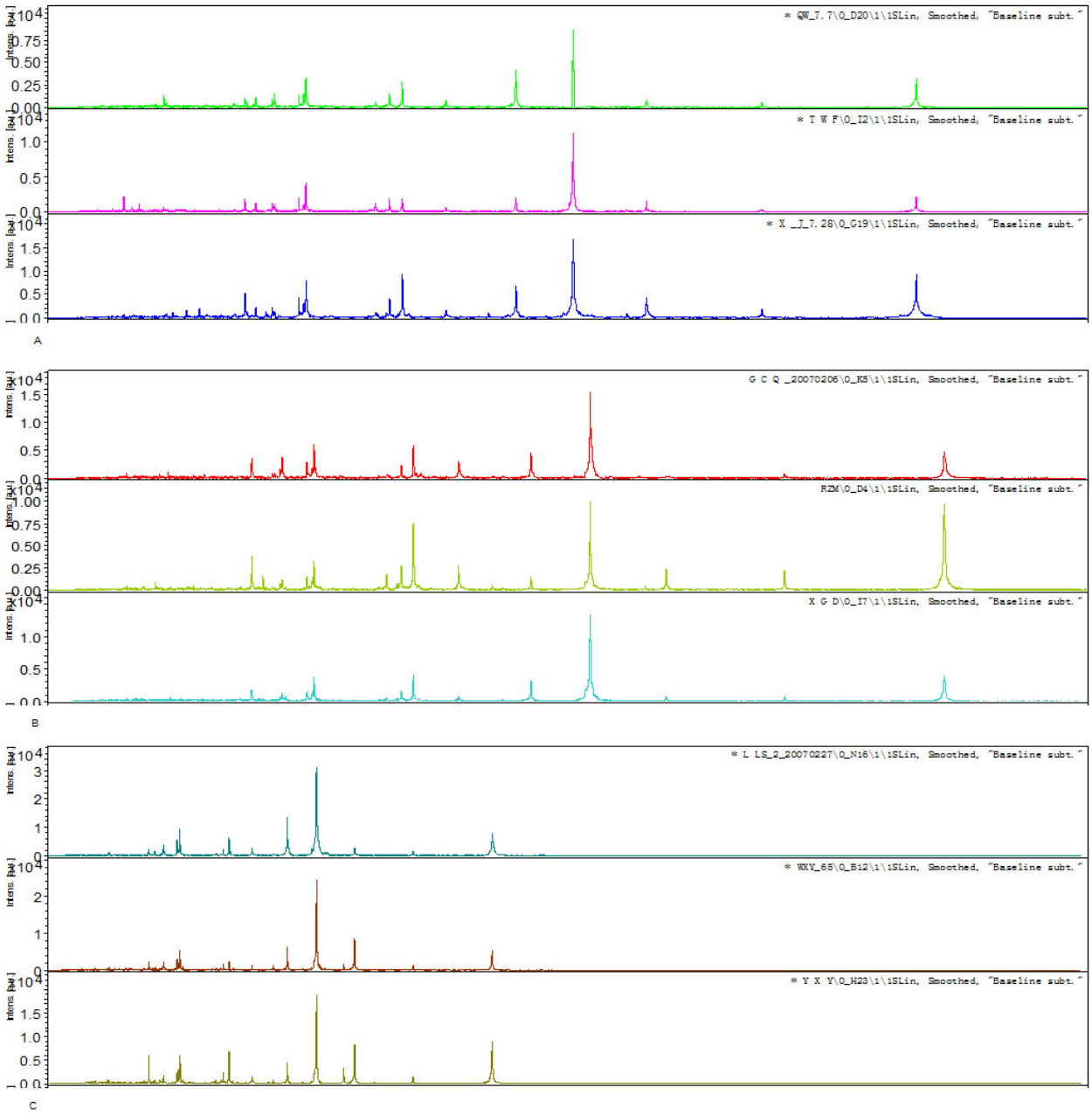

Figure 2. Typical WCX spectra for three groups. A: CON; B: DM; C: DPN. Each plot (A to C) shows three lines which represents three different serum sample profiles.

accuracy (AUC > 0.95) by ROC analysis (data not shown). These protein/peptide fragments with high specificity and sensitivity may be good serum biomarkers for DM and DPN. Later studies in a larger population group are necessary to confirm this finding. Further evaluation identified the 6631Da marker as fragment of Apolipoprotein CI by MS/MS.

Apolipoprotein CI (ApoCI) is a 6.6 kDa protein, which influences many proteins involved in the remodeling of lipoproteins in plasma. It is an inhibitor of lipoprotein binding to the LDL receptor, LDL receptor-related protein, and the VLDL receptor. It also is the major plasma inhibitor of cholesteryl ester transfer protein, and appears to interfere directly with fatty acid uptake (21). ApoCI is associated with decreased particulate uptake of apolipoprotein B-containing lipoproteins, leading to increased levels of several potentially atherogenic species, including cholesterol-enriched VLDL, IDL, and LDL (22, 23), which in turn lead to high blood pressure. Another study has been conducted to evaluate the effects of ApoCI overexpression on hepatic and peripheral insulin sensitivity in a mouse model, where obese mice with mild overexpression of ApoCI were generated and resulted in

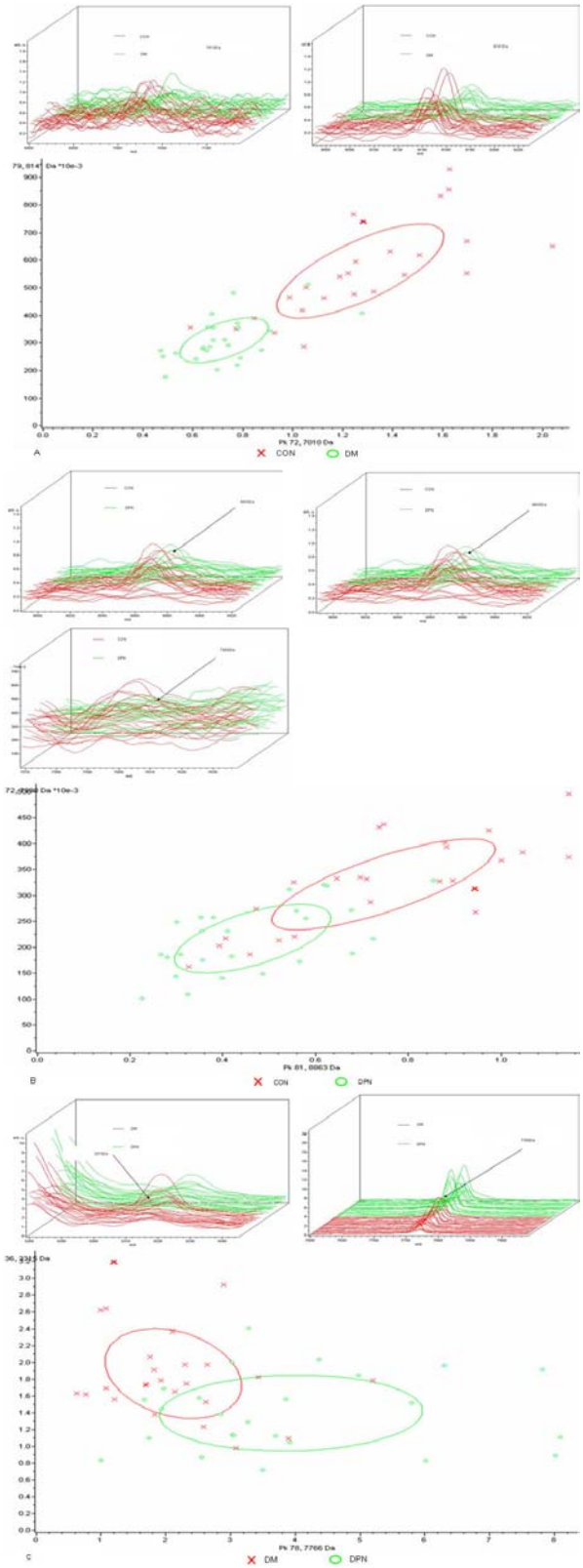

Figure 3. The first 2 significant peaks are marked with a star and short line along the average spectrums and the actual plot of the first 2 significant peaks in each two groups are displayed. A: CON vs DM; B: CON vs DPN; C: DM vs DPN.

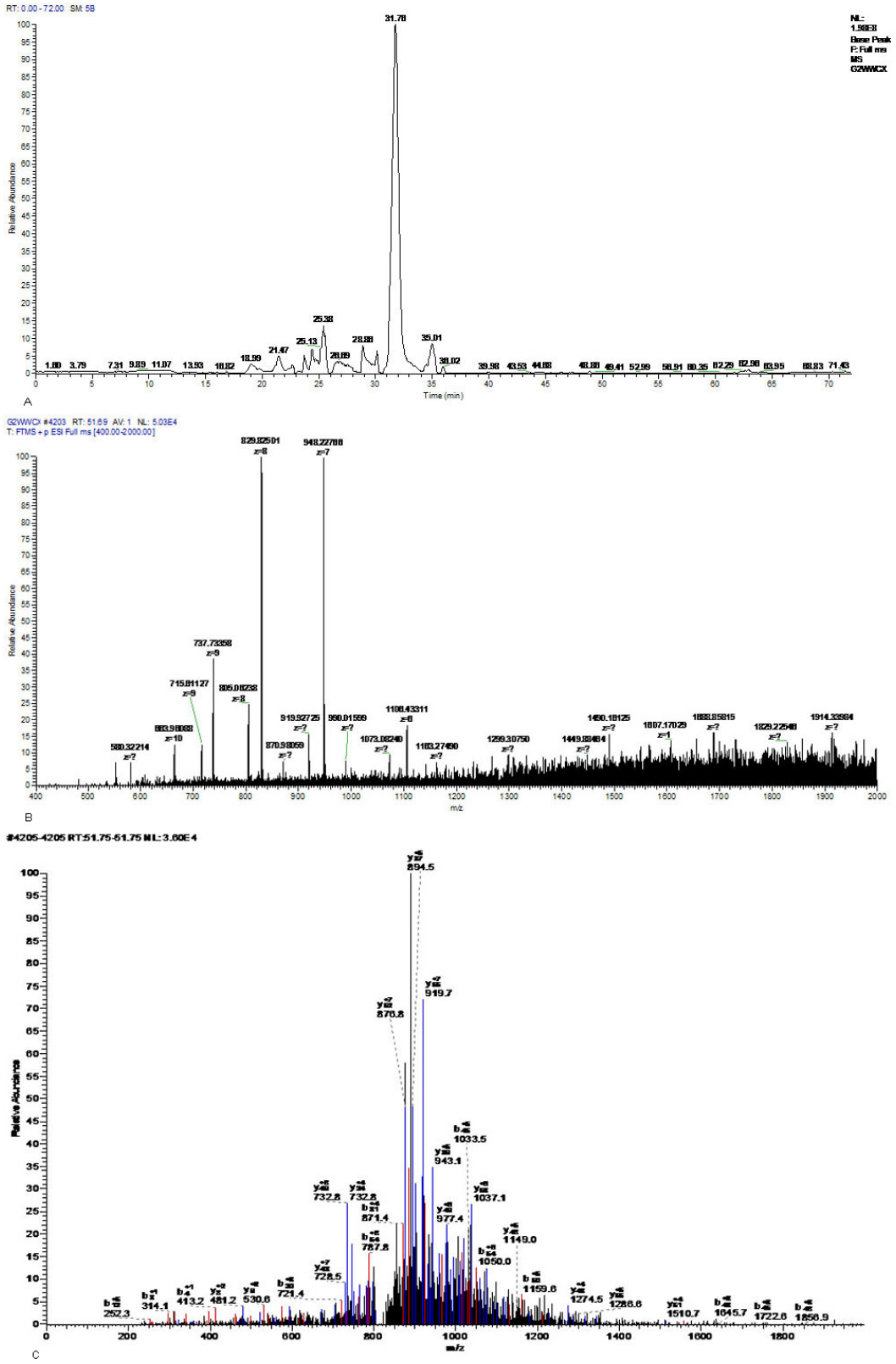

Figure 4. Identification of 6631Da peak by MS/MS analysis. (A) Peak spectrum based chromatography (G2W-WCX); (B) single scan fragmentation spectrum state of sera acquired in the orbitrap at 100,000 resolution (1106.4331 (6+), 948.2277 (7+), 829.8250 (7+), 737.7336(9+)); (C) MS/MS fragmentation spectrum analyzed in the orbitrap.

Table 3. Mass spectral characteristics of proteins/peptides with differential expression between patient and control subjects

M/Z	p Value [1]	Ave	Ave	SD N	SD T
		CON	DM	CON	DM
7010	< 0.001	34.36	19.61	9.05	4.78
8141	< 0.001	15.17	8.39	4.61	2.2
7598	<0.001	9.45	5.57	2.58	1.16
7646	<0.001	14.69	10.04	3.31	1.75
3315	<0.001	31.44	51.1	8.94	14.73
8863	<0.001	17.1	9.31	5.98	3.93
7766	<0.001	115.29	58.89	47.08	29.24
9062	<0.001	24.36	12.91	9.51	5.56
7922	<0.001	16.19	11.15	4.34	2.47
7833	<0.001	11.59	7.41	3.81	2.02
6631	<0.001	85.84	232.41	51.23	188.43
4644	<0.001	202.98	119.7	98.84	52.49
9289	0.0016	960.32	592.91	365.62	294.18
8565	0.0045	8.4	6.61	1.64	1.7
3302	0.0053	9.7	12.94	3.31	2.92
9430	0.0078	45.82	31.61	19.61	17.99
1984	0.0419	20.01	23.92	5.07	4.7
		CON	DPN	CON	DPN
8835	<0.001	9.83	6.56	2.26	2.11
7598	<0.001	8.95	6.04	2.44	1.84
8863	<0.001	20.81	12.95	6.71	4.69
9021	0.00251	7.08	4.71	2.18	1.66
7564	0.00625	9.78	7.5	2.02	1.98
7010	0.00723	31.42	21.85	8.33	8.15
9063	0.00728	24.5	15.56	9.55	7.65
8677	0.00872	12.07	8.78	3.68	3.2
6631	0.0268	86.27	169.81	51.53	118.23
1944	0.0467	17.74	12.49	7.88	4.73
		DM	DPN	DM	DPN
7766	0.029	58.65	109.05	29.13	56.21
3315	0.0363	51.7	38.69	14.81	12.53
3884	0.0363	17.82	24.24	4.76	8.07
8142	0.0363	12.29	17.05	2.73	6.13

[1] Mann-Whitney U-test adjusted by the Benjamini and Hochberg method

Table 4. Specificity and sensitivity for the SNN model

Group	Recognition Capability	Peaks for model		Veracity rate	Cross Validation
CON vs DM	100%	7010, 3315, 1867, 7597.7, 1466, 2863, 2281, 1944, 661, 1692	CON	100%	74.47%
			DM	100%	79.25%
CON vs DPN	100%	3192, 1062, 1618, 1944	CON	100%	100%
			DPN	100%	100%
DM vs DPN	100%	3315, 7766, 1865.91, 1331, 4964, 1692	DM	100%	68.09%
			DPN	100%	60.38%

hepatic steatosis and severe hepatic insulin resistance (24). The increase of ApoCI has been reported to reflect the levels of related apolipoproteins CIII and B, as well as insulin-like growth factor (IgF) binding protein. High plasma apoCI is also positively related to proinflammatory response in patients experiencing endotoxemia which is associated ($P<0.05$) with increased perioperative levels of TNF-alpha.

DPN is a common cause of neuropathic syndrome and produces significant morbidity. Successful treatment can be difficult and relies on modification of the underlying disease with maintenance of euglycemia and normal body weight and lipid levels as well as a multitude of symptomatic therapies. Clinical data indicates that lipid metabolism disorders in patients with type 2 diabetes mellitus complicated with DPN are important risk factors., as lipid metabolic disorders may be involved in the process of occurrence and development of DPN. For DPN patients, serum total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) were significantly higher (25). Apolipoproteins on the lipoprotein subclasses of all the major metabolic pathways have their own unique influence. The experimental results show that DM and DPN groups compared with healthy controls in patients with serum levels appeared significantly higher in ApoCI trends. This is consistent with previous findings, which may partially explain the developmental process of DPN. Advances in our understanding of the pathophysiology of PDN are providing potential new avenues for prevention and treatment targeted at neuronal transmission, fatty acid production, inflammation, antioxidants, the polyol pathway, protein kinase C, and others. Continuing research into the underlying pathophysiology of DPN will ultimately lead to more effective and better-tolerated therapies. Additional analysis of a larger set of individual samples in combination with more traditional immunoassays such as ELISA are required to further confirm whether high serum ApoCI levels increased odds ratios (ORs) of DPN in a nested case-control sample of type 2 diabetic individuals such as those observed in this study.

6. ACKNOWLEDGEMENTS

We thank Huazong Zeng, Jilong Liu, Xiaohui Hu, Jing Ren, Xiaomin Wang for valuable dedication to this manuscript. The authors acknowledge the financial support for the work by National Key Basic Research Program (NO. 2005CB523304); Science and Technology Commission's Program for Key Research of Shanghai Municipality (NO. 04DZ19503); Natural Science Foundation of Shanghai (No. 10ZR1439400). The authors have declared no conflict of interest.

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Key Words: Diabetic Peripheral Neuropathy, Serum, Diagnostic Model, Magnetic Beads, CLINPROT

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