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Original Article

Proteomic profiling of posterior longitudinal ligament of cervical spine

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Abstract: Objective: To identify putative biomarkers for ossification of posterior longitudinal ligament (OPLL). Material and methods: Proteomic analysis was performed in 4 ligament samples from OPLL patients and healthy controls. RT-PCR was used to further verify the proteomic analysis results. Results: A total of 50 differentially expressed spots were detected in 2-D electrophoresis between the two groups. In protein/peptide analysis, 21 proteins or peptides were finally identified. Besides 13 hematic proteins and 2 unknown proteins, 6 other proteins were differentially expressed. Among them, carbonic anhydrase I, NAD(P) dependent steroid dehydrogenase-like, billiverdin reductase B and alpha-1 collagen VI were down-regulated, while osteoglycin and nebulin-related anchoring protein were up-regulated. The results of NAD(P) dependent steroid dehydrogenase-like, alpha-1 collagen VI and nebulin-related anchoring protein were validated by RT-PCR. Conclusion: These differentially expressed proteins could play a role in the onset and progression of OPLL.

Keywords: Ossification of posterior longitudinal ligament, 2-dimensionl electrophoresis, diagnosis, biomarker

Introduction

Posterior longitudinal ligament lies behind the posterior surface of spinal vertebral bodies and from the foramen magnum to the sacrum. Ossification of posterior longitudinal ligament (OPLL) is a pathological condition that causes ectopic bone formation, which occurs frequently in cervical part. OPLL initially forms a hyperplastic mass in the ligament. This mass continually grows and finally compresses the spinal cord. It is called myelopathy. The incidence of this disorder is 0.4-3% in Asian population and 0.1-1.7% in whites [1]. OPLL has been recognized as a disease with unknown etiology for a long time. Multiple factors were hypothesized to be related to the pathological process, such as genetic background, hereditary transmission, hormonal abnormality, abnormal calcium metabolism, and an association with diabetes mellitus [2-5]. The diagnosis methods like computed tomography (CT) make OPLL more frequently to be identified, and also make it possible for surgical treatment [6]. For seeking more convenient and economic diagnosis of OPLL, serological biomarkers have been investigated [7-10].

Proteomics is a method studying a large-scale of proteins. This method was used to identify novel biomarkers in pathological tissues and biological fluids [11]. Like many other diseases processes, OPLL is also considered to be associated with specific changes in quantity and function of proteins. Proteomics thereby is a helpful technique in discovering biomarkers in OPLL. Proteomic analysis was performed using serum samples from OPLL patients [10]. However, a common shortcoming of serum proteomic analysis is that the most significantly altered proteins are albumin and hemoglobin, two abundant proteins in serum. These two proteins severely interfere in the ability of this method in separating less abundant serum proteins. To avoid the interference of highly abundant proteins, a novel way is introduced to dis-

cover biomarkers in pathological tissues, which contain less protein diversity. In the present study, to assess potential biomarkers and pathogenic proteins of OPLL, we analyzed proteins from ossified ligaments using two-dimensional difference gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) peptide sequencing.

Materials and methods

Ethics statement

This study was approved by the Institutional Review Boards of Changzheng Hospital and Xinhua Hospital. Written informed consent was obtained from all involved patients.

Ligament samples acquisition

Cervical posterior longitudinal ligaments from OPLL patients were collected during surgeries. Control samples were acquired from age- and sex-matched trauma patients without OPLL who received anterior cervical surgery. OPLL was diagnosed by CT and MRI. The ligaments were washed in saline three times and maintained at -80°C until use.

Ligament sample preparation

Ligaments were pulverized, and the frozen ligament powder was homogenized by ultrasonication in a lysis solution containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and a protease inhibitor cocktail (Complete Mini plus EDTA, Roche, Basel, Switzerland) at 4°C. Samples were centrifuged at 15,000 g for 45 min. The supernatant was collected, and protein concentrations were determined with spectrophotometric (Bradford) method. Bovine gamma globulin was used as a standard. Samples were stored at -80°C until use.

Minimal labeling of ligament proteins with CyDyes

A total of 50 μg protein from each sample was labeled with CyDye (CyDye Minimal Labeling Kit, GE Healthcare Piscataway, NJ). For paired samples, one sample was randomly selected and labeled with Cy3 dye. The other was labeled with Cy5 dye. The dye amount was 400 pg per 50 μg protein. Another 50 μg protein from each ligament was mixed together and labeled with Cy2 minimal dye to serve as internal standard. The labeled paired samples and 50 μg of internal standard were mixed. Then, an equal volume of rehydration buffer (8 M urea, 2% CHAPS, 18 mM DTT, 0.5% Pharmalyte, pH 3-10) was added. One micrograms of mixed sample was loaded onto an Immobiline strip (13 cm, pH 3-10, GE Healthcare Piscataway, NJ) after 12 h of rehydration.

2-D electrophoresis (2-DE) and DeCyde analysis

The first dimension electrophoretic separation was performed with an IPGphor apparatus (GE Healthcare Piscataway, NJ). The isoelectrofocusing was performed at constant temperature of 20°C and run at 30 V for 12 h, 550 V for 1 h, 1000 V for 1 h, 8000 V for 8 h, and then 500 V for 4 h. Before the second dimension electrophoretic separation, separation strips were incubated in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 100 mM DTT, and 0.01% bromophenol blue) for 15 min. For protein alkylation, strips were incubated in equilibration solution containing 100 mM iodoacetamide for another 15 min. The strips were then loaded onto the top of 12.5% polyacrylamide gels and sealed with 0.5% agarose. The second dimension electrophoretic separation was performed with an Ettan Daltsix Electrophoresis System (GE Healthcare Piscataway, NJ) at 20°C. Run was set at constant 15 mA per gel for the initial 30 min and then 30 mA per gel until bromophenol blue reached the bottom of the gel. Signals were detected at excitation wavelengths of 488, 532, and 633 nm for Cy2-, Cy3-, and Cy5-labeled samples respectively, using a Typhoon 9410 Variable Mode Imager (GE Healthcare Piscataway, NJ). Gels were scanned with a pixel size of 100 and analyzed using the Decyder 2-D 6.5 software (GE Healthcare Piscataway, NJ). Fluorescence intensity was normalized to the amount of protein and presented as an average ratio of each protein. Here, the ratio was equal to the amount of protein from OPLL samples divided by that from control samples.

Non-labeled sample (1 mg) was loaded on a preparative gel, separated simultaneously, and visualized by Coomassie brilliant blur staining. Spots selected for protein identification after Decyder analysis were matched and picked by hand from the preparation gel.

Table 1. Characteristics of OPLL patients

	Usage	Age	Sex	Diagnosis	Family history of OPLL
1	2D-DIGE	73	Male	OPLL (C3-7), mixed	No
2		58	Female	OPLL (C3-6), mixed	No
3		53	Male	OPLL (C4-6), continuous	No
4		48	Male	OPLL (C5-7), segmental	Yes
5		72	Male	C5 fracture	No
6		56	Female	C4-5 dislocation	No
7		54	Male	C4, C5 fracture	No
8		44	Male	C4/5, 5/6 traumatic disc herniation	No
9	RT-PCR	58	Male	OPLL (C5-7), continuous	No
10		42	Male	OPLL (C3-5), segmental	No
11		46	Male	OPLL (C2-3, C5-7), mixed	No
12		63	Female	OPLL (C6), segmental	No
13		62	Female	OPLL (C3-5), mixed	No
14		59	Female	C5, C6 fracture	No
15		67	Male	C5 fracture	No
16		60	Male	C6 fracture	No
17		53	Male	C5-6 dislocation	No
18		45	Male	C3-4 dislocation	No

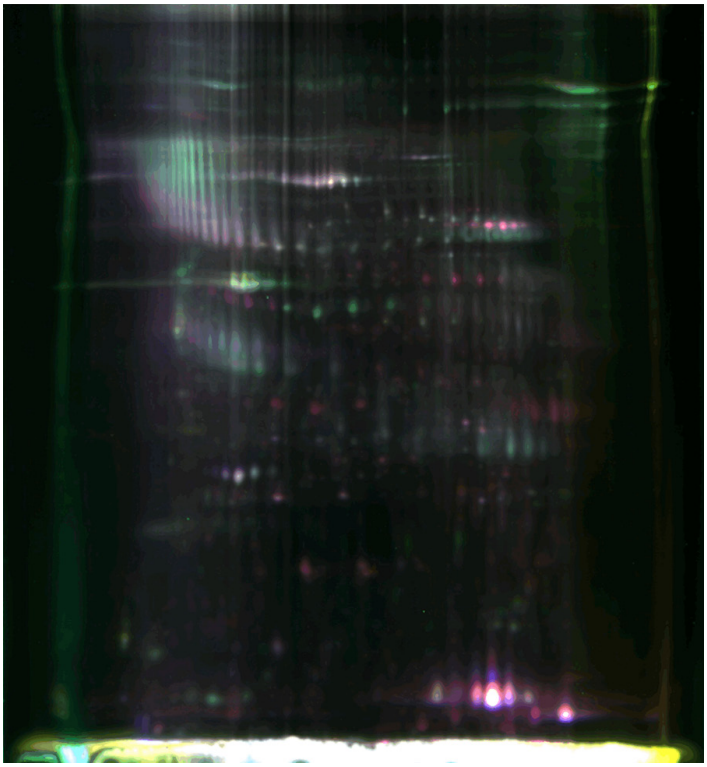

Figure 1. Representative gel from 2D-DIGE experiments.

In-gel digestion

Gel pieces excised from preparation gels were washed with 400 μL 30% ACN containing 40 mM NH_4HCO_3. Each gel piece was dried, added 1.5 mM NH_4HCO_3 containing 50 ng trypsin (BIO-RAD), and incubated at 37°C. Peptides were extracted with 60% acetonitrile and 0.1% trifluoroacetic acid (TFA), desalted using Zip Tips (Millipore, Billerica, USA) and dried.

Mass-spectrometry and protein identification

Dried samples were analyzed with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS, Applied Biosystems, Foster City, CA) for peptide mass fingerprinting. The spectra obtained were analyzed with the Sequest Search Engine (Bioworks 3.2 software, Thermo Finnigan, San Jose, CA).

RT-PCR

Total RNAs of ligaments were extracted using total RNA kits (Fermentas, Thermo Fisher Scientific, Pittsburgh, PA, USA). First-strand cDNA was synthesized using oligo (dT) 15 primers and M-MuLV reverse transcriptase. The resultant cDNAs were amplified with PCR using spe-

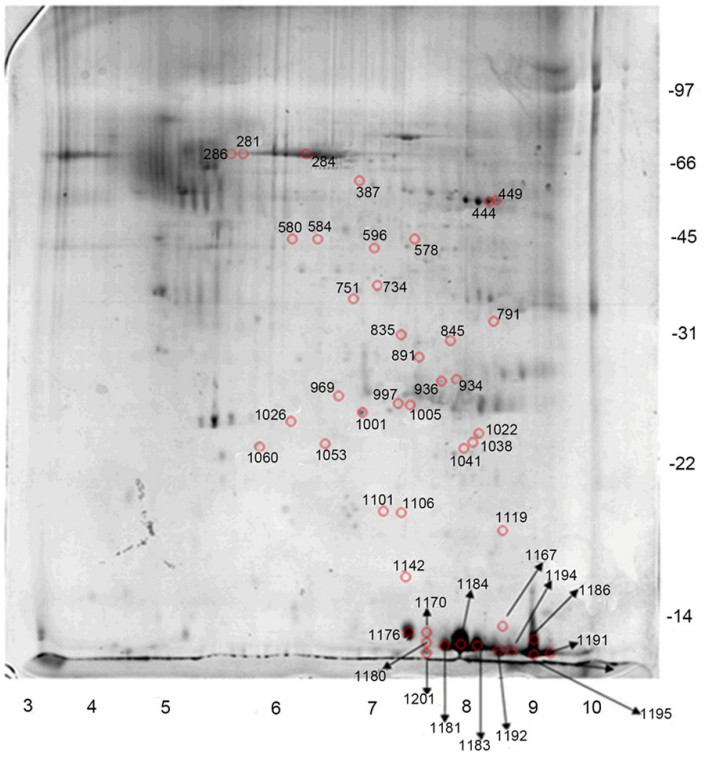

Figure 2. Selected spots from a gel.

cific PCR primers. PCR products were separated on 2% agarose gels, stained by ethidium bromide, and visualized under UV light. The optical density of DNA bands were measured by the TotalLab software (version 2.01). All chemicals were purchased from Invitrogen (Grand Island, NY, USA).

Statistics

The results are presented as means ± SEM. The data were evaluated using Student's t-test. Probability levels of < 0.05 were considered statistically significant.

Results

Ligament sample processing for proteomic analysis

Protein concentration was 1.39-7.01 μg/μl in 46 collected ligament samples. Of them, the majority of concentration was 3.36-5.62 μg/μl in 35 samples. To secure sufficient amount of protein for the large format 2-D DIGE gel and minimal labeling, 100 μg of protein from each sample was necessary. Totally, 5 OPLL samples and 8 control samples reached this requirement. To minimize individual variations, four samples per group from age- and sex-matched subjects were selected (**Table 1**).

2-D DIGE with minimal labeling of ligaments

Using 2-D DIGE with a minimal labeling proteomic platform, we compared protein patterns between 4 pairs of ligament samples from OPLL and control subjects. Approximate 1100 protein spots were observed in each sample (**Figure 1**). Proteins with an average ratio > 1.5 were selected for identification. Fifty differentially expressed protein spots were detected (**Figure 2**). Forty-five protein spots were excised from the preparative gel and were further subjected to in-gel trypsin digestion and protein identification by MALDI-MS. Finally, 21 proteins were identified from 32 (**Table 2**). Among them, the expression of 3 proteins was up-regulated and others were down-regulated in OPLL samples as compared to control samples.

Differentially expressed ligament proteins

Functions of the 21 differentially expressed proteins were summarized in **Figure 3**. Thirteen were hematic proteins, including hemoglobin and albumin, and 2 were unknown proteins. The other 6 proteins were nebulin-related anchoring protein (N-RAP), osteoglycin (OGN), biliverdin reductase B (BVRB), the complex between carbonic anhydrase I and the phosphonate antiviral drug foscarnet (CA1), NAD (P) de pendent steroid dehydrogenase-like (NSDHL), and collagen VI alpha-1 (VIα1). N-RAP isoform S and OGN were up-regulated. All other proteins were down-regulated.

Verification of proteomic analysis

Total RNA was extracted from 10 ligament samples (**Table 1**) for RT-PCR to examine mRNA expression of the 6 differentially expressed proteins. mRNA expression of N-RAP was up-regulated in OPLL pathological tissues, while

Table 2. Protein identification summary

		spots	protein	NCBI
1	Up-regulated	891,934	OGN	33150528
2		1005	Ig kappa chain NIG26 precursor-human	7438711
3		578	nebulin-related anchoring protein isoform S	29887963
4	Down-regulated	1191,1194	Chain A, Structure Of Hemoglobin In The Deoxy Quaternary State With Ligand Bound At The Alpha Hems	229751
5		449	fibrinogen beta chain, isoform CRA_d	119625338
6		284,286	Chain A, Crystal Structure Of The Ga Module Complexed With Human Serum Albumin	55669910
7		580	ALB protein	27692693
8		751	HP protein	47124562
9		835	alpha-1 collagen VI (AA 574-1009)	30030
10		387	NAD (P) dependent steroid dehydrogenase-like, isoform CRA_a	119593303
11		936	Chain A, X Ray Structure Of The Complex Between Carbonic Anhydrase I And The Phosphonate Antiviral Drug Foscarnet	158428858
12		997,1176	Chain A, Solution Structure Of Domain 3 From Human Serum Albumin Complexed To An Anti-Apoptotic Ligand Directed Against Bcl- Xl And Bcl-2	71042087
13		1001,1026	albumin, isoform CRA_j	119626073
14		444	fibrin beta	223002
15		1022	billiverdin reductase B (flavin reductase (NADPH))	4502419
16		1060,1186,1195	Chain A, T-To-Thigh Quaternary Transitions In Human Hemoglobin: Desarg141alpha Deoxy Low-Salt	56967331
17		1101	hypothetical protein	5262611
18		1170,1180,1181	Chain B, Oxygen Affinity Modulation By The N-Termini Of The Beta Chains In Human And Bovine Hemoglobin	999565
19		1183,1184,1201	Chain B, T-To-T (High) Quaternary Transitions In Human Hemoglobin: Deshis146beta Deoxy Low-Salt	61679604
20		281	unnamed protein product	28590
21		1192	mutant beta globin	13432059

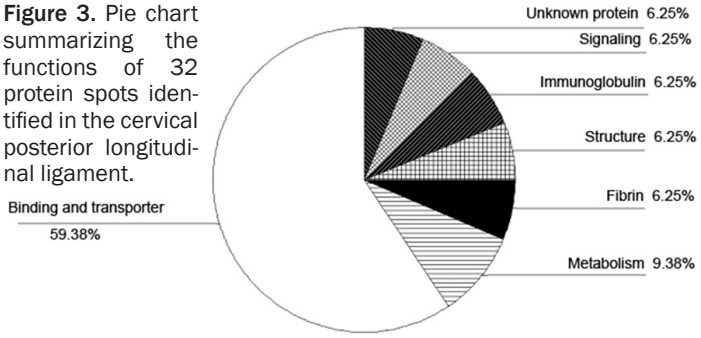

Figure 3. Pie chart summarizing the functions of 32 protein spots identified in the cervical posterior longitudinal ligament.

mRNA expressions of NSDHL and collagen VIα1 were down-regulated. This is consistent with the 2D-DIGE results. mRNA expressions of OGN, CA1 and BVRB was not statistically different between OPLL and control samples (**Table 3**).

Discussion

Two-dimensional electrophoresis of ligament tissues

Approximate 1100 protein spots were observed in all 2-D gel images, which demonstrates that our protocol is stable, high sensitive and reproducible. A primary protein map from the posterior longitudinal ligament was created and can be used as a reference in future study. The results prove that the methods of protein extraction and gel separation used in this study were feasible. However, the conditions remain less than ideal. In preliminary experiments, improvement of the conditions reduced image background noise but failed to remove vertical stripes, which may be caused by thiourea [12].

Differentially expressed ligament proteins

Mass spectrometry identified 21 proteins or peptides that were differentially expressed in pathological ligaments from OPLL patients as compared to control samples. Among these prteins/peptides, high-abundance blood proteins including hemoglobin and albumin, accounted for a large proportion. This is possibly caused by blood contamination. The vertebral artery and vein, especially the anterior internal vertebral plexus, runs through the posterior longitudinal ligament [13]. This anatomical characteristic results in inevitable blood contamination, even though the ligaments were cut into pieces and completely washed in saline. Total plasma protein concentration is 60-80 mg/ml. It is 100 times higher than that in cerebrospinal fluid (CSF) [14-16]. Such a great gap of concentrations between plasma and CSF significantly reduces the reliability of DIGE experiments. As the high sensitivity of DIGE, small changes in low abundant proteins can be detected while the normal fluctuation of highly abundant proteins was also considered as differential regulation. The most abundant proteins in serum included hemoglobin, albumin, IgG and IgA. This sample characteristic may explain why traces of hemoglobin, albumin and immunoglobulins were detected (**Table 2**).

Sequential extraction or affinity purification is usually used to remove highly abundant proteins. But, these methods can produce a loss of sample protein. In the present study, ligament specimens were only approximately 0.5 cm^3 in size and the protein concentrations was 3-4 mg/ml. For such a small amount of protein, purification procedures-produced proteins loss may finally lead to the loss of trace proteins. Based on the above considerations, we did not perform additional studies aiming at albumin, hemoglobin, and immunoglobulin.

Identification of protein features

N-RAP, a 185-kDa actin-binding LIM protein, was recently discovered in murine skeletal and cardiac muscle tissues [17]. N-RAP serves as a link between myofibril terminal actin and cell membrane protein complexes and thus serves as an organizing center in the initial phase of myofibril assembly [18]. N-RAP is also found in adult Human muscle, heart and brain tissues, and plays a crucial role in myofibrillogenesis [19]. Our study, at the first time, reported detectable N-RAP in human ligaments.

OGN is a small proteoglycan that contains tandem leucine-rich repeats. OGN is a key regulator of the left ventricular mass in rats, mice and humans, and modifies the hypertrophic response to extrinsic factors, such as hypertension and aortic stenosis [20]. OGN regulates type I collagen fibrillogenesis, and this ability is potentiated by BMP-1 [21].

Table 3. Relative quantity* of differentially expressed proteins

	OPLL samples	Control samples	*P* value
NSDHL	33.22259	63.69427	0.049
VIα1	3.676471	10.55966	0.024
N-RAP	161.2903	14.28571	0.041
OGN	5.428882	10.92896	0.375
CA1	5.060729	12.43781	0.569
BVRB	0.663482	0.367769	0.107

*Relative Quantity = Quantity of Sample/Quantity of Internal Reference. NSDHL: NAD (P) dependent steroid dehydrogenase-like; VIα1: collagen VI alpha-1; N-RAP: nebulin-related anchoring protein; OGN: osteoglycin; CA1: the complex between carbonic anhydrase I and the phosphonate antiviral drug foscarnet; BVRB: billiverdin reductase B.

Billiverdin reductase B (BLVRB) is an enzyme response for converting billiverdin to bilirubin in adults. Recently, BLVR has been recognized as a regulator of glucose metabolism, cell growth, and apoptosis due to its dual-specificity kinase characteristics [22].

Carbonic anhydrase (CA) exists in various cells and catalyzes the conversion of carbon dioxide and water to bicarbonate and protons. CAI has been speculated as a good biomarker for diabetes mellitus because its activity variations are proportional to diabetes severity [23]. Extracellular CAI was reported to induce microenvironment alkalinization, increase kallikrein activity, promote factor XIIa production, and broaden the relevance to neurovascular edema [24].

NAD (P)-dependent steroid dehydrogenase-like (NSDHL), also known as 3-beta-hydroxysteroid dehydrogenase, is an enzyme involving cholesterol synthesis. Mutations in the X-linked NSDHL gene caused CHILD syndrome in human.

Collagen VI is an extracellular matrix protein and possesses a triple-helical domain as a common structural element. Collagen VI regulates normal and transformed mesenchymal cell proliferation *in vitro*, induces tyrosine phosphorylation of paxillin and focal adhesion kinase, and activates MAP kinase ERK2 in fibroblasts [25, 26]. Collagen VIα-1 is the alpha 1 subunit of type VI collagen (alpha 1 [VI] chain), and its single-nucleotide polymorphisms of encoded gene were reported to be strongly associated with OPLL [27, 28].

Verification of differentially expressed proteins

mRNA expressions of N-RAP, NSDHL and collagen VI were consistent with their protein expressions, which further confirmed the role of these proteins in the pathological process of OPLL. Among the three proteins, collagen VI may be more promising for further study, as it is strongly associated with OPLL. Our findings at the first time demonstrate that collagen VI is differentially expressed between OPLL patients and healthy controls in protein and mRNA expression. No statistical differences were observed in mRNA expression of OGN, CA1 and BVRB between OPLL and control samples, which conflicts with the DIGE results. This result may be due to a small sample size or the experimental error of DIGE. Further verification with other independent methods may solve this question.

Conclusion

We performed proteomics to identify putative OPLL biomarkers in ligament tissues. Methods developed in this study include sample preparation, proteomic profiling, and protein identification. Using these methods, we conclude that 6 proteins, alone or in combination, are putative disease biomarkers. Among them, three proteins were further confirmed by RT-PCR. The relatively small number of samples with sufficient protein amount is a significant limitation for 2-DE combined with MALDI-MS/MS. Increasing sample size and cross validating these biomarkers with biological and immune parameters are necessary in further study.

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Disclosure of conflict of interest

None.

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