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RESEARCH ARTICLE

iTRAQ-based proteomics reveals novel biomarkers of osteoarthritis

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Abstract

Objective: We performed comprehensive proteomic analyses of articular cartilage by using the isobaric tags for relative and absolute quantitation (iTRAQ) method, and searched for candidate biomarkers for osteoarthritis (OA).
Methods: Articular cartilage was collected from patients with OA or femoral neck fracture for the control group. Molecular variations were detected by the iTRAQ method, and quantitative analyses were performed by western blot.
Results: Using the iTRAQ method, we identified 76 proteins with different expression levels in OA patients and the control group. Among these proteins, we selected LECT2 (leukocyte cell-derived chemotaxin-2), BAALC (brain and acute leukemia, cytoplasmic), and PRDX6 (peroxiredoxin-6), which had not been reported as biomarkers for OA.
Conclusions: Use of these proteins in combination with conventional OA biomarkers may better reflect the grade and prognosis of OA.

Keywords

BAALC, iTRAQ, LECT2, osteoarthritis, PRDX6, proteomics

History

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Introduction

Because patients with osteoarthritis (OA) outnumber those with rheumatoid arthritis (RA) or even osteoporosis, there is a need to identify molecular markers and develop new treatments against this disease. The primary pathology of OA is degeneration of the articular cartilage matrix. Recent epidemiological investigations have revealed that acetabular dysplasia is the most important factor involved in OA onset in the hip joint (Jingushi et al., 2011). Obesity has also been identified as closely associated with OA in knee and hip joints. Such mechanical stresses cause abnormalities in the metabolism of articular cartilage, leading to degeneration of the cartilage matrix. Although this degeneration has been attributed to various possible mediators, including proteases, hypoxia inducible factor-2α (Hirata et al., 2012; Saito et al., 2010; Saito & Kawaguchi, 2010), reactive oxygen species (ROS) (Xie et al., 2012), and the complement system (Wang et al., 2011a), the precise molecular pathogenic mechanism of OA remains unknown. Accordingly, current treatment practices are limited to surgical procedures incorporating prosthetic joint replacement with medical therapies limited to symptom control (Yamada et al., 2009). Curative non-surgical treatments remain to be established. OA is associated with a particularly high morbidity rate among the elderly. Because surgical procedures can be major physical challenges for the elderly owing to complications from cardiovascular disease and diabetes, the demand for the development of medical therapies for OA is also crucial from the perspective of health economics.

Biomarkers within the blood and urine can reflect the status and possible future progression of a disease. As indicators of normal biological and pathological processes and as pharmacological indicators of therapeutic interventions, biomarkers allow measurements and evaluations to be made objectively (Gharbi et al., 2011). Currently, biomarkers are utilized clinically in the diagnosis of musculoskeletal diseases such as RA and osteoporosis. Moreover, biomarkers are used to monitor disease progression, make prognoses and evaluate the effectiveness of new drug treatments. Measurements of biomarkers in bodily fluids such as blood or urine are a simple means of evaluating a disease condition. In recent years, there has been a high demand for exploration of biomarkers for a variety of diseases (Kraus, 2011).

Recently, an increasing number of studies have investigated the diagnosis and prognosis of OA by measuring biomarkers present in the blood serum, urine, or synovial fluid of OA patients (Kraus, 2011). Biomarkers allow diagnosis of OA in early stages when macroscopic changes are few and difficult to detect, which enables highly objective diagnoses, compared to diagnoses using radiography, and provides quantitative evaluation of disease severity. Any protein with a differential expression between regular healthy cartilage and OA cartilage is a candidate diagnostic marker of OA. Cartilage oligomeric matrix protein (COMP) has been studied as a diagnostic marker, because it is a cartilage matrix component that is released into the blood and urine during cartilage degeneration (Kato et al., 2005). Fragments of C-terminal cross-linked telopeptide type II collagen (CTX-II), type II procollagen carboxy-propeptide (CPII), type II

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collagen-related neoepitope (C2C) and hyaluronic acid (HA) are examples of other possible candidates of diagnostic markers for OA. CTX-II and C2C are products of sequential degradation by several proteases originating from the cartilage matrix, including matrix metalloproteinase (MMP)-1, MMP-8 and MMP-13 (Conrozier et al., 2012).

Proteomic analysis is a research method to catalog all proteins inside cells and organisms. This method can elucidate protein structure and function as well as protein interactions inside the cell. In the general method of proteomics, samples are electrophoresed and the differential proteins are picked up as spots. After trypsin digestion, proteins are analyzed by mass spectrometry (Chambers et al., 2000). Proteins that are not included in spot areas cannot be detected. Furthermore, detected proteins with a low abundance cannot be used in quantitative analysis by conventional methods.

The isobaric tags for the relative and absolute quantitation (iTRAQ) method allow a more comprehensive analysis. Whole samples undergo trypsin digestion and then labeling with an iTRAQ reagent for analysis by mass spectrometry based on shotgun proteomics (Aggarwal et al., 2006; Ross et al., 2004). More than two digested samples can be labeled with separate reagents to perform quantitative analysis. This method has a high sensitivity and it is possible to detect low-abundance protein.

General proteomic analysis frequently identifies abundant proteins (ribosomal, proteasomal and cytoskeletal proteins as well as molecular chaperons) as biomarkers. However, it is well known that rare proteins such as secretory proteins and kinases are also important for various biological functions. In this study, among the lysates eluted through a random peptide column, we detected molecular variations in low-abundance proteins that were difficult to identify by conventional methods.

Proteomic analysis of serum from OA patients has revealed increased levels of the C-terminal end product of the V65 vitronectin subunit, C3f peptide, and connective tissue-activating peptide III (CTAPIII) (de Seny et al., 2011), as well as HPT (Fernandez-Costa et al., 2012). COMP, apolipoprotein, haptoglobin precursor, calgranulins, defensins and thymosins have been detected in synovial tissue (Kong, 2012; Kriegsmann et al., 2012). Furthermore, OA cartilages have been analyzed by the MALDI-based imaging technique. As a result, OA-related peptides and proteins were detected in deep cartilage (Cillero-Pastor et al., 2013). In previous studies, serum biomarkers have been identified by iTRAQ in OA patients (Fernandez-Puente et al., 2011; Song et al., 2008). In comprehensive proteomic analyses, because it is possible that diseases other than OA in patients can affect components in serum and urine, we used cartilage as samples in the current study. This is the first report of iTRAQ methods to assess OA and control cartilages to find new molecular markers for OA.

Materials and methods

This study was approved by the steering committee and conducted under the guidelines for clinical studies of Fujita Health University. Human cartilage from OA patients and control subjects was obtained with informed consent.

Patients and articular cartilage

Control samples were obtained from 6 patients with femoral neck fracture (4 male and 2 female patients). The samples were collected from femoral head cartilage during bipolar femoral head replacement at Fujita Health University Hospital. OA cartilage samples were obtained from 17 patients with OA (5 male and 12 female patients). These samples were collected from the femoral heads, femoral condyles, and tibial plateaus of patients with primary OA, who underwent total hip or knee replacement surgery at Fujita Health University Hospital (Table 2).

Protein preparation

Samples were snap frozen at $-80\,^{\circ}C$ and cut into 10 μm sections by using a cryo-microtome (CM1900; Leica, Grove, IL). The sectioned samples were homogenized by sonication (T10 basic Disperser/Homogenizer; IKA) in RIPA buffer (10 mL of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS/g cartilage). Then, the samples were centrifuged at $20\,000 \times g$ at $4\,^{\circ}C$ for 10 min, and the supernatant was used for analysis. Amicon Ultra Centrifuge Filters 10 K (Millipore, Billerica, MA) were used to concentrate proteins by centrifugation at $5000 \times g$. Concentrated samples were stored at $-80\,^{\circ}C$ until use. A random peptide column (Proteominer; BIO-RAD, Hercules, CA) was used to prepare samples for iTRAQ. High-abundance proteins in these samples were excluded, while rare proteins were collected. Eluted samples were concentrated by Amicon Ultra Centrifuge Filters 10 K by centrifugation at $14\,000 \times g$.

iTRAQ method

The protein samples were buffer exchanged and concentrated using 4-mL spin concentrators with a 5-kDa molecular weight cut-off according to supplied protocols (Agilent Technology, Santa Clara, CA). Briefly, the samples were centrifuged at $2600 \times g$ for 20–30 min at $4\,^{\circ}C$. The protein samples were then buffer exchanged in the appropriate buffer containing 0.5 M triethylammonium bicarbonate for further analysis. The protein concentration of each sample was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL). From each sample, 100 μg of protein was reduced, alkylated, and digested prior to labeling with iTRAQ reagent according to the manufacturer's instructions (AB Sciex, Framingham, MA). Digested proteins prepared from each sample were labeled with iTRAQ reagent, and then pooled and washed according to the manufacturer's instructions (AB Sciex, Framingham, MA). Peptides were subjected to a trap and reverse-phase (RP) analytical column by using a gradient of 0–50% solvent B in solvent A over 140 min (solvent A: 0.1% trifluoroacetic acid [TFA] and 2% acetonitrile; solvent B: 0.1% TFA and 70% acetonitrile) and 50–100% solvent B for 5 min at a flow rate of 300 nL/min. The RP analytical column eluent was spotted onto a MALDI sample plate by using a DiNa Direct Nano-flow LC/MALDI system (KYA Tech, Tokyo, Japan) and analyzed by a 4800 mass spectrometer (AB Sciex, Framingham, MA). Relative protein abundance was determined using MS/MS scans of the iTRAQ-labeled

peptides. iTRAQ-labeled peptides were fragmented under collision-induced dissociation conditions to generate fragment ions that provided sequence information for the peptide and reporter ions. Thus, the identity of the protein from the analyzed peptide was confirmed, and the ratios of the peak areas of iTRAQ reporter ions were used to compare the relative abundance of the protein identified in the sample. ProteinPilot v4.0 (AB Sciex, Framingham, MA) was used for data analysis using NCBI and SWISS-PROT databases.

Western blot analysis

Protein samples (35 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 10% goat serum for 1 h at room temperature. Then, the membranes were probed with anti-LECT2 (ab89835, 1/250 dilution; Abcam), or anti-BAALC (H00079870-M01, 1/100 dilution; Abnova) antibodies for 1 h at room temperature, followed by incubation with a horseradish peroxidase-conjugated secondary antibody, and then chemiluminescence detection by ECL plus. Captured images were analyzed using a LAS4000 (Fuji Film) and Multi Gauge v2.0 (Fuji Film). For the anti-PRDX6 (MAB3490, 1/250 dilution; R&D SYSTEMS) antibody, the membrane was blocked in 5% skim milk. An XL-SAP Kit (APRO Science) was used for secondary antibody reactions. As internal controls, 35 μg protein samples (control: $n = 5$; OA: $n = 16$) were separated by SDS-PAGE, and then, SYPRO ruby protein gel staining (Invitrogen, Carlsbad, CA) was performed according to the manufacturer's instructions. Gel images were taken by a Typhoon scanner (GE Healthcare, Tyrone, PA). Total intensity measurements of 10–200 kDa proteins were analyzed by image analysis software (ImageJ, Wayne Rasband (NIH), DC). For quantification of western blot data, each protein signal was quantitated and normalized by the total protein intensity.

Kellgren–Lawrence grade (Kellgren & Lawrence, 1957)

Grade 0: normal
Grade 1: doubtful narrowing of the joint space and possible osteophytic lipping
Grade 2: definite osteophytes and definite narrowing of the joint space
Grade 3: moderate multiple osteophytes, definite narrowing of the joint space, and some sclerosis and possible deformity of the bone contour
Grade 4: large osteophytes, marked narrowing of the joint space, and severe sclerosis and definite deformity of the bone contour.

Results

For the iTRAQ method, a control sample was obtained from a patient with femoral neck fracture (female, 46 years old). An OA sample was also obtained from a patient with hip OA (female, 59 years old). These samples were collected during arthroplastic surgeries at Fujita Health University Hospital.

Before applying the iTRAQ method, we used random peptide columns (BIO-RAD, Hercules, CA, Proteominer) to concentrate rare proteins in extracts from control and OA cartilages. Eluted proteins from random peptide columns were then analyzed by iTRAQ (Figure 1A).

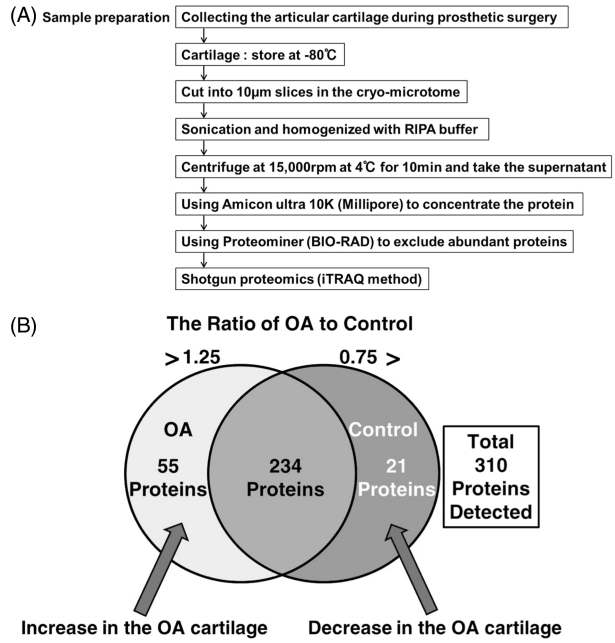

Figure 1. Result of shotgun proteomics based on iTRAQ method. (A) Experimental scheme. (B) Venn diagram showing the ratio of proteins that were included in OA and control cartilages based on the results of iTRAQ method. The diagram defines increased proteins for which OA/control ratio was more than 1.25 and decreased proteins for which the OA/control ratio was less than 0.75.

We detected a total of 310 proteins (Table 1), of which 55 proteins showed increased levels in OA cartilage and 21 proteins showed decreased levels in OA cartilage compared with the levels in the control group (Figure 1B). We selected 3 proteins (LECT2, BAALC and PRDX6) from the 76 proteins with variable levels, which had not been previously reported as biomarkers for OA and could be detected by western blotting with available antibodies. We next performed statistical analysis of western blot data of these proteins. Control samples were obtained from 5 patients with femoral neck fracture (4 male and 1 female patients). OA samples were obtained from 16 patients (5 male and 11 female patients) (Table 2).

We found that the expression levels of LECT2 (leukocyte cell-derived chemotaxin-2) were significantly higher in OA cartilage (LECT2: control, $n = 5$; OA, $n = 16$; $p = 0.005$). BAALC (brain and acute leukemia, cytoplasmic) expression was increased in OA cartilage compared with that in control cartilage (BAALC: control, $n = 5$; OA, $n = 16$; $p = 0.051$). PRDX6 (peroxiredoxin-6) expression was significantly reduced in OA cartilage compared with that in controls (PRDX6: control, $n = 5$; OA, $n = 16$; $p = 0.011$) (Figure 2A and B).

We next investigated whether the expression levels of LECT2, BAALC and PRDX6 proteins in OA patients were associated with the trends in each parameter, including age, Kellgren–Lawrence grades, height, body mass index (BMI), gender and joint site (Figure 3A–F). People over 65 years of age were defined as elderly in Japan. There was a significant difference in age and LECT2 expression in patients over 65

Table 1. The articular cartilage proteome.

No.	Accession	Name	Ratio of OA to control
Top 30 of increased protein in OA cartilage			
1	gi|179665	Complement component C3	2.681
2	gi|119605141	Cartilage oligomeric matrix protein, isoform CRA_a	2.391
3	gi|158257468	Unnamed protein product	1.911
4	gi|158255148	Unnamed protein product	1.821
5	gi|1000745	Pro-a2(XI)	1.791
6	gi|62205353	Matrix Gla protein	1.758
7	gi|193787240	Unnamed protein product	1.756
8	gi|118573093	Ovochymase-1	1.656
9	gi|4220894	Transcriptional co-activator CRSP70	1.652
10	gi|50295448	Platelet phospholipase A2	1.633
11	gi|194377958	Unnamed protein product	1.632
12	gi|119220552	Protein sidekick-1 precursor	1.623
13	gi|119604277	Pleiotrophin	1.596
14	gi|119587667	hCG2045237	1.573
15	gi|940106	T-cell receptor delta chain	1.571
16	gi|34364882	Hypothetical protein	1.527
17	gi|49113217	HHIP-like 2	1.524
18	gi|348041283	C-type lectin domain family 3 member A isoform 1	1.514
19	gi|62420929	Actin-like protein	1.503
20	gi|118763987	PRSS3 protein	1.471
21	gi|193786062	Unnamed protein product	1.435
22	gi|18307851	Angiogenin	1.433
23	gi|62897225	Transforming growth factor, beta-induced, 68 kDa variant	1.425
24	gi|68563369	Solute carrier family 4, anion exchanger, member 1	1.414
25	gi|21757045	Unnamed protein product	1.411
26	gi|59806345	Leukocyte cell-derived chemotaxin-2 precursor	1.389
27	gi|1017427	Elastic titin	1.381
28	gi|119609838	Phosphatidylserine receptor, isoform CRA_d	1.376
29	gi|609342	Nucleophosmin-anaplastic lymphoma kinase fusion protein	1.369
30	gi|21755876	Unnamed protein product	1.341
Top 30 of decreased protein in OA cartilage			
1	gi|1340142	Alpha1-antichymotrypsin	0.402
2	gi|13171812	Immunoglobulin heavy chain variable region	0.415
3	gi|189054134	Unnamed protein product	0.554
4	gi|67190163	Proteoglycan 4 isoform A	0.598
5	gi|332164775	Pyruvate kinase isozymes M1/M2 isoform c	0.608
6	gi|56205585	S100 calcium binding protein A1	0.629
7	gi|51094573	Procollagen C-endopeptidase enhancer	0.632
8	gi|53690154	Small nuclear ribonucleoprotein polypeptide B	0.649
9	gi|114676256	Hyaluronan and proteoglycan link protein 4	0.661
10	gi|296011023	Stromal cell-derived factor 1 isoform delta	0.685
11	gi|114656687	Protein disulfide-isomerase A3 isoform 1	0.685
12	gi|14595132	Raichu404X	0.695
13	gi|312005	Small nuclear ribonucleoprotein E	0.702
14	gi|194374101	Unnamed protein product	0.702
15	gi|119622424	Aggrecan 1	0.722
16	gi|189069324	Unnamed protein product	0.728
17	gi|134254698	Peroxiredoxin 6	0.741
18	gi|54303904	Aging-associated gene 6 protein	0.754
19	gi|62898165	Biglycan preproprotein variant	0.759
20	gi|119586853	hCG2044782, isoform CRA_b	0.761
21	gi|32891795	Clusterin	0.761
22	gi|48146501	IL17B	0.762
23	gi|662841	Heat shock protein 27	0.771
24	gi|194382308	Unnamed protein product	0.772
25	gi|95115683	Hemoglobin alpha 1-2 hybrid	0.774
26	gi|215274249	Fibulin-1	0.784
27	gi|30354619	YWHAZ protein	0.787
28	gi|2204207	Glutathione S-transferase	0.791
29	gi|74761482	Epithelial splicing regulatory protein 2	0.793
30	gi|21264602	Laminin subunit alpha-5 precursor	0.801

The top 30 proteins that were increased or decreased in OA cartilage on iTRAQ method were extracted.

years of age ($p = 0.0012$). BAALC and PRDX6 showed almost no difference in expression (Figure 3A). In terms of Kellgren–Lawrence grade, there were almost no differences in LECT2, BAALC and PRDX6 expression (Figure 3B). To compare height, we divided the patients into groups of >160 cm or <160 cm. According to the annual health report of the Ministry of Health, Labour and Welfare of Japan in 2011, 160 cm was considered as the standard height for men

Table 2. Characteristics of the OA patients and controls in this study.

No	Sample	Age	Gender	Height	Weight	BMI	K-L Grade	Joint
A								
Patients list that was used in the iTRAQ analysis								
1	Control	46	F	156	55	23	–	–
2	OA	59	F	150	51	23	IV	Hip
B								
Patients list that was used in the Western Blot analysis								
1	Control	70	M	165	32.7	12	–	–
2	Control	70	M	158	52.7	21	–	–
3	Control	31	F	162	46	17	–	–
4	Control	39	M	180	81.5	25	–	–
5	Control	77	M	164	52	19	–	–
6	OA	75	M	159.5	70	27	III	Hip
7	OA	60	F	153	49	20	III	Hip
8	OA	56	F	156.8	56.2	22	IV	Hip
9	OA	52	M	173	73.8	24	III	Hip
10	OA	63	F	159	59	23	IV	Hip
11	OA	67	F	165	62.5	22	III	Knee
12	OA	74	F	147.8	54	24	IV	Knee
13	OA	87	F	151	50	21	IV	Knee
14	OA	82	F	135	49.7	27	IV	Knee
15	OA	79	F	143.7	37.7	18	III	Knee
16	OA	76	M	152	52	22	IV	Knee
17	OA	75	F	153	57.4	24	IV	Knee
18	OA	77	F	146.5	64	29	III	Knee
19	OA	63	M	162	69.1	26	III	Knee
20	OA	77	M	174.3	59.7	19	III	Knee
21	OA	81	F	149	50.3	22	II	Knee

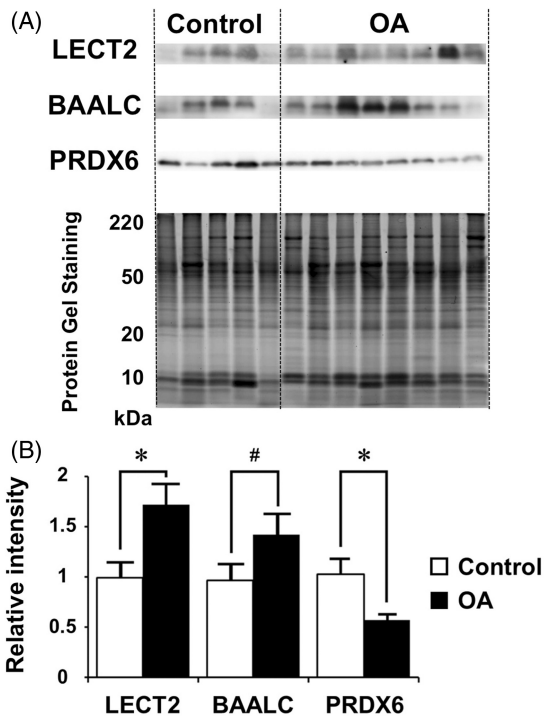

Figure 2. Western blot analysis of LECT2, BAALC and PRDX6. (A) We confirmed the protein expression levels of LECT2, BAALC and PRDX6 in OA and control. Protein gel was stained with SYPRO ruby. (B) Statistical analysis of western blot. Quantities are indicated relative to the average value of each protein level in controls. $*p<0.05$, statistically significant differences between OA and control, as determined by Student's *t*-test. Error bars indicate SEM. LECT2 (control, $n=5$; OA, $n=16$; $p=0.005$), BAALC (control, $n=5$; OA, $n=16$; $p=0.051$), PRDX6 (control, $n=5$; OA, $n=16$; $p=0.011$). (#) $p=0.051$.

and women over 20 years of age. We found a mild correlation in the LECT2 protein level between patients in the two groups. LECT2 expression tended to be up-regulated in patients over 160 cm ($p=0.057$). There were almost no differences in BAALC and PRDX6 expression (Figure 3C). On the other hand, BMI calculated according to the criteria of JASSO (the Japan Society for the Study of Obesity, 2002), which defines a BMI of >25 kg/m^2 as obese. LECT2, BAALC and PRDX6 showed almost no differences in expression (Figure 3D). In evaluation by gender, there was almost no difference in LECT2, BAALC and PRDX6 expression (Figure 3E). Next, we compared differences between joint sites (knee versus hip). BAALC expression was higher in knee joints, but it was not statistically significant (Figure 3F).

Discussion

In this study, we identified three new molecules that show expression differences between patients with OA and those with femoral neck fracture.

LECT2 is a neutrophil chemotactic factor expressed mainly in the liver (Yamagoe et al., 1996). It is a multifunctional protein involved in liver regeneration and immunocompetence. LECT2 is an effector of β-catenin-induced hepatitis that can progress to a precancerous lesion of hepatocellular carcinoma (Anson et al., 2012). A correlation has been observed between vascular invasion of hepatocellular carcinomas and a decline in LECT2 expression (Ong et al., 2011). There is a link between the disease severity of RA and LECT2 polymorphism in Japan (Kameoka et al., 2000), and LECT2 is an inhibitor of collagen-induced arthritis in mice (Okumura et al., 2008). LECT2 is also present in cartilage and it is identical to chondromodulin-II that has been purified from bovine epiphyseal cartilage. Moreover, LECT2 stimulates chondrocyte growth and matrix formation *in vitro* (Hiraki et al., 1996; Shukunami et al., 1999). In the present study, a significant increase in LECT2 expression was observed in OA cartilage ($p=0.005$).

The receptor for LECT2 is C-type lectin DC-SIGN (CD209), an antigen receptor expressed in dendritic cells (DCs) of the skin, mucosal tissues and lymphatic tissues such as the tonsils, lymph nodes and spleen. It is also expressed in monocyte-induced DCs (Chen et al., 2010a). CD209 is highly expressed in inflammatory cells of the synovial membrane in RA patients, and has been found in the synovial membrane of OA patients (van Lent et al., 2003). The expression of CD209 depends on interleukin-4, and is inhibited by interferon, transforming growth factor-β, and anti-inflammatory drugs (Relloso et al., 2002). These observations indicate an involvement of LECT2 in the pathophysiologies of OA and RA.

The Wnt/β-catenin pathway is known to positively regulate LECT2 expression in the liver (Ovejero et al., 2004). An OA-like phenotype is induced by increased expression of the β-*catenin* gene in the cartilage of mouse models, suggesting that the Wnt/β-catenin pathway is associated with OA (Wu et al., 2010). Therefore, an association between increased LECT2 expression in OA and the Wnt/β-catenin pathway is possible. However, iTRAQ performed in the present study did not detect Wnt or β-catenin proteins. Therefore, the cause of the variation between control and OA groups could not be determined.

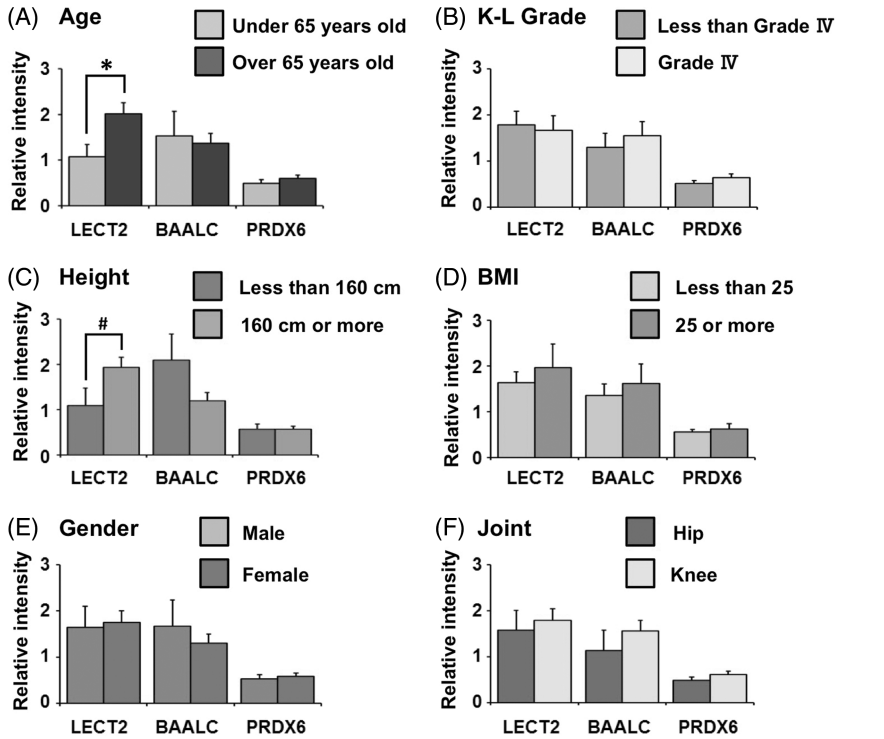

Figure 3. Experimental correlation between LECT2 protein levels and Age in OA patients. (A) Age (>65, $n=11$; <65, $n=5$; LECT2, $p=0.012$; BAALC, $p=0.397$; PRDX6, $p=0.166$). (B) K-L Grade (>IV, $n=8$; <III, $n=8$; LECT2, $p=0.401$; BAALC, $p=0.282$; PRDX6, $p=0.131$). (C) Height (>160 cm, $n=4$, <160 cm, $n=12$; LECT2, $p=0.057$; BAALC, $p=0.107$; PRDX6, $p=0.482$). (D) BMI (>25, $n=4$; <25, $n=12$; LECT2, $p=0.298$; BAALC, $p=0.301$; PRDX6, $p=0.330$). (E) Gender (male, $n=5$; female, $n=11$; LECT2, $p=0.420$; BAALC, $p=0.284$; PRDX6, $p=0.304$). (F) Hip or Knee (hip, $n=5$; knee, $n=11$; LECT2, $p=0.338$; BAALC, $p=0.217$; PRDX6, $p=0.106$). *$p<0.05$, statistically significant differences between >65 and <65, as determined by Student's *t*-test. Error bars indicate SEM. (#) $p=0.057$. Quantities are indicated relative to the average value of each protein level in controls.

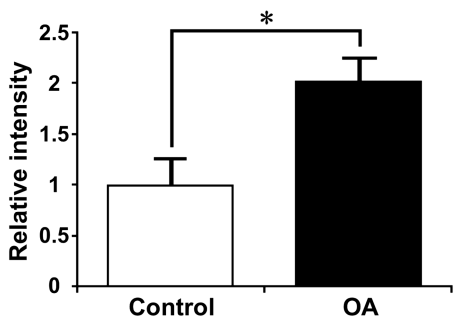

Figure 4. The level of LECT2 in control and OA patient at over 65 years of age. We recalculated expression level of LECT2 in OA and control cartilage by excluding the case of under 65 years of age. LECT2 was increased in OA with a significant difference compared with control (Control $n=3$, OA $n=11$). *$p<0.05$, as determined by Student's *t*-test. Error bars indicate SEM.

Although further investigation is required to determine whether increased LECT2 expression in OA operates positively or negatively during disease onset and progression, we propose that identification of another molecule that shows increased expression in OA patients is highly valuable. LECT2 may represent a possible diagnostic marker of OA.

When the expression level of LECT2 was compared against various parameters in OA patients, LECT2 levels were found to be increased significantly in patients over 65 years of age ($p=0.012$). When considering patients over 160 cm in height ($p=0.057$), no significant difference in LECT2 levels was found, but a tendency toward increased expression was apparent. Although no correlation was observed among height, age, and other OA biomarkers, a relationship has been suggested between *GDF5*, which has been associated with OA, and height in humans (Williams et al., 2011). Therefore, the level of LECT2 may rise according to age. For this reason, we recalculated the expression level of LECT2 in OA and control cartilages by excluding cases in which the patients were under 65 years of age. As a result, we found significant up-regulation of LECT2 expression in the OA group compared with that in the control (Figure 4).

BAALC includes 6 isoforms and is abundant in acute myelogenous leukemia (AML) cells and the brain. Currently, it is being studied extensively as a diagnostic or prognostic marker for AML. There have also been reports of a correlation between BAALC and AML prognosis (Langer et al., 2008). Histone post-translational modifications such as H3K9K14 acetylation, H3K4 trimethylation and H3K23 trimethylation are involved in the regulation of BAALC expression in AML cells (Franzoni et al., 2012). In animal models, inhibitory effects on OA have been observed after intra-articular injection of trichostatin A, an inhibitor of histone deacetylase (Chen et al., 2010b). These observations show that cartilage cell degeneration might lead to epigenetic changes, resulting in altered BAALC levels in OA patients. Thus, OA prognosis and its correlation with BAALC are areas of current interest.

Although there have been no previous studies regarding the relationship between BAALC and OA, a study has shown variation in microRNA abundance based on differences in weight loads for each layer of articular cartilage in a bovine model. Non-weight-bearing regions show down-regulated expression of miR-148a, a microRNA that is associated with leukemia, compared with the expression level in weight-bearing regions (Dunn et al., 2009). miR-148a expression shows a negative correlation with *BAALC* gene expression (Langer et al., 2008), and high *BAALC* gene expression correlates with the presence of an early-stage marker of precursor cells. This observation suggests that cell proliferation is stimulated in weight-bearing regions of articular cartilage. Therefore, an increase in the amount of BAALC in OA patients ($p=0.051$) could modulate cell proliferation in damaged cartilage.

PRDX6 is an enzyme that has both glutathione peroxidase and lysosomal-type phospholipase A2 activities, and also possesses an antioxidant activity. The link between OA and ROS is well known with ROS increasing in OA patients (Koorts et al., 2012; Xie et al., 2012). The expression of PRDX5, which belongs to the same peroxiredoxin family, increased significantly in OA cartilage. In other studies, the expression of PRDX5 has been suggested to increase as a protective measure against tissue damage caused by ROS (Wang et al., 2002).

In a proteomic analysis of cartilage extracts from mice, PRDX6 was detected, but the correlation between PRDX6 and OA was not studied (Wilson et al., 2008). However, we found that the amount of PRDX6 was significantly reduced in OA cartilage, compared with the control group, suggesting that protection against tissue damage from ROS by PRDX6 is prevented in OA. Taken together, these results strongly suggest that PRDX family members are involved in protection against damage caused to cartilage by ROS, and that these proteins may be applicable in therapeutic intervention. Our study also identified the utility of PRDX6 as a marker. No correlation was found between PRDX6 abundance and age, height, BMI, gender or Kellgren–Lawrence grade.

Based on previous studies, a correlation between OA and BMI is well known. The OA risk factor is a BMI of $\geq$30 kg/m^2 in WHO research (Gudbergsen et al., 2013; Wills et al., 2012). However, average weight and BMI in Japan are low compared with those in Europe and the United States (WHO expert consultation, 2004). In our current study, average weight and BMI were 56 kg and 22 kg/m^2, respectively. These values are obviously lower than the worldwide average. In addition, there was no correlation between OA and BMI because of the low number of patients with high BMI in the current study. For these reasons, we do not deny the correlation between OA and BMI.

In addition to cartilage-specific molecules such as CTX-II, CPII, C2C, HA, MMP-1, MMP-8 and MMP-13 (Conrozier et al., 2012), non-organ specific molecules such as fibulin-3 peptides (Henrotin et al., 2012), follistatin-like protein 1 (Wang et al., 2011b), and C3 (Wang et al., 2011a) are useful as OA biomarkers that can reflect OA pathology. In our current study, LECT2, BAALC and PRDX6 were not cartilage-specific proteins. Considering the above reports, we believe that these proteins may also be useful as OA biomarkers.

Because serum biomarkers are easy to measure continuously, they are considered useful for prognostication of OA. Furthermore, combined with diagnostic imaging results, the results of our study may assist OA diagnosis. LECT2 is a secreted protein that can be detected in serum (Sato et al., 2004). BAALC can also be detected in serum (Baldus et al., 2003). Although PRDX6 is also detected in serum, it is an enzyme and not a secreted protein (Zhang et al., 2009). As mentioned above, there is a high possibility that these proteins can be detected and measured in serum samples from OA patients. Therefore, they may be useful biomarkers for OA. As a next step, it will be important to perform large-scale research using serum from OA patients.

Of the previously identified OA markers such as CTX-II, C2C, COMP and HA, many are catabolic enzymes or fragments generated by proteolysis and/or degradation of the extracellular matrix. In addition to identifying COMP in OA cartilage, we confirmed variation in the expression of a molecule that could potentially function *via* autocrine or paracrine mechanisms in cartilage cells (LECT2), a molecule that could potentially function within cartilage cells (BAALC), and a molecule that has an antioxidant activity (PRDX6).

Conclusions

In developed countries with an aging population, the number of individuals with OA is predicted to continue to rise. Given the sheer number of such individuals, the utility of a marker that can be easily measured in blood, urine or synovial fluids cannot be ignored. In the future, efficient selection of patients with a high potential risk for progressive joint destruction and application of intensive prevention measures may drastically reduce the number of patients with severe joint destruction. To this end, identification and evaluation of an easily measurable biomarker is imperative. Combination of conventional markers with newly identified markers, the variation of which was confirmed in this study, may improve diagnosis of the OA disease state and the capacity for prognosis. We believe that it is necessary to perform large-scale clinical research using serum from OA patients to investigate useful biomarkers in the near future.

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Contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version for publication.

Dr Yamada had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ikeda, Ageta.
Acquisition of data. Ikeda, Ageta
Analysis and interpretation of data. Ikeda, Ageta.
Manuscript preparation. Ikeda, Ageta, Tsuchida, Yamada
Statistical analysis. Ikeda, Ageta.

Declaration of interest

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References

Aggarwal K, Choe LH, Lee KH. (2006). Shotgun proteomics using the iTRAQ isobaric tags. Brief Funct Genomic Proteomic 5:112–20.
Anson M, Crain-Denoyelle AM, Baud V, et al. (2012). Oncogenic beta-catenin triggers an inflammatory response that determines the

aggressiveness of hepatocellular carcinoma in mice. J Clin Invest 122: 586–99.

Baldus CD, Tanner SM, Kusewitt DF, et al. (2003). BAALC, a novel marker of human hematopoietic progenitor cells. Exp Hematol 31: 1051–6.

Chambers G, Lawrie L, Cash P, Murray GI. (2000). Proteomics: a new approach to the study of disease. J Pathol 192:280–8.

Chen J, Lu XJ, Yang HY, Shi YH. (2010a). An interaction between a C-type lectin receptor and leukocyte cell-derived chemotaxin 2 of ayu, *Plecoglossus altivelis*. Fish Shellfish Immunol 28:245–8.

Chen WP, Bao JP, Hu PF, et al. (2010b). Alleviation of osteoarthritis by Trichostatin A, a histone deacetylase inhibitor, in experimental osteoarthritis. Mol Biol Rep 37:3967–72.

Cillero-Pastor B, Eijkel GB, Kiss A, et al. (2013). Matrix-assisted laser desorption ionization-imaging mass spectrometry: a new methodology to study human osteoarthritic cartilage. Arthritis Rheum 65:710–20.

Conrozier T, Balblanc JC, Richette P, et al. (2012). Early effect of hyaluronic acid intra-articular injections on serum and urine biomarkers in patients with knee osteoarthritis: an open-label observational prospective study. J Orthop Res 30:679–85.

de Seny D, Sharif M, Fillet M, et al. (2011). Discovery and biochemical characterisation of four novel biomarkers for osteoarthritis. Ann Rheum Dis 70:1144–52.

Dunn W, DuRaine G, Reddi AH. (2009). Profiling microRNA expression in bovine articular cartilage and implications for mechanotransduction. Arthritis Rheum 60:2333–9.

Fernandez-Costa C, Calamia V, Fernandez-Puente P, et al. (2012). Sequential depletion of human serum for the search of osteoarthritis biomarkers. Proteome Sci 10:55.

Fernandez-Puente P, Mateos J, Fernandez-Costa C, et al. (2011). Identification of a panel of novel serum osteoarthritis biomarkers. J Proteome Res 10:5095–101.

Franzoni A, Passon N, Fabbro D, et al. (2012). Histone post-translational modifications associated to BAALC expression in leukemic cells. Biochem Biophys Res Commun 417:721–5.

Gharbi M, Deberg M, Henrotin Y. (2011). Application for proteomic techniques in studying osteoarthritis: a review. Frontiers Physiol 2:90.

Gudbergsen H, Boesen M, Christensen R, et al. (2013). Changes in bone marrow lesions in response to weight-loss in obese knee osteoarthritis patients: a prospective cohort study. BMC Musculoskelet Disord 14: 106.

Henrotin Y, Gharbi M, Mazzucchelli G, et al. (2012). Fibulin 3 peptides Fib3-1 and Fib3-2 are potential biomarkers of osteoarthritis. Arthritis Rheum 64:2260–7.

Hiraki Y, Inoue H, Kondo J, et al. (1996). A novel growth-promoting factor derived from fetal bovine cartilage, chondromodulin II. Purification and amino acid sequence. J Biol Chem 271:22657–62.

Hirata M, Kugimiya F, Fukai A, et al. (2012). C/EBPbeta and RUNX2 cooperate to degrade cartilage with MMP-13 as the target and HIF-2alpha as the inducer in chondrocytes. Hum Mol Genet 21: 1111–23.

JASSO, the Japan Society for the Study of Obesity. (2002). New criteria for 'obesity disease' in Japan. Circ J 66:987–92.

Jingushi S, Ohfuji S, Sofue M, et al. (2011). Osteoarthritis hip joints in Japan: involvement of acetabular dysplasia. J Orthop Sci 16:156–64.

Kameoka Y, Yamagoe S, Hatano Y, et al. (2000). Val58Ile polymorphism of the neutrophil chemoattractant LECT2 and rheumatoid arthritis in the Japanese population. Arthritis Rheum 43:1419–20.

Kato S, Yamada H, Terada N, et al. (2005). Joint biomarkers in idiopathic femoral head osteonecrosis: comparison with hip osteoarthritis. J Rheumatol 32:1518–23.

Kellgren JH, Lawrence JS. (1957). Radiological assessment of osteo-arthrosis. Ann Rheum Dis 16:494–502.

Kong MK. (2012). Evaluation of a pretreatment method for two-dimensional gel electrophoresis of synovial fluid using cartilage oligomeric matrix protein as a marker. J Microbiol Biotechnol 22:654–8.

Koorts AM, Levay PF, Hall AN, et al. (2012). Expression of the H- and L-subunits of ferritin in bone marrow macrophages of patients with osteoarthritis. Exp Biol Med (Maywood) 237:688–93.

Kraus VB. (2011). Osteoarthritis year 2010 in review: biochemical markers. Osteoarthritis Cartilage 19:346–53.

Kriegsmann M, Seeley E, Schwarting A, et al. (2012). MALDI MS imaging as a powerful tool for investigating synovial tissue. Scand J Rheumatol 41:305–9.

Langer C, Radmacher MD, Ruppert AS, et al. (2008). High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. Blood 111: 5371–9.

Okumura A, Saito T, Otani I, et al. (2008). Suppressive role of leukocyte cell-derived chemotaxin 2 in mouse anti-type II collagen antibody-induced arthritis. Arthritis Rheum 58:413–21.

Ong HT, Tan PK, Wang SM, et al. (2011). The tumor suppressor function of LECT2 in human hepatocellular carcinoma makes it a potential therapeutic target. Cancer Gene Ther 18:399–406.

Ovejero C, Cavard C, Perianin A, et al. (2004). Identification of the leukocyte cell-derived chemotaxin 2 as a direct target gene of beta-catenin in the liver. Hepatology 40:167–76.

Relloso M, Puig-Kroger A, Pello OM, et al. (2002). DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. J Immunol 168:2634–43.

Ross PL, Huang YN, Marchese JN, et al. (2004). Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3:1154–69.

Saito T, Fukai A, Mabuchi A, et al. (2010). Transcriptional regulation of endochondral ossification by HIF-2alpha during skeletal growth and osteoarthritis development. Nat Med 16:678–86.

Saito T, Kawaguchi H. (2010). HIF-2alpha as a possible therapeutic target of osteoarthritis. Osteoarthritis Cartilage 18:1552–6.

Sato Y, Watanabe H, Kameyama H, et al. (2004). Serum LECT2 level as a prognostic indicator in acute liver failure. Transplant Proc 36: 2359–61.

Shukunami C, Kondo J, Wakai H, et al. (1999). Molecular cloning of mouse and bovine chondromodulin-II cDNAs and the growth-promoting actions of bovine recombinant protein. J Biochem 125:436–42.

Song X, Bandow J, Sherman J, et al. (2008). iTRAQ experimental design for plasma biomarker discovery. J Proteome Res 7:2952–8.

van Lent PL, Figdor CG, Barrera P, et al. (2003). Expression of the dendritic cell-associated C-type lectin DC-SIGN by inflammatory matrix metalloproteinase-producing macrophages in rheumatoid arthritis synovium and interaction with intercellular adhesion molecule 3-positive T cells. Arthritis Rheum 48:360–9.

Wang MX, Wei A, Yuan J, et al. (2002). Expression and regulation of peroxiredoxin 5 in human osteoarthritis. FEBS Lett 531:359–62.

Wang Q, Rozelle AL, Lepus CM, et al. (2011a). Identification of a central role for complement in osteoarthritis. Nat Med 17:1674–9.

Wang Y, Li D, Xu N, et al. (2011b). Follistatin-like protein 1: a serum biochemical marker reflecting the severity of joint damage in patients with osteoarthritis. Arthritis Res Ther 13:R193.

WHO expert consultation. (2004). Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. Lancet 363:157–63.

Williams FM, Popham M, Hart DJ, et al. (2011). GDF5 single-nucleotide polymorphism rs143383 is associated with lumbar disc degeneration in Northern European women. Arthritis Rheum 63:708–12.

Wills AK, Black S, Cooper R, et al. (2012). Life course body mass index and risk of knee osteoarthritis at the age of 53 years: evidence from the 1946 British birth cohort study. Ann Rheum Dis 71: 655–60.

Wilson R, Belluoccio D, Little CB, et al. (2008). Proteomic characterization of mouse cartilage degradation in vitro. Arthritis Rheum 58: 3120–31.

Wu Q, Zhu M, Rosier RN, et al. (2010). Beta-catenin, cartilage, and osteoarthritis. Ann N Y Acad Sci 1192:344–50.

Xie L, Lin AS, Kundu K, et al. (2012). Quantitative imaging of cartilage and bone morphology, reactive oxygen species, and vascularization in a rodent model of osteoarthritis. Arthritis Rheum 64: 1899–908.

Yamada H, Yoshihara Y, Henmi O, et al. (2009). Cementless total hip replacement: past, present, and future. J Orthop Sci 14:228–41.

Yamagoe S, Yamakawa Y, Matsuo Y, et al. (1996). Purification and primary amino acid sequence of a novel neutrophil chemotactic factor LECT2. Immunol Lett 52:9–13.

Zhang XZ, Xiao ZF, Li C, et al. (2009). Triosephosphate isomerase and peroxiredoxin 6, two novel serum markers for human lung squamous cell carcinoma. Cancer Sci 100:2396–401.