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Research Article

A Comprehensive Proteomics Analysis of Blood Sera from Patients of Osteoarthritis-Comparative Study before and after Total Joint Replacement Surgery

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Abstract

To find candidate theranostic proteins for osteoarthritis (OA), we performed proteomic analysis of serum samples from patients with OA. We used serum samples from OA patients who underwent total hip or knee replacement surgery. We used 12 serum samples that were collected before (Pre) and 3 months after the surgical procedure (3M) from each of six patients with OA. We performed comprehensive proteomic analysis, and calculated the fold change between Pre and 3M samples for each identified protein.In the proteomic analysis, we identified 410 proteins. Among the identified proteins present in more than three patients' samples, there were 10 proteins whose 3M /Pre value was < 0.5. Among the proteins, five (lactotransferrin, neutrophil defending 1, actin cytoplasmic 1, keratin type I cytoskeletal 10, and heparanase) were differentially expressed when adjusted for fluctuations of albumin levels in a statistically significant way. The serum concentration of lactotransferrin was significantly decreased after the operation according to a volcano plot. Supporting this finding, the levels of lactotransferrin in the serum samples were decreased on western blots for each patient.Using comprehensive proteomic analysis, we found that lactotransferrin protein expression decreased significantly after total hip or knee replacement surgery.

Keywords: Lactotransferrin; Osteoarthritis; Serum; Proteomics; Replacement Surgery

Introduction

Osteoarthritis (OA) begins with the degeneration of articular cartilage and causes changes in tissues such as bone, ligaments and synovium. Obesity, age and sex are well known risk factors for OA. In recent years, it has been proposed that OA may be related to pathological conditions, such as metabolic syndrome and impaired glucose metabolism [1-3].The elderly comprises the majority of OA patients, where clinical symptoms such as pain resulting from OA cause a decline in activities of daily living (ADL), which can be a major obstacle to healthy life extension. A large-scale cohort study (Research on Osteoarthritis/Osteoporosis Against Disability Study) reported that the number of Japanese people afflicted with OA symptoms is estimated to be about 8 million [4]. Worldwide, it has been reported that 249 of every 100,000 individuals suffer from OA [5]. Excluding cases resulting from external injuries, OA is the principal disease causing physical disabilities in individuals aged ≥ 50 years, with characteristic OA lesions observed in at

least one joint in 70-80% of patients who are ≥ 65 years of age [6]. Because of population ageing,the number of people suffering from illnesses is increasing, including the number ofpeople suffering from OA.Extensive medical resources are required to prevent this increase in the number of OA patients, making OA important in terms of medical economics. Consequently, it is desirable to prevent OA progression by means of intervention in the form of early diagnosis and treatment.Blood and urine biomarkers are currently used to diagnose diseases, and these markers are a convenient way to monitor disease progression. Biomarkers have already been developed and used clinically for the diagnosisand monitoring of motor system diseases, such as osteoporosis and rheumatoid arthritis [7,8] Bone-specific alkaline phosphatase(BAP), a bone formation marker derived from osteoblasts, is used to assess osteoporosis treatment results. In addition, the autoantibody, anti-cyclic citrullinated peptide antibody (anti-CCP), is highly specific for the early diagnosis of rheumatoid arthritis. In recent years, there has been an increase in the number of studies reporting biomarkers for the diagnosis and evaluation of OA progression in blood, urine, and synovial fluid [9,10].

At present, biomarkers for articular cartilage damage include cartilage oligomeric matrix protein (COMP), matrix metalloproteinase (MMP), and the C-terminal telopeptide of type II collagen (CTX-II). COMP results from degenerating cartilage that is dispersed in the blood or urine, and is therefore being investigated asadiagnostic marker for OA [11]. MMP expression is increased inchondrocytes by stimulating cytokines, such as interleukin (IL) 1 and IL-6 [12]. CTX-II is the principal degradation product of type II collagen, which constitutes the cartilage tissues. Serum COMP and CTX-II have been shown to be an indication of OA progression [13]. It has also been reported that elevated levels of hyaluronic acid in the blood correlates with the OA disease stage in the knee and finger joints, and is visible on X-ray images, indicating a possible method for predicting OA progression [14]. Furthermore, research is underway to determine whether an increase in MMP expression generated type I collagen neo-epitope (C1M), C3M, and MMP-3; these collagen decomposition products caused by cytokine stimulation in synovial cell cultures can be used as OA markers [15]. CTX-II levels in the blood of professional indoor soccer players were measured and compared before and after one season of play. In comparison with levels measured before the season began CTX-II levels had increased after the season was finished, in 10 of the 14 study participants [16]. A large amount of research on both existing and new OA markers is currently ongoing. A comparative analysis of metabolites in the serum of knee joint OA patients and healthy controls was performed using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) [17].

The levels of 14 metabolites,includingthe metabolism of amino acids and purine, such as L-histidine, hypoxanthinewas significantly altered in OA patients, suggesting that these substances could serve as markers for the early diagnosis of OA. Even now, OA has been diagnosed using clinical symptoms and X-ray images. As it is difficult to repair articular cartilage once it has been destroyed, early diagnosis is crucial to OA treatment. Surgical methods are the only effective means to treat end-stage OA [18]. However, the development of useful OA markers has facilitated the early diagnosis and subsequent treatment of OA. It is also expected that OA symptoms can be controlled using non-invasive treatments and rehabilitation. If it is possible to measure marker levels to diagnose early-stage OA and start treatment early, it is thought that it will become possible to maintain long-term, high-quality ADL while performing minimally invasive treatments. Moreover, this will presumably reduce healthcare costs and extend the duration of a healthy life. In addition, markers that accurately reflect OA status can be used to select a suitable treatment that corresponds to a detailed OA status.

Inprevious researches, our laboratory used a random peptide column to concentrate articular cartilage samples from OA patient [19]. Proteomics analysis was performed using the isobaric tags for the relative and absolute quantitation (iTRAQ) method, and a comparison using cartilage from a femoral neck fracture patient as a control.Significantly higherlevels of leukocyte cell derived chemotaxin 2 (LECT 2) were found in the OA patient,in addition to a significant reduce in peroxiredoxin 6 (PRDX6) levels. The sera from the OA patients were also used to study the utility of LECT2 and PRDX6 as a marker. We also found that the serum levels of PRDX6 had significantly increased in OA patients three months after surgery when compared to its levels measured prior to surgery [20].

In recent years, proteomics has been frequently performed in screening for biomarkers. Recent studies have investigated markers that differentiate between childhood-onset multiple sclerosisand acquired demyelinating disease. Mass spectrometer (MS)analysis of spinal fluid collected from both types of patients showed that the levels of 14 proteins increased including amyloid-like protein 2 and neurofascin.In contrast, the levels of seven other proteins decreased such as apolipoprotein B-100 and C4b-binding protein alpha chain [21]. Chiou and Lee [22] also studied early diagnostic markers for hepatocarcinoma. MS analysis was performed using the urine of hepatocarcinoma patients in comparison with healthy control subjects, where the levels of S100 calcium binding protein A9 (S100A9) and granulin had increased.Although there have been numerous studies on OA biomarkers, almost all of them involved comparisonsbetween an OA patient group and a healthy control group. It is rare to compare the sera from the same subject

to highlight any differences before and after total joint replacement surgery in the same patient. Patients are distinct individuals with different medical, oral administration, and treatment histories,and disease severity. Therefore, itis not easy to compare individuals, even those with the same disease. In total joint replacement surgery, the degenerative cartilage and inflamed synovium are almost completely removed. To search for new proteins that maybe indicative of OA status, we compared each patient's serum taken before total joint replacement with the same taken after surgery by performing a comprehensive MS analysis, thereby identifying molecules that underwent pre-and post-surgical fluctuations.

Materials and Methods

This study was approved by a steering committee and conducted in accordance with the guidelines for clinical studies of Fujita Health University. The samples of blood serum from OA patients were obtained with informed consent.

Patients and blood serum

Blood serum samples used in this study were collected from six OA female patients (age 55–79 years), (Table 1).

No	Age	Gender	Height	Weight	BMI	K-L Grade	Joint	PH
1	68	F	159.0	46.5	18.4	2	Hip	-
2	55	F	158.0	45.3	18.1	4	Hip	-
3	67	F	140.0	43.5	22.1	4	Hip	HT
4	72	F	156.2	45.0	18.4	4	Hip	HT
5	73	F	140.0	51.8	26.4	4	Hip	HT
6	79	F	150.0	61.5	27.3	3	Knee	HT

Table 1: Characteristics of the patients in this study.K-L Grade; Kellgren-Lawrence grade (Kellgren and Lawrence, 1957).

with severe hip or knee OA who underwent total hip or knee replacement surgery at Fujita Health University. These patients have no history of severe diseasesor have only hypertension except OA. Patients with revision surgery were excluded. All the blood samples were obtained during hospitalisation and immediately centrifuged at 3300 rpm for 10 min at 4 °C and stored at -80 °C.

Protein Preparation

A random peptide column (Proteominer; Bio-Rad, Hercules, CA) was used to prepare samples before trypsin digestion. The concentrated samples were digested using a trypsin (PierceTM-Mass Spec Sample Prep Kit for Cultured Cells, #84840) digestion protocol. The resulting samples were analysed by liquid chromatography with mass spectrometry (LC-MS).

LC-MS Analysis

The samples were separated using the EASY-nLC 1000 resin (Thermo Scientific, San Jose, California, USA) on a PepMap RSLC C18 (3 mm, 75 mm × 15 cm) column (Thermo Scientific). Solvent A was high-performance liquid chromatography (HPLC)-grade water with 0.1% (v/v) formic acid, and solvent B was HPLC grade acetonitrile with 0.1% (v/v) formic acid. Separation was performed with stepwise gradient (0-35% B for 110 min, 35-95% B for 10 min, 95% B for 10 min) at 300 nL/min during 130 min. MS data were generated using an Orbitrap fusion Tribrid mass spectrometer (Thermo Scientific) operated at -2.0 kV (positive ions) applied to the central electrode. The peptides were analysed in data-dependent acquisition mode. A survey full scan MS (from m/z 300 to 1500) was run in the Orbitrap at resolution 120,000, target automatic gain control (AGC) values of 400,000, and maximum injection time of 50 ms. Most intense precursor ions with charge state 2-7 were isolated and fragmented using collision-induced dissociation (CID) fragmentation with 35% collision energy and detected in the linear ion trap. Monoisotopic precursor selection and dynamic exclusion (60 s duration, 10 ppm mass tolerance) were enabled.

Data Analysis

The tandem MS (MS/MS) searches were carried out using MASCOT (Version 2.5.5) and SEQUEST HT search algorithms against the Swiss-Prot human protein database using Proteome Discoverer (PD) 2.1 (Version 2.1.1.21 Thermo Scientific). The workflow for both algorithms included a spectrum selector, MASCOT, SEQUEST HT search nodes, percolator, ptmTS, event detector, and precursor ions' area detector nodes. Oxidation of methionine and phosphorylation at serine, threonine, and tyrosine (+79.966 Da) were set as variable modifications, and carbamidomethylation of cysteine was selected as a fixed modification. MS and MS/MS mass tolerances were set to 10 ppm and 0.6 Da, respectively. A maximum of two missed cleavage events was allowed. Target-decoy database searches were used for calculation of the false discovery rate (FDR), and for peptide identification, the FDR was set to 1%.Label-free quantification was also performed in PD 2.1 using precursor ions' area detector nodes.

Statistical Analysis

For a volcano plot, statistical analysis was conducted by means of the two-class paired SAM (significance analysis of microarrays) algorithm [23]. For a dot plot, we used Student's t test to compare the data on the proteins that we detected with serum albumin data; p values < 0.05 were assumed to indicate statistical significance.

Western Blot Analysis

Protein expression levels were also examined by western blotting. Protein samples (2.5 □g) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked in 10% goat serum overnight at 4 °C. The membranes were probed with an anti-lactotransferrin (GTX77521, 1/1000 dilution; Gene Tex) antibody for 1 h at room temperature. The signals were detected by using Trueblot (# 18-8816-31, 1/1000 dilution; ROCKLAND). The membrane was blocked in Tris-buffered saline (pH 7.4) with 0.1%Tween 20. The signal was visualised by chemiluminescence (ECL Plus; GE Biosciences) and LAS4000 (Fuji Film). Captured images were analysed by means of Multi Gauge v3.1 (Fuji Film).

Results

In our previous study, we used random peptide columns to concentrate rare proteins in extracts from OA patients' cartilage samples[19]. Control samples were from patients with femoral neck fracture. In the present study, we planned a comprehensive proteomic analysis of blood serum samples from OA patients who underwent total joint replacement surgery.At first, we used commercially available human serum (Millipore, Normal Human Serum, S1-100ML, Lot: 2491 117), which was concentrated on random peptide columns as a pilot experiment. These purified protein samples were separated by SDS-PAGE, and then stained with SYPRO ruby. As a result, the numbers of the protein bands in the concentrated samples obviously increased as compared with the no-treatment samples (Figure 1A). Moreover, protein analysis on an MS instrument (Orbitrap Fusion) of the same samples revealed that the number of identified proteins increased from 171 to 310 (Figure 1B).

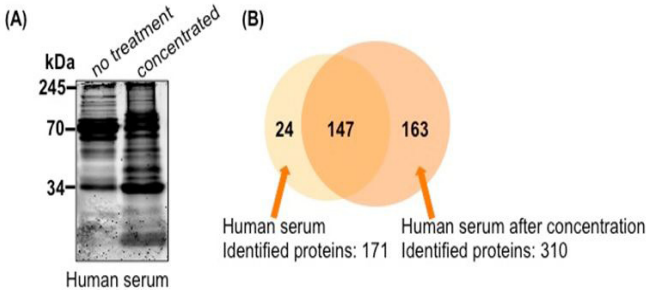

Figure 1: Pilot experiment of serum concentration.

(A) The numbers of confirmed protein bands increased in the concentrated samples.

(B) The numbers of identified proteins increased in the concentrated samples compared with the unconcentrated samples.

These results suggested that the concentration procedure on random peptide columns is suitable for comprehensive proteomic analysis.We next collected blood serum samples for the proteomic analysis. The serum samples were obtained before (Pre) and 3 months after the surgical procedure (3M) from each OA patient. Patients' clinical characteristics are shown in (Table 1). Age was 69 ± 3.29 (mean ± SE), height 150.5 ± 3.56, weight 48.9 ± 2.77, and the body-mass index (BMI) 21.7 ± 1.71. All the patients were females. Five patients underwent total hip replacement surgery,whereas only one patient underwent total knee replacement surgery (Table 1). We also ensured that all the serum samples were concentrated on the random peptide columns. Next, the purified serum samples were analysed by MS (Figure 2A). The MS analysis identified 410 proteins (FDR = high), among which 229 proteins were identified in more than three patients, 201 proteins in more than four patients, and 160 proteins in all six patients (Figure 2B).

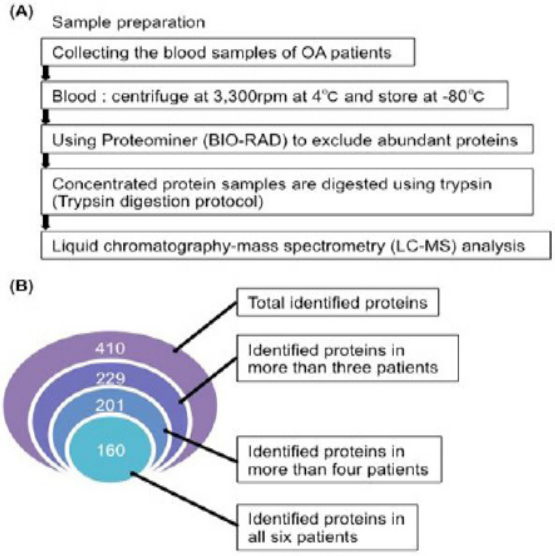

Figure 2: Result of LC-MS analysis.

(A) The experimental scheme.

(B) All the proteins that were identified. The number of differentially expressed proteins (between groups Pre and 3M) identified in all six patients' samples was 160.

According to this result, we calculated changes in expression of all the identified proteins in each patient before and after the operation. The peak area values for the identified proteins in the 3M groups were divided by the same identified protein peak area values in the Pre-group. We defined this value (3M/Pre) as a fold change for each protein. We then calculated the geometric

mean for all the identified proteins' fold changes between the two groups for each patient.

Among the 410 identified proteins in this analysis, the number of molecules was 16 with 3M/Pre-value> 2, and there were 19 molecules with the 3M/Pre-value< 0.5 (Table 2A).The list of the identified proteins whose 3M/Pre-value was >2 and <0.5 in more than three patients' samples is shown in Table 2B. There were 10 proteins whose 3M/Pre-value was <0.5 (Table 2B).

(A)	Fluctuate proteins	
	More than 1 patient	More than 3 patients
> 2fold	16	5
< 0.5fold	19	10

(B)

No	Description	Accession	Gene ID	Geometric mean
> 2				
1	Ig lambda chain V-IV region Hil	P01717		4.272
2	Periostin	Q15063-1	POSTN	3.375
3	Ig mu heavy chain disease protein	P04220		3.318
4	Ig heavy chain V-III region JON	P01780		2.607
5	Apolipoprotein A-V	Q6Q788	APOA5	2.083
< 0.5				
1	lactotransferrin	P02788	LTF	0.201
2	Neutrophil defensin 1	P59665	DEFA1; DEFA1B	0.318
3	Keratin, type 1 cyoskeletal 9	P35527	KRT9	0.349
4	Hemoglobin subunit beta	P68871	HBB	0.379
5	Actin, cytoplasmic 1	P60709	ACTB	0.402
6	Complement fector H-related protein 4	Q92496	CFHR4	0.445
7	Coagulation factor X	P00742	F10	0.449
8	Keratin, type 1 cyoskeletal 9	P13645	KRT10	0.453
9	Antithrombin-III	901008	SERPINCI	0.470
10	Heparanse	Q9Y251	HPSE	0.497

Table 2: The data of identified proteins.

(A) The number of proteins that were identified by LC-MS analysis.

(B) Ranking of proteins identified in more than three patients.

Among those 10 downregulated proteins, we compared the 3M/Pre-value of these proteins in each patient with the 3M/Pre value of serum albumin in each patient by Student's t test. Five proteins (lactotransferrin, neutrophil defending 1, actin cytoplasmic 1, keratin type I cytoskeletal 10, and heparanase) were differentially expressed between groups Pre and 3M when adjusted for albumins' value ($p < 0.05$); (Figure 3A) in a statistically significant way. We detected 5032 peptides in the LC-MS experiment on the OA patients' samples. We analysed all the detected peptides using a volcano plot. The volcano plot showed that 44 peptides were significantly downregulated, 27 peptides were significantly upregulated, and the peptides constituting lactotransferrin were located in downregulated peptides' area. There were no peptides from lactotransferrin in the upregulated peptides' area (Figure 3B).

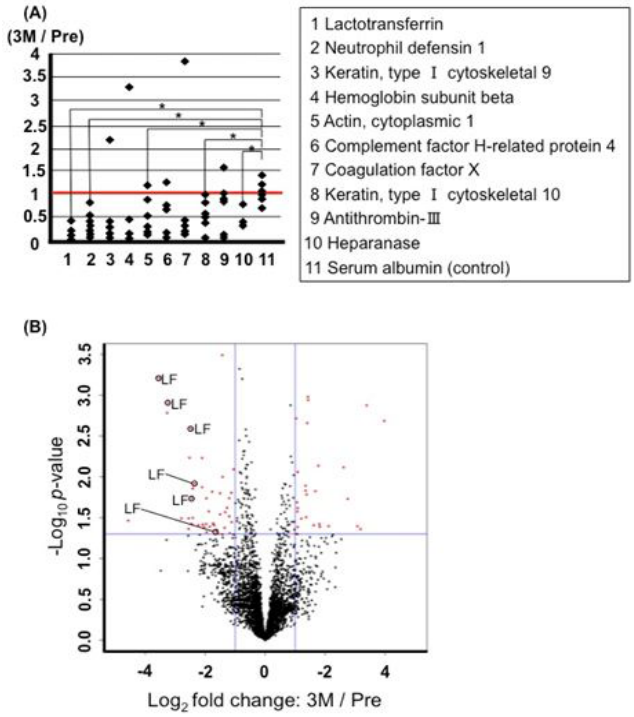

Figure 3: Result of dot plot and volcano plot.

(A) Comparison of the identified proteins and serum albumin. 5 proteins showed significant differences between groups Pre and 3M ($p < 0.05$).

(B) The volcano plot of peptides for groups Pre and 3M. The red points in the plot represent the differentially expressed peptides with statistical significance (lactotransferrin: LF). Red points on the left side represent downregulated peptides.

On the basis of this result, we next performed western blot analysis of lactotransferrin. As a result, we confirmed that the serum level of lactotransferrin decreased after surgery in all the samples (Figure 4).

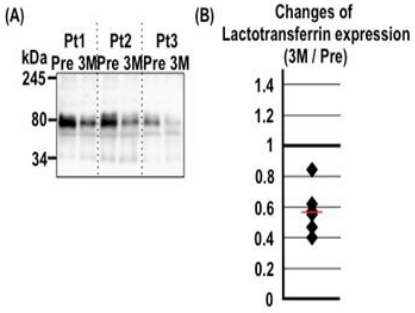

Figure 4: Western blot analysis of lactotransferrin.

(A) We compared the signals between groups Pre and 3M. The samples that were used in this analysis were three of the six patients with OA (Pt: Patient).

(B) Fold changes between groups Pre and 3M (red bar: mean = 0.56, n = 6). We analysed the captured images using LAS4000 (Fuji Film) and Multi Gauge v3.1 (Fuji Film).

Discussion

To search for proteins that could reflect OA pathology, we collected pre-and post-surgical sera from six OA patients, followed by concentratingprotein in the serum samples with a random peptide column. The concentrated protein samples were analysed using MS, and proteins were identified that could reflect the OA status.In our study,we used a random peptide column to concentrate proteins from human OA cartilage [19]. To verify whether this concentration method would also be effective for human serum samples,we concentrated human sera with a random peptide column and analysed it by MS. As a result, the total number of identifiable proteins increased from 171 to 310 (Figure 1B).This indicates that sample concentration using a random peptide column was just as effective for blood sera. Consequently, we determined that this protein concentration method was an effective sample concentration technique for comprehensive proteomic analysis experiments.

Lactotransferrin, is an 80 kDa iron-binding protein present in the blood and exocrine fluid, and is a member of the transferrin family [24]. The term 'lactoferrin' is widely known, and it is abundant in saliva, bile, pancreatic juices and tears. It is also found in bovine milk and human milk [25,26]. It is secreted from the granules of neutrophils [27] where it is released at the site of inflammation and inhibits bacterial growth by binding with iron ions [28]. Lactotransferrin is also known to have other effects such as the inhibition of tumour cell proliferation, protection of red blood cells from oxidation, and activation of macrophages [29]. It is a known as a biomarker for inflammatory bowel disease and colon cancer. After measuring lactotransferrin levels in ascitic fluid, Lee et al. [30] reported that they were significantly higher in patients with cirrhosis complicated by spontaneous bacterial peritonitis. In contrast, in patients with liver cirrhosis without bacterial peritonitis, high lactotransferrin levels in ascitic fluidare correlated witha higher incidence of hepatocellular carcinoma.

Hyaluronic acid and type IV collagen are used as diagnostic markers for liver cirrhosis, and they also reflect liver fibrosis. Therefore, it is generally considered that there is little correlation between these proteins and increases in lactotransferrin levels resulting from inflammation. In addition, lactotransferrin levels in the faeces of children with inflammatory bowel disease arereportedly to be significantly higher than those in healthy children, and are related to disease severity [31]. Sjögren syndrome is an autoimmune disease that targets exocrine glands at sites where the autoantibodies SS-A (also called Ro RNA particle) and SS-B (also called La snRNP)are produced [32]. Lacrimal and salivary glands are particularly vulnerable, and dry eye is the principal symptom. Lactotransferrin is also being researched as a biomarker for Sjögren syndrome [33]. Tears that were obtained from Sjögren patients and patients with only dry eye were analysed by MS and compared with those of dry eye patients. Fifty-six proteins were identified, including defensin α1, clusterin and lactotransferrin. From the above findings, we speculate that lactotransferrin levels in body fluids and exocrine fluids increase in response to local inflammation, and are therefore indicative of local inflammation. It is also possible that there is a correlation between the rateof this increase and the severity of a patient's symptoms.

Until now, not many reports relating to lactotransferrin levels and OA have been published. In recent years, the inhibition of IL-1β-induced chondrocyte apoptosis using200 □g/ml lactotransferrin has been reported [34],as well as the use of recombinant human lactotransferrin in inhibiting dexamethasone-induced chondrocyte apoptosis at similar concentrations [35]. Another study showed that in OA patients when chondrocytes were treated with camel lactotransferrin, IL-1β inducing activity, the nuclear import of nuclear factor kappa B p65, COX-2 mRNA and protein expression, and prostaglandin E2 production, were blocked [36]. Research on bovine lactotransferrin indicates that these results were due to the activation of ERK1/2, p38 and Aktby lactotransferrin [37]. In addition, a study in mice considered the possibility of oral OA treatment with 100% iron-saturated bovine lactotransferrin [38].

Lactotransferrin levels are also elevated in hepatocellular carcinoma, inflammatory bowel disease and colon cancer. As any of the aforementioned diseases can result in localised or full-body inflammation, it is thought that degranulation of neutrophils causes an increase in lactotransferrin that can be observed in bodily fluids, such as blood and ascitic fluid. It is also conceivable that the pre- and post-surgical fluctuations in lactotransferrin levels in the present experiment were influenced by inflammatory diseases other than OA. However, pre-surgical elevations did not suggest

the presence of such inflammatory diseases in any of the patients studied. Nonetheless, the fluctuations in lactotransferrin levels may have reflected extreme cases of localised inflammation that were not reflected in the blood collection data, similar to that seen for OA with a treatment-related decline.

The present our study demonstrated a significant decline in post-surgical lactotransferrin levels. LECT2 or brain and acute leukaemia, cytoplasmic (BAALC)were not found among the proteins identified in this experiment. PRDX6 was listed but was below the detection sensitivity threshold. In contrast to the presence of lactotransferrin in high levels in bodily fluids, LECT2 is primarily expressed mainly in the liver [39]. BAALC and PRDX6 are expressed in high levels in OA cartilage [19]. Because the molecules localises to specific cells and tissues, some molecules fall below the detection sensitivity threshold in serum samples, thus remaining undetected.

Until now, researchesrelated with OA have primarily been conducted by comparing OA patient groups with healthy control groups. Very few studies similar to ours, where comparisons were made between an individual patient's pre- and post-surgical samples. Based on the present within-subject comparison study, it can be concluded that proteins found in low levels in the disease groups may be overlooked,despite their usefulness as substances that reflect the OA status. In the future research, the proteins identified in this study should be validated using a cohort study with a larger sample size in order to consider theirapplication in determining OA status.

Conclusion

Using comprehensive proteomic analysis,we found that lactotransferrin may indicate OA status before and after total joint replacement surgery. Using western blot analysis, we confirmed that the concentration of lactotransferrin in serum decreased after the surgical procedure in all six patients. We need to study lactotransferrins' usefulness as an objective theranostic marker of OA. The serum level of lactotransferrin may reflect curative effects of OA treatment. It is necessary to confirm our finding using cohort samples.

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