

# Comparative analysis of serum proteomes: Identification of proteins associated with sciatica due to lumbar intervertebral disc herniation

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**Abstract.** Lumbar intervertebral disc herniation (LDH) is one of the most common orthopedic conditions that can cause lower back pain and sciatica. However, the pathogenesis of LDH is poorly understood. The aim of the present study was to use proteomic analysis of blood samples to establish whether there are serum proteins associated with LDH, which may be useful in elucidating LDH pathogenesis. The ultimate aim was to develop a simple technique for the diagnosis of LDH based on the blood samples of patients with sciatica. The study used comparative analysis of serum proteomes associated with sciatica due to LDH. A total of 30 LDH patients with sciatica, receiving treatment between August and December 2007, were selected as the experimental group (or LDH group). A total of 2 ml of blood was obtained from each of the 30 patients in the LDH group and from 30 healthy volunteers, who constituted the control group. Two-dimensional electrophoresis of the blood samples was conducted, distinct protein spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and proteins associated with LDH were detected. An enzyme-linked immunosorbent assay (ELISA) was developed to screen for the LDH proteins and was tested on the sera of a second test and control group that included 10 patients with LDH and 10 healthy subjects, respectively. Based on signal intensity, the expression levels of 6 proteins on the dielectrophoretogram were found to be significantly associated with LDH. The identities of the LDH proteins were upregulated apolipoprotein-L1 (APO-L1) and two types of serum albumin precursors, and downregulated

apolipoprotein M (APO-M), tetranectin (TN) and immunoglobulin light chain (IGL). Further ELISA experiments confirmed that there were increased serum levels of 4 out of the 6 proteins in patients with sciatica due to LDH, which was statistically different compared to the healthy subjects. In conclusion, these results suggest that serum APO-L1, TN, APO-M and IGL may serve as LDH biomarkers.

## Introduction

Lumbar intervertebral disc herniation (LDH) is one of the most common orthopedic conditions that can cause lower back pain and sciatica. It is estimated that ~70% of the population experiences lower back pain during their lifespan, and at any given time, 55% suffer from lower back pain associated with radicular syndromes (1-3). Sciatica symptoms are extremely persistent and up to one-third of all patients with sciatica undergo lumbar surgery (4). A number of studies have discussed the mechanical and biochemical factors involved in the induction of radicular pain due to LDH (5) and numerous trials of potential molecular and biological therapies have been conducted in this context. Previous studies have evaluated three main biochemical changes: Decreased matrix synthesis, increased catabolism, and changed levels of growth factors and cytokines associated with altered disc cell phenotypes during disc aging and degeneration (6-11). However, sciatica of a radicular origin represents an undetermined clinical problem. The study by Bogduk (12) stated that discogenic pain cannot be diagnosed clinically and relies on supposition. Thus far, the pathogenesis of LDH is poorly understood and, aside from surgical intervention, no effective therapy is available.

Proteomic techniques are powerful tools that can provide detailed information regarding changes to the expression of protein profiles. Studies on proteomics have been carried out in certain diseases, including malignant tumors and neuropathy (13-17). A specific marker for a particular disease can be identified by comparing the protein spectrum of a patient with the disease with that of a healthy subject or with the protein spectrum from the gene bank (18). Proteomics may become one of the best measures for tracking disease markers and drug targets. Although there are certain studies describing the use of proteomics for marker detection and evaluation of

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pathogenesis in rheumatoid arthritis, few studies have been published regarding proteomics research in the osteoarthritis system (19,20), particularly in LDH. The pathogenesis and development of LDH undergoes a multi-stage process, which is based on gene expression changes. Proteins are the key bridges that connect genes with the biological function outcomes. Therefore, more proteomic studies are required to help improve the understanding of LDH pathogenesis. Proteomic analysis studies of the cerebrospinal fluid (CSF) of patients with LDH have documented quantitative differences in the expression of CSF proteins from patients with LDH compared to controls. Thus, these changes can be considered to be biomarkers of a condition, such as LDH (21). However, it is difficult to obtain the CSF and therefore it is difficult to identify such biomarkers. Therefore, the aim of the present study was to use proteomics to identify differentially expressed proteins in the sera of LDH patients compared to control subjects. The validity of the differential protein expression in the patients with LDH was further examined and confirmed with an enzyme-linked immunosorbent assay (ELISA). We suggest that these differentially expressed proteins may be useful as biomarkers for LDH, and in addition, they may lead to the development of targeted therapies for this disease.

## Materials and methods

*Diagnostic criteria for LDH.* The diagnosis of LDH was based on the symptoms and objective signs of sciatica. Lower back pain, severe lower leg pain and the Lasègue sign were observed in all the patients. In addition, magnetic resonance imaging confirmed LDH. Subjects with liver or kidney diseases were excluded from the study. Three orthopedic surgeons conducted independent clinical diagnoses on the basis of the physical and radiological examinations, which were confirmed by surgery.

*Subjects.* A total of 30 patients with LDH (15 females and 15 males,  $28.1 \pm 2.3$  years old, who had never received any prior surgical treatment and had not possessed any disease-related or predisposing risk for LDH) and 30 healthy subjects who lacked any symptoms of disc herniation (15 females and 15 males,  $25.2 \pm 3.1$  years old) were included in the proteomics study. Another 10 patients with LDH (5 females and 5 males,  $26.3 \pm 2.3$  years old) and 10 healthy subjects (5 females and 5 males,  $30.1 \pm 3.3$  years old) were enrolled for the ELISA test.

*Serum samples.* Venous blood samples were collected from all subjects and phenylmethylsulfonyl fluoride was added to the samples. The serum was separated from the samples and stored at  $-80^{\circ}\text{C}$  until use according to the instructions of the ProteoPrep Blue Albumin and IgG Depletion kit (Sigma) and 2-D clean up kit (Amersham Biosciences). To minimize the variation among individuals, the gender and age of the patients were matched in the control and LDH groups in the proteomic study. The study was approved by the local ethics committee of the Third Affiliated Hospital of Sun Yat-sen University and it was carried out in compliance with the principles stipulated by the Declaration of Helsinki. All patients and control subjects gave their informed consent prior to enrollment in the study.

A  $30\text{-}\mu\text{l}$  serum sample was loaded onto a 1-ml column. According to the manufacturer's instructions, a

ProteoPrep<sup>®</sup> Blue Albumin and immunoglobulin G (IgG) Depletion kit (Sigma-Aldrich, St. Louis, MO, USA) was used to remove serum albumin and IgG. A 2-D clean-up kit (Amersham Biosciences Corp., Piscataway, NJ, USA) was utilized to discard the salt and sulfatide in the serum. Protein concentrations in the serum were determined prior to conducting two-dimensional electrophoresis (2-DE), using Bradford's method (22) with bovine serum albumin as the standard.

*Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).* 2D-PAGE was performed according to the method of Görg *et al.* (23) and the manufacturer's instructions of the Bio-Rad (Hercules, CA, USA) electrophoresis device. Non-linear immobiline pH 4-7 gradient (IPG) strips (Bio-Rad) were used for isoelectric focusing (IEF). A 0.3-mg sample of the total protein was loaded onto the IPG strips. The strips were rehydrated at 50 V for 12 h, followed by IEF at 150 V for 1 h, 500 V for 3 h, 1,000 V for 1 h, 5,000 V for 1 h, 7,000 V for 2 h and 10,000 V until the total voltage x hours of exposure reached 50,000 Vh. Following this, the strips were equilibrated for 15 min in equilibration buffer I [6 M urea, 2% sodium dodecyl sulfate, 50 M Tris-HCl (pH 8.8), 30% glycerol, 2% dithiothreitol (DTT) and bromophenol blue]. The strips were subsequently equilibrated in the same buffer containing 3% iodoacetamide instead of DTT for 15 min, and were then transferred to a 12% glycerol gradient gel for separation. The second dimensional separation was performed at 12 mA per strip for 30 min, subsequently switching to 20 mA per strip until the bromophenol blue indicator reached the bottom of the gel. Following 2-DE, the proteins in the gel were visualized by silver staining (20).

*Image analysis.* The stained gel was scanned by an Image Scanner type II (Amersham Biosciences Corp.) and the 2-D electrophoregram was analyzed with the application of ImageMaster 2D Elite 5.0 software (Amersham Biosciences Corp.). The data were statistically processed on the Windows program SPSS v15.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

*Identification of proteins by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).* Protein spots exhibiting significant expressional differences (up- or downregulated by  $>3$ -fold) were excised from the gel and sliced into sections of  $\sim 1\text{ mm}^3$ . The spots were destained and dehydrated. Trypsin digestion solution, which was treated with N-tosyl-L-phenylalanine chloromethyl ketone, was added to the tube containing the spots for digestion overnight at  $37^{\circ}\text{C}$ . Following digestion, the peptides were obtained from the extraction buffer, which contained 50% acetonitrile and 0.1% trifluoroacetic acid (TFA), and were dried under  $\text{N}_2$ . The extracted peptides were mixed with  $\alpha$ -cyano-4-hydroxycinnamic acid solution in 0.1% TFA and 50% acetonitrile. The solution obtained was placed on the metal plate of the MALDI-TOF target and dried at room temperature. The samples were analyzed using MALDI-TOF-MS (Applied Biosystems, Foster City, CA, USA). The spectrum was recorded in the reflector positive ion mode. The standard conditions consisted of a 355-nm

Table I. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of differentially expressed proteins in patients with lumbar intervertebral disc herniation (LDH) compared to healthy controls.

Spot no.	Accession no.	Protein name	MW, Da	pI	Protein score	Optical density intensity in LDH
A1	645213	Apolipoprotein M	13042.4	7.66	210	Decreased
A2	796167	Immunoglobulin light chain	14669.1	6.25	207	Decreased
A3	384697	Serum albumin precursor	47329.7	5.97	371	Increased
A4	745872	Serum albumin precursor	69321.5	5.92	107	Increased
A5	9028	Tetranectin	22552.3	5.52	307	Decreased
A6	514475	Apolipoprotein L1	43900.0	5.84	234	Increased

MW, molecular weight; and pI, isoelectric point.

wavelength laser and an accelerating voltage of 20,000 V. The matrix and peptides were selected in the mass range of 700-3,500 Da. Subsequent to MS analysis, the peptides that were different from the matrix peptide mass fingerprint were selected and the mass spectrum/mass spectrum was achieved.

**Database retrieval.** The mass spectrum obtained from the MALDI-TOF was assessed by searching the Mascot software from NCBI (www.matrixscience.com) with the following restrictions to the initial retrieval: Species, *Homo Sapiens*; mass range, 800-4,000 Da; isoelectric point (pI) range, 4-7; maximum tolerance error of peptide fragment,  $\pm 0.5$  Da; ion selection,  $(M+H)^+$  and monoisotopic; a minimum of 4 matched peptides; fixed modifications, carbamidomethyl; and variable modifications, oxidation.

**ELISA.** The levels of the aforementioned identified proteins (associated with LDH pathology) were measured in the blood serum samples using an ELISA kit (BMA Biomedicals AG, Augst, Switzerland) according to the manufacturer's instructions. The blood serum was obtained from another 10 patients with LDH and 10 healthy subjects.

## Results

**Analysis of the protein profiles.** The protein profiles from the sera of 30 patients with LDH and 30 healthy subjects were analyzed by 2-DE with silver-staining of proteins in the gel. Several protein spots were separated on 2-DE gels from the experimental and control groups, totaling  $950 \pm 50$  and  $920 \pm 50$  spots, respectively. The majority of protein spots were clustered between 10 and 120 kDa and between pI 4-7. The overall pattern of the protein expression in all 2-DE gels was extremely similar. The proteins that were up- or downregulated by  $>3$ -fold and appeared in all patients with LDH pathology were selected for identification by peptide mass fingerprinting, using MALDI-TOF-MS and protein database searching. The results from this identification were of high confidence if the protein had a significant score and high-sequence coverage. A total of 6 protein spots were identified as being associated with LDH pathology (Table I). The identities of these 6 spots were found to be upregulated apolipoprotein-L1 (APO-L1) and two serum albumin precursors, and downregulated

apolipoprotein M (APO-M), tetranectin (TN) and immunoglobulin light chain (IGL). The 6 proteins were found to be significantly and consistently different in the sera of patients with LDH compared to healthy subjects, according to 2-DE gel separation and proteomic analysis (Fig. 1).

The protein expression of APO-L1 was not detected in the sera of healthy subjects but was only found in patients with LDH pathology. In addition, the protein expression levels of APO-M, TN and IGL in the sera from patients with LDH were all downregulated by  $22 \pm 3$  ( $P < 0.01$ ),  $37 \pm 5$  ( $P < 0.01$ ) and  $27 \pm 3\%$  ( $P < 0.01$ ), respectively, compared to the sera from healthy subjects.

The ELISA results support the proteomic analysis results. The ELISA demonstrated that the mean serum concentrations of APO-M, TN and IGL were significantly lower in LDH patients compared to the healthy control group ( $P < 0.05$ ). By contrast, the plasma levels of APO-L1 in LDH patients were significantly higher than the values recorded for the healthy control group ( $P < 0.01$ ).

## Discussion

Proteomics is a powerful tool in the analysis of protein expression and composition of various tissues, as it provides useful information regarding biochemical pathways and biological processes and connects the relevant genes with protein expression. Proteomics primarily involves the application of 2-DE, MS and database retrieval systems (24). Due to its reproducibility, accuracy and high-throughput, proteomics has been extensively used in studies pertaining to tumors, inflammation and immunity, as well as other complex areas of study. Although proteomics is becoming increasingly available to investigators, the application of proteomics to LDH is only in the initial stages (21).

In the present study, proteomics was applied to identify specific biomarkers associated with the pathogenesis of LDH. A total of 6 proteins were found that were differentially expressed in the sera of 30 patients with LDH compared to 30 healthy subjects. Furthermore, 4 of these 6 proteins were statistically different in terms of their serum level between the experimental and control groups as determined by the ELISA measurements on the serum samples. These 4 proteins were APO-L1, TN, APO-M and IGL.

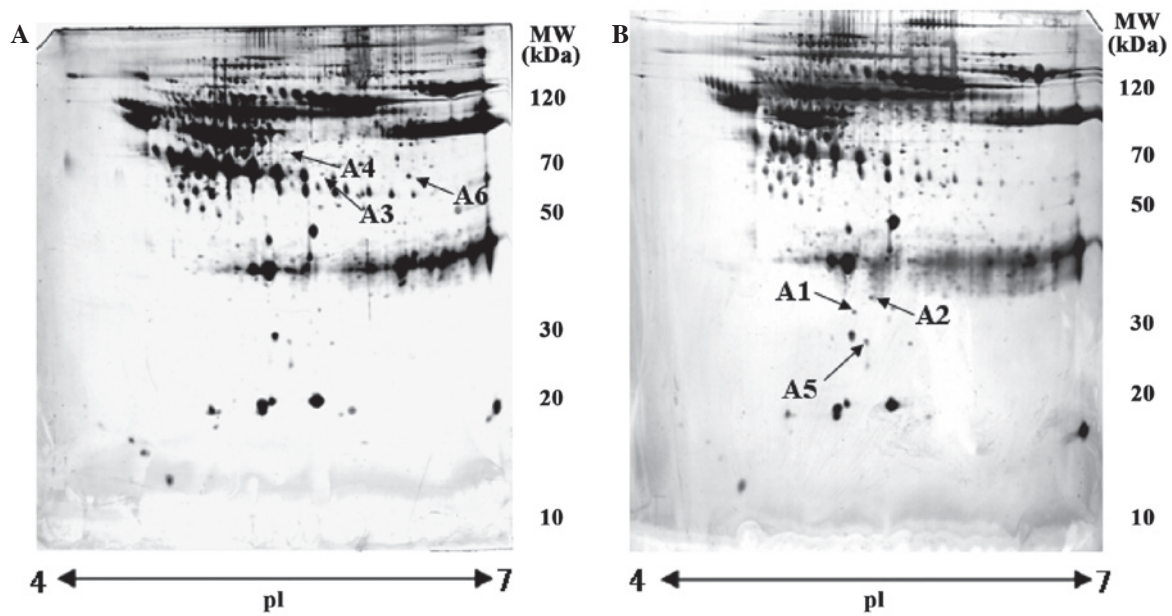


Figure 1. Human serum proteins separated on two-dimensional electrophoresis gels; (A) from a patient with LDH and (B) a healthy control. Total protein (300  $\mu\text{g}$ ) was loaded on the immobilized pH gradient strip (pH 4-7, non-linear) for isoelectric focusing and separated in the second dimension. Subsequently, the spots were silver-stained. The arrows indicate 6 distinctly expressed protein spots. LDH, lumbar intervertebral disc herniation.

Extremely little is known with regard to APO-L, a protein first characterized in 1997 as a new human high-density lipoprotein (25). APO-L was found only in the pancreas and is described as a 383-amino acid residue protein with no significant homology to any other known protein sequence. In healthy subjects, free APO-L is not detected in the plasma; instead, it binds to other proteins, mainly large APO-A1-containing lipoproteins. The cloning of CG12\_1 in 1999 suggested that other APO-L proteins may exist. APO-L is specifically expressed in endothelial cells lining healthy atherosclerotic iliac arteries and the aorta, and it is responsive to changes in the tumor necrosis factor (TNF)- $\alpha$  level (26). The study by Horrovoets *et al* (26) also identified and classified novel members of the APO-L protein family. The similarities of these proteins suggest that they have arisen through local gene duplication. Although they do not possess classical signal peptide sequences, evidence exists that at least APO-L1 is secreted in the plasma (25,27). It is believed that TNF- $\alpha$  plays a key role in the inflammation induced by LDH (28-30). However, Brisby *et al* (31) found that the concentration of TNF- $\alpha$  in the serum of LDH patients is not significantly different compared to healthy patients. Therefore, the level of APO-L1 may be a reflection of the TNF- $\alpha$  secreted into the serum and may serve as a biomarker for LDH. Additionally, APO-L1 is associated with the lysis of lysosomes and the appearance of an APO-L version with a signal peptide represents a novel component of innate immunity (32,33). Herniation of the intervertebral disc is associated with human autoimmunity. In addition, APO-L1 is a novel Bcl-2 homology domain 3-only protein, which when overexpressed and accumulated intracellularly, induces autophagic cell death in cells as characterized by the increasing formation of autophagic vacuoles (34). Thus, APO-L1 may be the signal peptide of LDH.

TN is a plasminogen-binding protein, that can be encountered in the plasma and in the extracellular matrix

(ECM) (35,36). In addition to its plasminogen-binding properties, TN can bind to heparin, APO-A, tissue plasminogen activator, hepatocyte growth factor and angiostatin (37-39). TN expression has been detected in various endocrine tissues, as well as in epithelial and mesenchymal cells, including fibroblasts, monocytes and neutrophils (40-42). TN also constitutes a crucial component of the ECM in osteogenesis, muscle development and regeneration, suggesting its important physiological role in tissue remodeling (43,44). Serum TN is decreased following trauma or acute myocardial infarction, during pregnancy and in patients with liver cirrhosis, rheumatoid arthritis or malignant tumors (37,45-47). Although the exact biological function of TN has yet to be established, certain evidence suggests that TN plays an important role in tissue remodeling. TN increases the tissue-type plasminogen activator-catalyzed activation of plasminogen in the presence of poly-d-lysine (35) and activated plasminogen is considered to play a key role in the degradation of the ECM. In this study, serum TN was also decreased. As the majority of the LDH cases are caused by degeneration of the lumbar disc, in the process of which apoptosis of the nucleus pulposus cell is first observed and is subsequently followed by the imbalance of lumbar disc remodeling. In this way, the serum TN level is reduced in patients with LDH, which is considered to be associated with regeneration of the lumbar disc, leading to a decrease in the synthesis and secretion of TN.

Human APO-M was found and initially isolated from chylomicrons in the 1999 study by Xu and Dahlbäck (48). Xu *et al* (49) have reported that transforming growth factor- $\beta$  can also downregulate APO-M expression and secretion in HepG2 cells. These data indicate that APO-M may be associated with the host defense response as the APO-M gene is located in the histocompatibility complex III region on chromosome 6 (50). A number of genes in this region are associated with the immune responses and the APO-M

gene is extremely close to the TNF- $\alpha$  gene and lymphotoxin genes (50). Therefore, APO-M may also be associated with the immune system, or regulated by cytokines or other inflammatory factors. The detailed association between APO-M and LDH remains unclear and requires further investigation. The function of IGL in LDH patients also remains unclear. Thus, it can be used clinically for screening or monitoring LDH requires further studies.

Previously, no direct association between the pathogenesis of LDH and the 4 proteins that were differentially expressed in the LDH group has been found. To the best of our knowledge, the present study is the first to report on this association and is unique as the serum molecular analysis of LDH was accomplished using a proteomics approach. Thus, proteomic analysis is an efficient technique for identifying the diagnostic biomarkers of LDH. In conclusion, the 4 proteins that were differentially expressed in LDH patients compared to healthy subjects were recognized. These findings contribute to the advancement of knowledge in this area of study.

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