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PAPER

Proteomic identification of plasma biomarkers in uterine leiomyoma

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Recent progresses in quantitative proteomics have offered opportunities to discover plasma proteins as biomarkers for tracking the progression and for understanding the molecular mechanisms of uterine leiomyomas. In the present study, plasma samples were analyzed by fluorescence two-dimensional differential gel electrophoresis (2D-DIGE) and differentially expressed proteins were identified by matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). In total, 20 proteins have been firmly identified representing 13 unique gene products. These proteins mainly functioned in transportation (such as apolipoprotein A-I) and coagulation (such as fibrinogen gamma chain). Additionally, our quantitative proteomic approach has identified numerous previous reported plasma markers of uterine leiomyomas such as alpha-1-antitrypsin. On the contrary, we have presented several putative uterine leiomyomas biomarkers including afamin, apolipoprotein A-I, carbonic anhydrase 1, fibrinogen beta chain, fibrinogen gamma chain, gelsolin, hemopexin, leucine-rich alpha-2-glycoprotein, serotransferrin and vitamin D-binding protein which have not been reported and may be associated with the progression and development of the disease. In summary, we report a comprehensive patient-based proteomic approach for the identification of potential plasma biomarkers for uterine leiomyomas. The potential of utilizing these markers for screening and treating uterine leiomyomas warrants further investigations.

Introduction

Uterine leiomyoma is a benign neoplasm deriving from the myometrial compartment of the uterus and is the most widespread gynaecological problem in women. This benign tumor causes significant troubles such as abnormal uterine bleeding and reproductive dysfunction.[1] However, 60% of the patients with uterine leiomyoma do not exhibit a cytogenetic abnormality.[2] It leads to the difficulty to prognose this disease in advance. Recently, although numerous uterine leiomyoma-related genetic markers have been studied, many of the molecular mechanisms related to the uterine leiomyoma pathophysiology are still waiting for elucidation. Moreover, many investigations on uterine leiomyoma pathogenesis lack physiological relevance due to these studies being solely based on investigation of fewer genes and cell lines.[3,4]

Two-dimensional gel electrophoresis (2DE) and MALDI-TOF MS have 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 2DE 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-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

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facilitate the data accuracy in normalization and increase statistical confidence in relative quantity across gels.[5–9]

In this study, developing new techniques to detect uterine leiomyoma is essential for both early diagnosis and eventual prognosis. Proteomics is a powerful tool for the analysis of complex mixtures of proteins and the identification of biomarkers. To examine differentially expressed levels of plasma proteins associated with uterine leiomyoma, we developed a proteomics-based approach involving immunodepletion of high-abundance proteins, 2D-DIGE analysis, and subsequent MALDI-TOF MS analysis to obtain a panel of plasma proteins, differentially expressed between uterine leiomyoma patients and healthy donors.

Materials and methods

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.

Plasma sample collection and purification

From Jan 2009 to Dec 2009, thirteen donors in a single hospital (Chiayi Christian Hospital, Chiayi, Taiwan) were enrolled in the study. Those included in the study were divided into uterine leiomyoma patients ($n = 5$) and healthy donors group ($n = 8$). The criteria to assess the presence of uterine leiomyoma were based on the pathological diagnosis and guidelines proposed by the World Health Organization. Healthy individuals were selected with similar age and with no uterine leiomyoma diagnosed clinically. The clinical data of patients were measured in the clinical laboratory and are summarized in Table 1. 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 accordance with the manufacturer's instructions. The depleted plasma samples were precipitated by adding 1 volume of 100% TCA (at −20 °C) to 4 volumes of sample and incubated for 10 min at 4 °C. The precipitated protein was then recovered by centrifugation at 13 000 rpm for 10 min, and the resulting pellet was washed twice with ice-cold acetone. Air-dried pellets were resuspended in 2DE lysis containing 4% w/v CHAPS, 7 M urea, 2 M thiourea, 10 mM Tris–HCl, pH 8.3, 1 mM EDTA.

Table 1 Clinical parameters for leiomyoma patients participating in this investigation (values are mean and the range). The values in this table are calculated with the non-parametric test. The abbreviations used in this table: GOT, glutamate oxaloacetate transaminase; GPT, glutamic pyruvic transaminase; BUN, blood urea nitrogen

	Case ($N = 5$)	Control ($N = 8$)	P-value
Age/years	41 (37–48)	48 (41–50)	0.0749
GOT/ U l^{-1}	18 (17–20)	18.5 (9–20)	0.9257
GPT/ U l^{-1}	13 (10–20)	22.5 (12–31)	0.0814
BUN/ mg dl^{-1}	12.6 (11–15.9)	11.3 (7.9–16.9)	0.4286
Creatinine/ mg dl^{-1}	0.7 (0.6–0.7)	0.7 (0.66–1.1)	0.0989

Sample preparation for 2D-DIGE and gel image analysis

The plasma protein pellets were dissolved in 2DE lysis buffer and protein concentrations were determined using a coomassie protein assay reagent (BioRad). Before performing 2D-DIGE, plasma samples from 5 uterine leiomyoma patients and 8 healthy donors were pooled, individually. After protein quantification with the Bradford assay, 50 μg of protein samples from patients and healthy donors were labeled with 125 pmol of either Cy3 or Cy5 for triplicate comparison on three 2DE, respectively. 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 quantified on multiple 2DE. 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, pH 3–10 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 non-linear pH gradient (IPG) strips (pH 3–10, 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 6 M urea, 30% (v/v) glycerol, 1% SDS (w/v), 100 mM Tris–HCl (pH8.8), and 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 × 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 2DE gels 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 2D 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 γ 1.5 average-fold increase or decrease in abundance with a p-value <0.05 were selected for protein identification.[10,11]

Protein staining

Colloidal coomassie blue G-250 staining was used to visualize CyDye-labeled protein features in 2DE. Bonded gels were

fixed in 30% v/v ethanol, 2% v/v phosphoric acid overnight, washed three times (30 min each) with ddH_2O and then incubated in 34% v/v methanol, 17% w/v ammonium sulfate, 3% v/v phosphoric acid for 1 h, prior to adding 0.5 g l^{-1} 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.

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 h 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 h 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_2O, and stored at −20 °C prior to MS analysis.

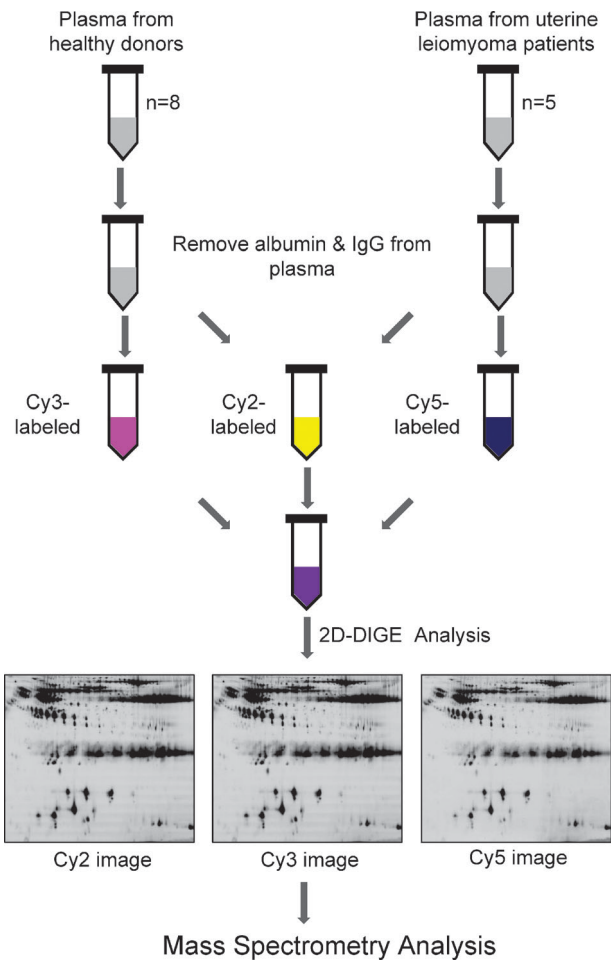

Fig. 1 Experimental design to perform 2D-DIGE/MALDI-TOF MS-based quantitative plasma proteomics.

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 (Sophisticated Numerical Annotation Procedure). 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; Median-Level: 0.5. The spectrometer was also calibrated with a peptide

A

	Cy2 (50µg)	Cy3 (50µg)	Cy5 (50µg)
Gel1	Pool	Uterine leiomyoma	Healthy donors
Gel2	Pool	Healthy donors	Uterine leiomyoma
Gel3	Pool	Uterine leiomyoma	Healthy donors

B

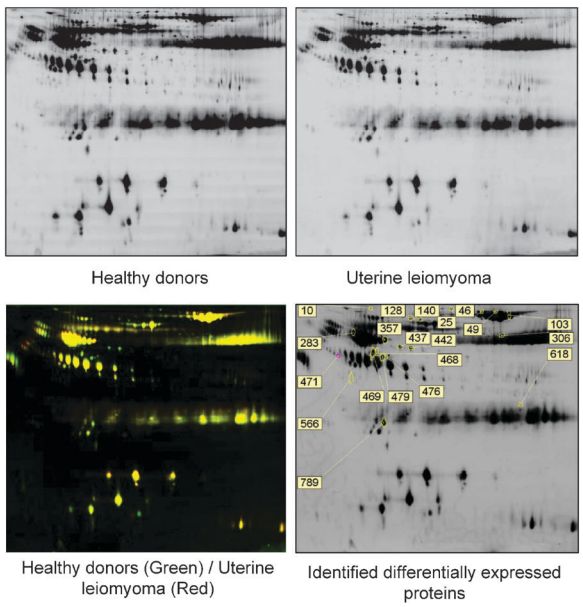

Fig. 2 2D-DIGE analysis of uterine leiomyoma-induced differentially expressed proteins. (A) Plasma samples arrangement for a triplicate 2D-DIGE experiment. (B) Plasma samples (50 µg each) were labeled with Cy-dyes and separated using 24 cm, pH 3–10 non-linear IPG strips. 2D-DIGE images of the plasma samples from uterine leiomyoma patients and healthy individuals at appropriate excitation and emission wavelengths were shown (upper images) as well as the 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).

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-2010) with 521 016 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 2DE.

Immunoblotting

Immunoblotting was used to validate the differential abundance of mass spectrometry identified proteins. Aliquots of 20 μg of plasma proteins were diluted in Laemmli sample buffer (final concentrations: 50 mM Tris pH 6.8, 10% (v/v) glycerol, 2% SDS (w/v), 0.01% (w/v) bromophenol blue) and separated by 1D-SDS-PAGE following standard procedures. After electroblotting separated proteins onto 0.45 μm Immobilon P membranes (Millipore), the membranes were blocked with 5% w/v skimmed milk in TBST (50 mM Tris pH 8.0, 150 mM NaCl and 0.1% Tween-20 (v/v)) for 1 h. Membranes were then incubated in primary antibody solution in TBS-T containing 0.02% (w/v) sodium azide for 2 h. Membranes were washed in TBS-T (3 × 10 min) and then probed with the appropriate horseradish peroxidase-coupled secondary antibody (GE Healthcare). After further washing in TBS-T, immunoprobed proteins were visualized using an enhanced chemiluminescence method (Visual Protein Co.).

Enzyme-linked immunosorbent assay (ELISA) analysis of plasma

EIA polystyrene microtitration wells were coated with 50 μg of protein samples and incubated at 37 °C for 2 h. The plate was washed for three times with phosphate buffered saline-tween 20 (PBST) and three times with PBS. After the uncoated space was blocked with 100 μl of 5% skimmed milk in PBS at 37 °C for 2 h, the plate was washed three times with PBST. Antibody solution was added and incubated at 37 °C for 2 h. After washing with PBST and PBS for 10 times in total, 100 μl of peroxidase-conjugated secondary antibodies in PBS was added for incubation at 37 °C for 2 h. Following 10 washings, 100 μl of 3,3′,5,5′-tetramethyl benzidine (Pierce) was added. After incubation at room temperature for 30 min, 100 μl of 1 M H_2SO_4 was added to stop the reaction followed by measured absorbance at 450 nm using a Stat Fax 2100 microtiterplate reader (Awareness Technology Inc., FL, USA).

Results

2D-DIGE and mass spectrometry analysis of the immunodepleted plasma proteome

Because albumin and immunoglobulin G account for around 70–80% proteins in human plasma, these high-abundance

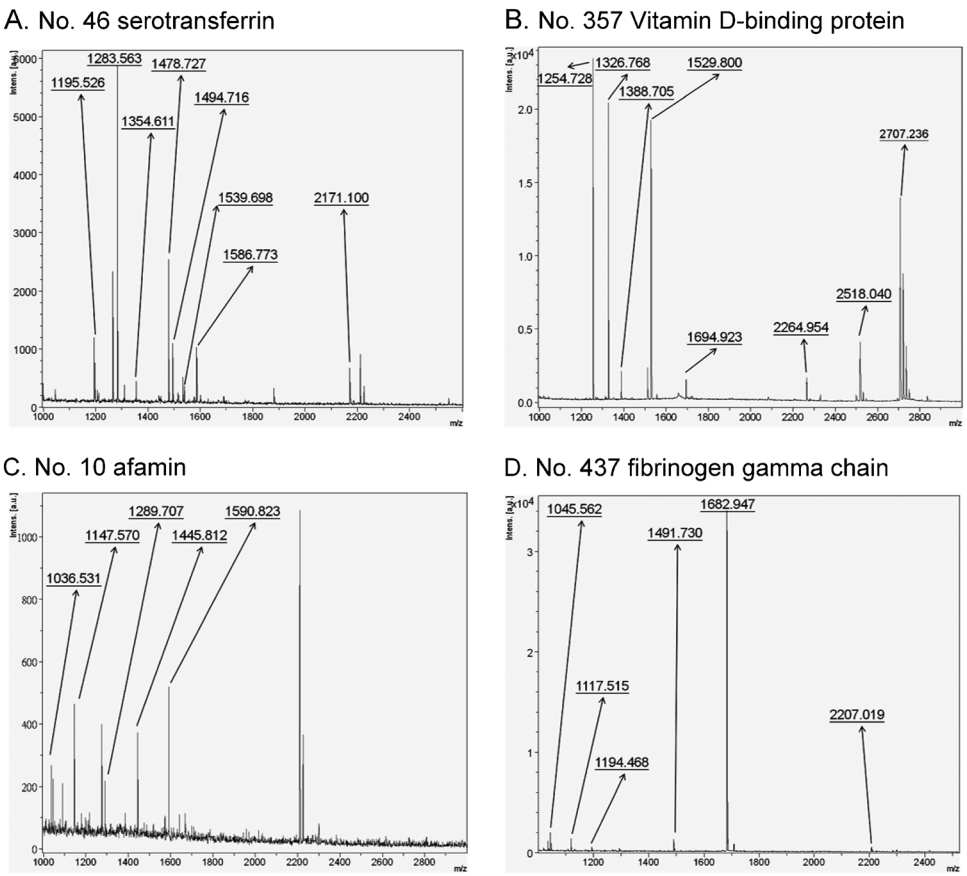

Fig. 3 Peptide finger printing of identified proteins (A) serotransferrin, (B) vitamin D binding protein, (C) afamin and (D) fibrinogen gamma chain.

proteins are the obstacle for 2DE 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 accurate analysis. In this study, the high-abundance proteins were removed before performing 2D-DIGE analysis.

In order to study the alteration of plasma protein in the uterine leiomyoma patients, comparative proteomics analysis was performed between uterine leiomyoma patients and healthy donors. The 2DE 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

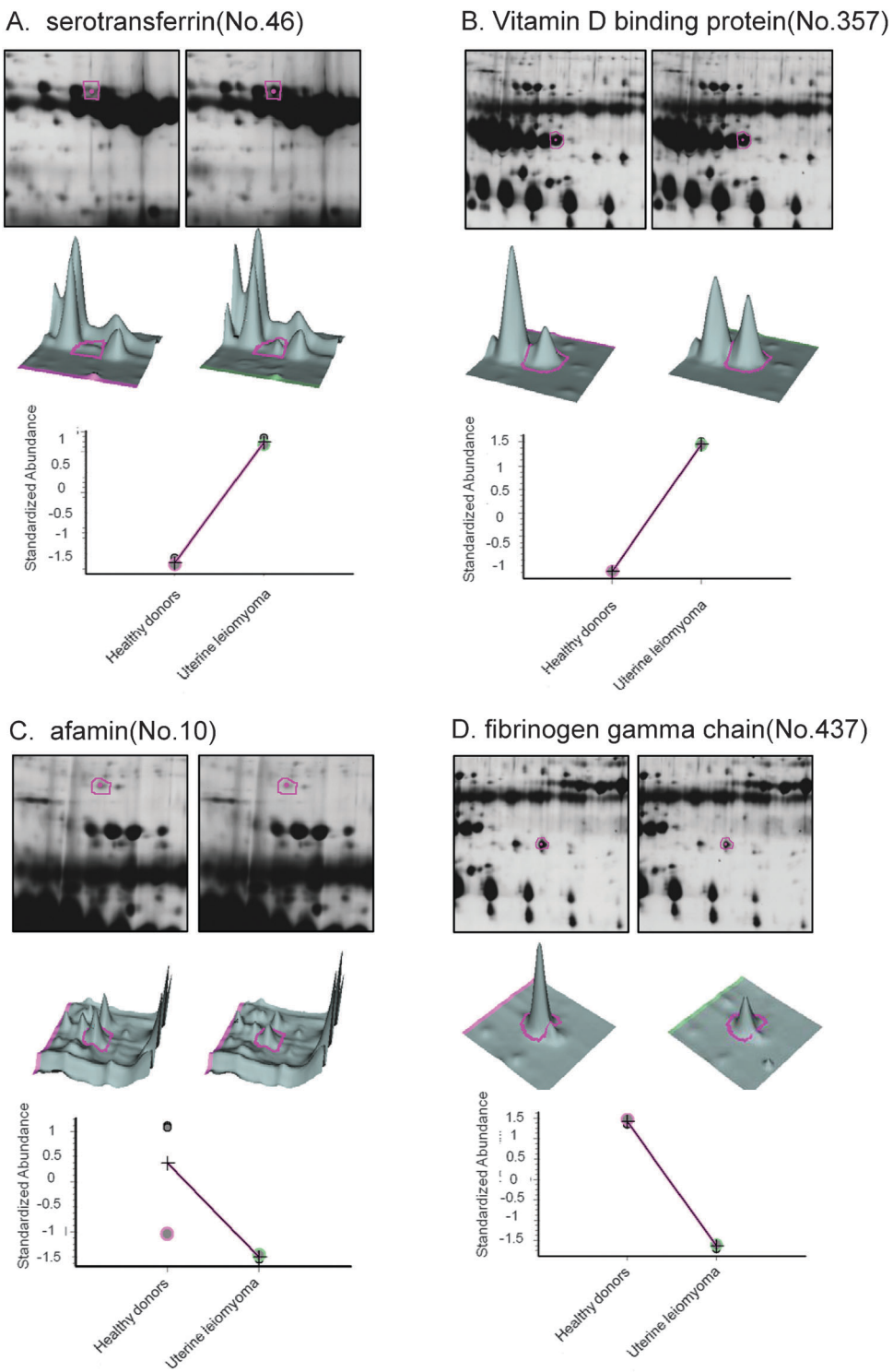

Fig. 4 Differential abundance of plasma proteins ((A) serotransferrin, (B) vitamin D binding protein, (C) afamin and (D) fibrinogen gamma chain) between uterine leiomyoma patients and healthy individuals.

Table 2 Alphabetical list of differentially expressed plasma proteins between uterine leiomyoma patients and healthy individuals as identified by MALDI-TOF peptide mass fingerprinting after 2D-DIGE analysis. Identified proteins highlighted with gray color demonstrated these proteins have not been reported as uterine leiomyoma markers

Spot No.	Swissprot No.	Protein name	Pred. MW	Pred. PI	Coverage (%)	MASCOT score	No. of peptides match/supplied	*T*-test	Ave. ratio (uterine leiomyoma/ normal)	peptide sequence	Location	Function
479	Q96HG5	Actin, cytoplasmic 1	42 052	5.29	29	67/56	9/29	1.40×10^{-6}	−3.24	AVFPSIVGRPRGYSFTTTAER	cytoplasm	cytoskeleton
476	Q96HG5	Actin, cytoplasmic 1	42 052	5.29	35	76/56	10/31	0.0002	−2.49	AVFPSIVGRPRGYSFTTTAER	cytoplasm	cytoskeleton
10	P43652	Afamin	70 963	5.64	9	70/56	7/13	0.00076	−1.58	HPDLSIPELLRRLCFFYNK.	secreted	transportation
283	Q86U18	Alpha-1 antitrypsin	46 878	5.37	16	74/56	6/12	7.10×10^{-5}	1.52	FLENEDRR GKWERPFEVK	secreted	Inflammatory response
789	P02647	Apolipoprotein A-I	30 759	5.56	30	90/56	11/36	0.0002	−1.57	THLAPYSDELR VQPYLDDFQK	secreted	transportation
618	P00915	Carbonic anhydrase 1	28 909	6.59	23	58/56	4/9	0.028	1.53	ESISVSSEQLAQF GGPFSDSYR	cytoplasm	transportation
306	Q9UC63	Fibrinogen beta chain	52 106	5.37	35	139/56	18/42	0.0023	−1.56	QDGSVDFGR SILENLR	secreted	coagulation
442	Q9UC63	Fibrinogen gamma chain	52 106	5.37	36	134/56	12/27	2.70×10^{-6}	−2.39	QSGLYFIKPLK VGPEADKYR	secreted	coagulation
437	Q9UC63	Fibrinogen gamma chain	52 106	5.37	18	80/56	7/15	1.20×10^{-5}	−2.32	TSEVKQLIK VGPEADKYR.	secreted	coagulation
469	Q9UC63	Fibrinogen gamma chain	52 106	5.37	18	109/56	7/8	0.00032	−1.76	DNCCILDER VELEDWNGR	secreted	coagulation
468	Q9UC63	Fibrinogen gamma chain	52 106	5.37	18	109/56	7/8	0.0015	−1.58		secreted	coagulation
25	P06396;	Gelsolin	86 043	5.9	11	65/56	7/16	0.0014	−2.96	EPGLQIWR TGAQELLR	secreted	cytoskeleton
140	P02790	Hemopexin	52 385	6.55	30	109/56	11/25	6.30×10^{-5}	−1.54	YYCFQGNQFLR D YFMPCPGR	secreted	transportation
128	P02790	Hemopexin	52 385	6.55	24	87/56	9/21	0.0012	−1.52	NFPSPVDAAFR DYFMPCPGR	secreted	transportation
471	P02750	Leucine-rich alpha-2-glycoprotein	38 382	6.45	17	65/56	4/11	5.60×10^{-5}	1.63	DLLLPQPDLR VAAGAFQGLR	secreted	Inflammatory response
566	P02787	Serotransferrin	79 280	6.81	16	115/56	11/27	6.40×10^{-5}	−1.64	EGYYGYTGAFR APNHAVVTR	secreted	signal transduction
49	P02787	Serotransferrin	79 280	6.81	8	72/56	6/10	0.011	1.55	MYLGYEYVTAIR WCALSHHER	secreted	signal transduction
103	P02787	Serotransferrin	79 280	6.81	12	86/56	9/26	0.0014	1.74	SCHTAVGR APNHAVVTR	secreted	signal transduction
46	P02787	Serotransferrin	79 280	6.81	15	120/56	10/19	0.00023	2.16	MYLGYEYVTAIR WCALSHHER	secreted	signal transduction
357	P02774	Vitamin D-binding protein	54 526	5.4	28	122/56	11/26	6.00×10^{-6}	1.8	VMDKYTFELSR RTHLPEVFLSK	secreted	cytoskeleton

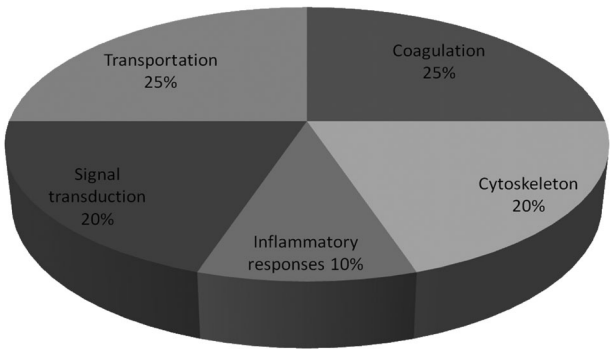

Fig. 5 Percentage of plasma proteins identified from albumin and immunoglobulin G depleted plasma by 2D-DIGE/MALDI-TOF MS for uterine leiomyoma according to their biological functions.

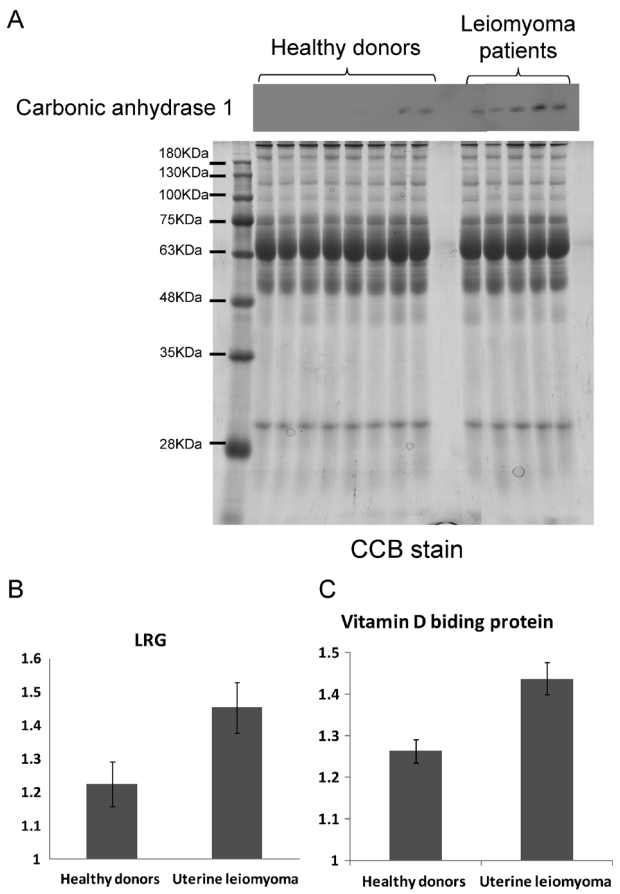

Fig. 6 Representative immunoblotting and ELISA analysis of carbonic anhydrase 1, leucine-rich alpha-2-glycoprotein and vitamin D binding protein for selected differentially expressed plasma proteins identified by proteomic analysis in patients with and without uterine leiomyoma. 20 μg of the albumin and immunoglobulin-depleted plasma were loaded and resolved by SDS-PAGE followed by immunoblotting with carbonic anhydrase 1 or staining with colloidal coomassie blue G-250 as an internal loading control. Additionally, the levels of leucine-rich alpha-2-glycoprotein and vitamin D binding protein were confirmed by ELISA.

labeling with Cy2 as an internal standard to run on all gels to facilitate image matching across gels (Fig. 1). The plasma samples arrangement for a triplicate 2D-DIGE experiment is shown in Fig. 2A. Thus, the triplicate samples resolved in different gels can be quantitatively analyzed by means of the internal standard on multiple 2DE. After resolving protein samples with the 2D-DIGE technique, the DeCyder image analysis software indicated that 84 protein features were showing greater than 1.5-fold change in expression level with the Student *t*-test (*p*-value) less than 0.05. MALDI-TOF MS identification revealed that 20 proteins (corresponding to 13 unique proteins) were differentially expressed (Fig. 2B, Fig. 3, Fig. 4 and Table 2). Most of these identified proteins are functionally involved in transportation (25%), coagulation (25%), cytoskeleton (20%), signal transduction (20%) and inflammatory responses (10%) (Fig. 5).

Representative examples for the evaluation by DeCyder of alteration in spot intensities using the 2D-DIGE system are displayed in Fig. 4. To display visual alterations in corresponding spot intensity proportions, selected identified spots (serotransferrin, vitamin D binding protein, afamin and fibrinogen gamma chain) are shown as 3-D images as well as the associated graph views of standardized abundances of the selected spots and spot locations (Fig. 4).

Validation by immunoblotting and ELISA

To verify the abundances of proteins deduced from the results of 2D-DIGE and MALDI-TOF MS, the abundance levels of identified carbonic anhydrase, leucine-rich alpha-2-glycoprotein and vitamin D binding protein were investigated by immunoblotting and ELISA, respectively. As shown in Fig. 6, 29 kDa of carbonic anhydrase was increased in the plasma of patients. Additionally, 38 kDa of leucine-rich alpha-2-glycoprotein and 55 kDa of vitamin D binding protein were significantly increased in the plasma of patients with uterine leiomyoma. These immunoblotting and ELISA results are consistent with the data from the 2D-DIGE and MALDI-TOF MS, and further suggest that they may be employed as potential markers for the early diagnosis of uterine leiomyoma.

Discussion

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 20 altered expressions of plasma proteins corresponding to 13 unique plasma proteins (Table 2). A majority of altered proteins belong to two major functional groups, transportation and coagulation, while other affected category includes cytoskeleton, signal transduction and inflammatory responses (Fig. 5). Of these, actinin[12,13] and alpha-1-antitrypsin[14] have been reported as uterine leiomyoma markers in previous studies. In contrast, afamin, apolipoprotein A-I, carbonic anhydrase 1, fibrinogen beta chain, fibrinogen gamma chain, gelsolin, hemopexin, leucine-rich alpha-2-glycoprotein, serotransferrin and vitamin D-binding protein have not been reported as uterine leiomyoma markers to our knowledge. Further investigation indicated that the combination of these identified proteins has not yet been described as cancer markers in other cancer types. Accordingly, the combination of these identified proteins might be further evaluated as uterine leiomyoma specific markers.

Afamin, a vitamin E-binding protein, has been reported as a ovary cancer marker[15,16] and cervix cancer marker.[17] However, afamin is down-regulated in this study, implying that there are differentially regulated mechanisms across ovary cancer, cervix cancer and uterine leiomyoma. In addition, afamin was highly expressed in the later stages of ovary cancer but not in the early stages of the disease, suggesting that its expression might correlate to the level of tumorigenesis.

Apolipoprotein A-I is a component of the high density lipoprotein responsible for the cholesterol transport into the liver.[18] In numerous cancers such as bladder cancer, oesophageal cancer, cholangiocarcinoma, ovarian cancer and colon cancer, the apolipoprotein A-I level is up-regulated;[19–23] however, the reduced plasma apolipoprotein A-I level is associated with gastric tumor.[24] Our current 2D-DIGE analysis revealed that the apolipoprotein A-I level is down-regulated in uterine leiomyoma. These previous reports and our results noted that the regulation of the apolipoprotein A-I level during tumorigenesis is complicated and needs further clarification.

Serum fibrinogen is a regulator during blood coagulation and has been recognized as a metastatic and an angiogenic predictor in numerous cancers; meanwhile, it is an indicator for malignant tumors.[25–29] Since uterine leiomyomas are benign neoplasms, the low expression level of serum fibrinogen is reasonable to compare with malignant tumors.

Gelsolin is a key regulator of actin filament assembly and disassembly and has been widely identified as a novel invasion modulator in many types of cancer including pancreatic cancer, bladder cancer, lung cancer and breast cancer.[30–33] Thus, our current 2D-DIGE analysis is consistent with these observations showing down-regulation in uterine leiomyomas.

Leucine-rich alpha-2-glycoprotein has been shown to be involved in cell adhesion and has been recognized as a biomarker for certain diseases including microbial infections, ovarian cancer and pancreatic cancer.[34,35] Our result is the first time to support leucine-rich alpha-2-glycoprotein as a marker for uterine leiomyomas.

Vitamin D-binding protein is a secreted transportation protein which carries the vitamin D sterols and prevents polymerization of actin. In clinical diagnosis, vitamin D-binding protein has been widely used as a biomarker for breast cancer, thyroid cancer, lung cancer, and oral carcinoma.[36–39] Our plasma proteomic analysis is the first report to evidence vitamin D-binding protein as a marker for uterine leiomyomas.

The isolation of low-abundance proteins from plasma is frequently complicated because of the existence of high-abundance proteins such as serum albumin and immunoglobulin. Serum albumin and immunoglobulin are the most (50–70%) and second (10–20%) abundant protein in plasma, respectively. These proteins will mask the low-abundance proteins and limit the amount of the total plasma proteins that can be applied and resolved by proteomic analysis. To remove these two high-abundance proteins, we used an albumin and IgG depletion kit from Sigma-Aldrich, which contains prepacked spin columns and allows removal of 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.

Our 2D-DIGE experiment is based on fluorescence-based quantitation which can detect sub-nanogram of dye-labeled proteins; however, our post-staining experiment is based on colloidal coomassie blue staining with sensitivity around 10–100 nanograms.[40] Hence, numerous differentially expressed dye-labeled low-abundance plasma proteins can be captured by a fluorescent scanner but failed to be imaged with colloidal coomassie blue staining. This is the reason why only less than half of 84 differentially expressed features on 2DE can be picked for MALDI-TOF analysis with only 20 proteins identified. Additionally, the plasma proteins are largely glycosylated in the circulation system. The high level of glycosylation in plasma proteins has been reported to interfere trypsin digestion and MALDI-TOF MS analysis.[41] Consequently, glycosylation of plasma proteins might contribute to a low-identification rate in this study.

In conclusion, the quantitative plasma proteomics analysis provided a valuable impact for uterine leiomyoma research. Our quantitative proteomic approach has identified numerous previous reported plasma markers of uterine leiomyoma such as apolipoprotein A-I. Additionally, we have presented several putative uterine leiomyoma biomarkers which may be associated with the progression and development of the disease and have 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 uterine leiomyoma warrants further investigation.

Abbreviations

2DE	two-dimensional gel electrophoresis
Ab	antibody
CCB	colloidal coomassie blue
CHAPS	3-([(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate)
CLI	critical limb ischemia
ddH_2O	double deionized water
DIGE	differential gel electrophoresis
DTT	dithiothreitol
MALDI-TOF MS	matrix assisted laser desorption ionization–time of flight mass spectrometry
TFA	trifluoroacetic acid

Declaration of competing interests

The authors confirm that there are no conflicts of interest.

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