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Comparative Proteomic Analyses of Synovial Fluids and Serums from Rheumatoid Arthritis Patients

Ran Noh[1], Sung Goo Park[1], Ji Hyeon Ju[2], Seung-Wook Chi[1], Sunhong Kim[1], Chong-Kil Lee[3], Jeong-Hoon Kim[1]*, and Byoung Chul Park[1]*

[1]*Medical Proteomics Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-333, Republic of Korea*
[2]*Rheumatism Research Center, Catholic Research Institute of Medical Science, Catholic University of Korea, Seoul 137-701, Republic of Korea*
[3]*Department of Pharmacy, Chungbuk National University, Cheongju 361-763, Republic of Korea*

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**Corresponding authors*
B.C.P.
Phone: +82-42-860-4260;
Fax: +82-42-860-4269;
E-mail: parkbc@kribb.re.kr
J.-H.K.
Phone: +82-42-860-4264;
Fax: +82-42-860-4269;
E-mail: jhoonkim@kribb.re.kr

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Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disorder that primarily affects the flexible joints and may also affect a number of tissues and organs. The progression of RA involves an inflammatory response of the capsule around the joint, swelling of synovial cells with excess synovial fluid (SF), and the development of fibrous tissue in the synovium. Since the progressive pathology of the disease often leads to the irreversible destruction of articular cartilage and ankylosis of the joint, early diagnosis of RA is essential. Thus, we undertook a comparative proteomic approach to investigate novel biomarkers for early diagnosis using SFs and serums from RA patients. As a result, we identified 32 differentially expressed spots in SFs and 34 spots in serums. The differential expression of the STEAP4 and ZNF 658 proteins were validated using immunoblotting of the SFs and serums, respectively. These data suggest that differentially expressed proteins in SFs and serums could be used as RA-specific biomarkers for the diagnosis and monitoring of RA. Furthermore, these findings advance our understanding of the molecular etiopathogenesis of RA.

Keywords: Rheumatoid arthritis, synovial fluid, serum, proteomic analyses

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovial membrane (synovium) that surrounds the joint, often leading to the destruction of the cartilage, bone, and ligaments, causing deformity of the joints [13, 11]. The progression of RA can ultimately result in joint destruction, functional declines, work disability, and enhanced mortality [13, 11]. Although the etiology of RA is multifactorial, genetic risk factors are estimated to account for roughly 50% of the etiology [4, 10]. The human leucocyte antigen (HLA) alleles have been demonstrated to be one of the genetic risk factors associated with RA, even though the association between HLA and RA is still incompletely understood [3, 8, 13, 18, 27]. The remaining etiologies can be attributed to environmental factors, such as infectious agents, oral contraceptives, and smoking [18, 27].

The diagnosis of RA is based on clinical examination of symptoms, patient history, and serological laboratory tests [2]. Serological diagnostic testing is of growing importance in the early detection and differentiation of RA [9, 25, 26]. Most RA patients generate rheumatoid factor (RF), which is an abnormal autoantibody against immunoglobulin G (IgG) [20, 24]. RF was the first autoreactive element identified in the serums of RA patients [24]. Thus, it was commonly used as a blood test for the diagnosis of RA in the past. However, the specificity of RF is limited, as it is also found in patients with other autoimmune diseases and infectious diseases, and even in healthy individuals [24]. In order to develop novel serological markers for RA, several autoantibodies have been investigated in the serums of RA patients. Autoantibodies such as anti-RA33, anti-calpastatin, anti-Sa, anti-perinuclear factor (APF), and anti-keratin antibodies (AKA) have been well studied, and are routinely observed in RA [26, 27]. However, their clinical use is

limited because of their lower specificity and sensitivity in the diagnosis of RA.

The identification of biomarkers related to RA progression would be of great clinical importance, not only for the early and differential diagnosis of RA in comparison with other diseases showing arthritis characteristics, but also for its treatment and the development of experimental and clinical therapeutic trials [4]. Furthermore, RA biomarker identification may further our understanding of the disease. Synovial fluid (SF) is in contact with the primary tissues affected by RA (cartilage and synovium) and has been implicated in disease pathophysiology; it is therefore an excellent source for the discovery of biomarkers [4, 19]. In addition, the use of serum is fast and easy and offers practical advantages in the diagnosis of RA.

Here, we used proteomic approaches using SFs and serums from RA patients and non-RA patients in order to discover novel diagnostic and/or prognostic markers for RA. We carried out two-dimensional (2D) gel electrophoresis followed by in-gel proteolytic (enzymatic) digestion and mass spectrometric analyses. For this purpose, SF and serum samples derived from 38 individuals were analyzed.

Materials and Methods

Patients and Sample Preparation

All of the human SF and serum samples used in this study were provided by the Catholic University of Korea. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Approval by the ethics committee of Seoul St. Mary's Hospital (Seoul, Republic of Korea) was obtained for all procedures. The SF samples were obtained from 11 RA patients and 15 non-RA patients and the serum samples were obtained from four normal individuals, four early RA patients, and four active RA patients whose ages ranged from 27 to 59 years. Each sample was pooled and centrifuged at 12,000 rpm for 30 min at 4°C to remove the precipitates. Pooled SF and serum samples were loaded onto a Multi Affinity Removal Spin Cartridge (Agilent, USA) according to the manufacturer's instructions. Depleted samples were precipitated using trichloroacetic acid (TCA) to concentrate the proteins and remove salts and detergents.

Two-Dimensional Gel Electrophoresis Analysis

The protein samples were prepared for 2D electrophoresis (2DE) using a 2D Clean-Up Kit (GE Healthcare, USA) to remove salt content. Electrophoresis in the first dimension was carried out in an IPGPhor system (GE Healthcare). IPG strips (linear pH 4–7, 13 cm) were rehydrated overnight in 250 µl of rehydration buffer (9 M urea, 2% CHAPS, 4% thiourea, IPG buffer pH 4–7, 40 mM DTT). Isoelectric focusing (IEF) was carried out using a multistep protocol (1 min at 300 V, 90 min at 3,500 V, 15–16 h at 3,500 V, 30 min at 300 V). Before electrophoresis in the second dimension, each strip was equilibrated in two steps (15 min each) in 7 ml of freshly prepared sample buffer (1.5 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) supplemented with 450 µl of DTT and 0.17 g of iodoacetamide, respectively. Second-dimension electrophoresis was carried out at 40 V on an SDS-polyacrylamide gel using the Tris-glycine system. The samples were run until the bromophenol blue dye reached the bottom of the gel. Finally, the gels were silver-stained. The stained gels were scanned as 8-bit grey-scale tiff images with ImageScanner III, which is a modified version of Epson Expression 10000 XL (GE Healthcare). For 2DE gel image analysis, the Progenesis Samespots software (Nonlinear Dynamics, UK) was used. Protein spots in the 2DE gels were quantified by normalizing spot volumes using this software. After matching the enhanced spot images, the spots were excised manually.

In-Gel Enzymatic Digestion of Proteins

Before digestion, the excised spots were destained using destaining buffer (30 mM $K_3[Fe(CN)_6]$ and 100 mM $Na_2S_2O_3$). The spots were then washed with HPLC water. The excised spots were subjected to in-gel digestion with trypsin. The gel pieces were washed with 50 mM NH_4HCO_3 solution and 100% acetonitrile (ACN) for the reduction of DTT and alkylation of IAA. After the wash step, the peptide samples were dried in a speed-vac system. Next, a solution of 50 mM NH_4HCO_3 solution containing trypsin was added. The samples were then incubated at 37°C for 1 h. Subsequently, 200 µl of 50 mM NH_4HCO_3 solution was added, and the samples were incubated at 37°C overnight. The peptides were further extracted by adding 100% ACN and 5% formic acid (FA) solutions. After the extraction of the peptides, the samples were dried in a speed-vac and cleaned using ZipTip pipette tips (10 µl pipette tip, Millipore), which contained a C18 reversed-phase column for desalting, purifying, and concentrating peptides and proteins. The samples were then dried once again in a speed-vac and subsequently dissolved in a 3% ACN and 0.1% FA solution prior to matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS) analysis.

MALDI-TOF-MS Analysis

Mass spectrometry (MS) was performed at the Yonsei Proteome Research Center (YPRC, Korea). SF and serum peptide profiles were analyzed with a MALDI-TOF/TOF mass spectrometer (4800 ABSciex, USA). For sample preparation, 70% ACN was allowed to flow through the porous tip to remove impurities attached to the resin. Next, a solution of 2% formaldehyde was passed through the column to create acidic conditions. Following this pretreatment, the samples to be analyzed were passed through the column. Subsequently, the wash buffer (2% FA) was used to remove impurities such as salt and chemicals. Finally, the peptides attached to the resin were eluted in a drop-by-drop manner using the elution buffer.

Database Search and Protein Identification

MS and MS/MS data were used in subsequent searches by Mascot software (Matrix Science; http://www.matrixscience.com) using the MSDB protein sequence database for human proteins.

Antibodies and Immunoblotting

The anti-STEAP4 antibody was purchased from Sigma (USA; PRS4313) and the anti-ZNF658 antibody was purchased from Abcam (UK; ab140090). Immunoblotting was performed according to the manufacturer's instructions.

Results and Discussion

Immunodepletion of Abundant Proteins from SFs and Serums

Proteins such as albumin, immunoglobulin, and apolipoproteins are abundant in the human body fluids such as blood, plasma, and SFs [12] and they may interfere the identification of other potentially important spots of interest in 2DE analyses. To circumvent this issue of interference, we decided to remove common proteins that are abundant in human SFs and serums. The SFs from RA patients (n = 11) or non-RA patients (n = 15) were pooled and then cleared using a cartridge that removes 14 abundant proteins (albumin, IgG, IgA, antitrypsin, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin). Likewise, serums derived from early RA patients (n = 4), active RA patients (n = 4), and normal individuals (n = 4) were pooled for analysis, and a cartridge known to remove six abundant proteins (albumin, IgG, IgA, antitrypsin, transferrin, and haptoglobin) was used. To check the efficiency of the immunodepletions, the protein profiles of non-depleted and depleted samples were analyzed by SDS-PAGE (Figs. 1A and 1B, respectively). Through this step, the albumin, IgG, and other highly abundant proteins in the samples were successfully removed.

Identification of RA-Specific Proteins in SFs by 2DE Proteomic Analysis

Proteomic analyses of SFs from arthritis patients suffering from RA or non-RA were analyzed by 2DE in order to identify novel biomarkers for the early diagnosis and prognosis of RA. Based on our 2DE preliminary results, pH 4–7 strips were used for this purpose. Since the SF consisted of relatively few proteins, very small quantities of proteins were observed in the 2D image (Figs. 2A and 2B). For reproducibility, experiments were conducted at least three times; the spots that were reproducible and differed between the test groups were selected. In the RA SFs, 29 differentially expressed spots were observed, whereas three spots were observed in the non-RA synovial fluids. These protein spots were identified using MALDI-TOF/TOF MS and a subsequent MASCOT database search (Table 1). Among the 29 RA-specific proteins, four proteins were identified as an immunoglobulin heavy-chain variable region, which is consistent with the chronic inflammation observed in RA patients. Interestingly, STEAP4 was specifically found in RA SF (Table 1). STEAP4, a homolog of TIARP, has been found to be overexpressed in synovium

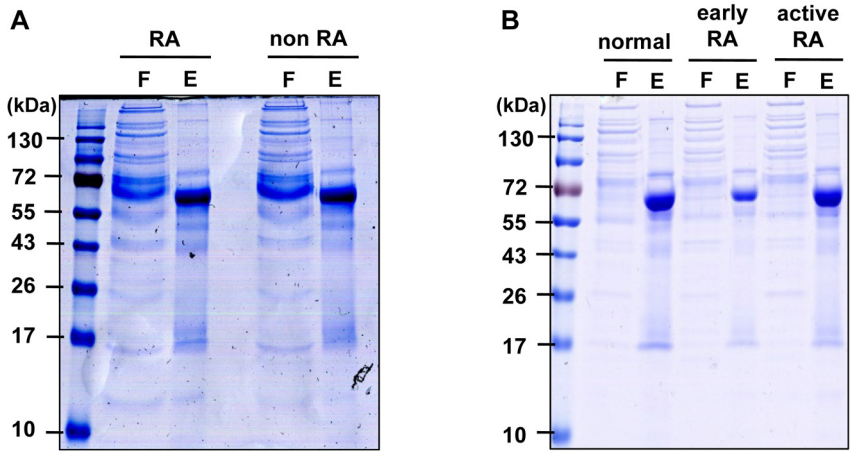

Fig. 1. Immunodepletion of SFs and serums from Rheumatoid arthritis(RA) and non-Rheumatoid arthritis(non-RA) patients.
The immunodepletion steps were confirmed by SDS-PAGE and subsequent Coomassie blue staining. (**A**) SFs from RA and non-RA patients were immunodepleted using Multi Affinity Removal Spin Cartridges. (**B**) Serums from RA and non-RA patients were immunodepleted using Multi Affinity Removal Spin Cartridges. F, flow-through fraction; E, elution.

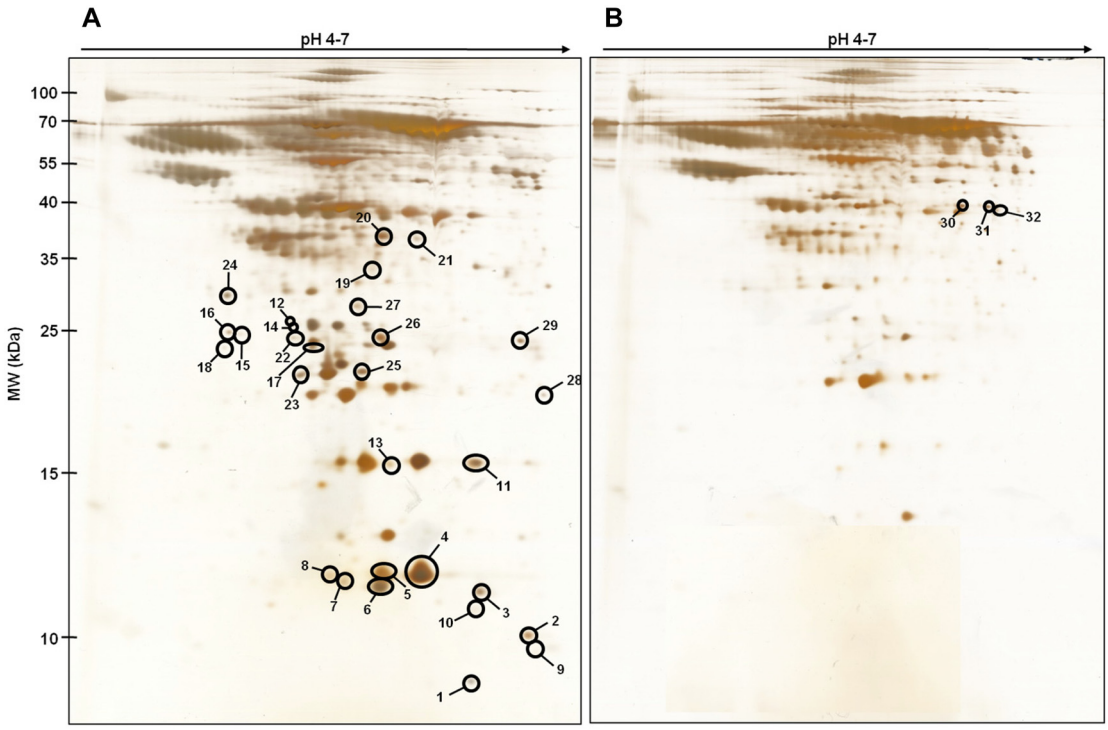

Fig. 2. Two-dimensional gel image of SFs from RA and non-RA patients.
Immunodepleted proteins (100 μg) from RA and non-RA patients were separated by 2DE and stained with silver staining. A total of 32 protein spots were visualized. (**A**) Twenty-nine different spots were observed to be RA-specific. These protein spots identified using MALDI-TOF/TOF MS are indicated by numbers at their spot positions in the gel (Table 1). (**B**) Three spots were observed in the non-RA samples.

from RA patients, and is known to regulate IL-6, IL-8, and cell proliferation [7, 22]. Overexpressed STEAP4 might be released upon RA-associated chronic inflammation. In addition, major histocompatibility complex (MHC) class I antigen was identified only in RA SF, whereas MHC class II antigen was identified in non-RA SF. Based on the mass spectrometry results, this difference could be attributed to the differences in antigen processing and presentation between RA and non-RA immune systems (Table 1). There are two pathways for antigen processing and presentation in cells. In the MHC class I pathway, peptides generated from the degradation of cytosolic proteins by proteasomes are bound to an MHC class I molecule, and ultimately presented to cytotoxic T cells ($CD8^+$) [5, 15]. On the other hand, in the MHC class II pathway, peptides generated from the degradation of endogenous proteins by proteases from endosomes and lysosomes are bound to MHC class II molecules, and consequently presented to the helper T cells ($CD4^+$) [1, 23].

The molecular weight of the identified proteins was lower than expected. Presumably, the proteins may be cleaved or degraded as a result of cell lysis, which is mediated by the chronic inflammation. The 23 differentially expressed proteins in the SFs of RA and non-RA patients found by 2DE are listed in Table 1.

Identification of RA-Specific Proteins in Serums by 2DE Proteomic Analysis

In order to examine the change in proteomic patterns during RA progression, serums from four normal individuals, four patients with early RA, and four patients with active RA were analyzed in the same manner as described for SF. A total of 34 different spots were observed in the 2DE analysis (Fig. 3). Fifteen spots disappeared during RA progression, 11 spots were found in normal serum, 6 spots were found in early RA serum, and 2 spots were found in active RA serum. These spots were identified using MALDI-TOF/TOF MS analysis (Table 2). Of the 15 spots that disappeared during RA progression, 8 were identified as keratins (cytokeratin 9, keratin 1, or keratin 16), whereas keratin was only identified once in the other groups. Vimentin also disappeared during RA progression. This result is intriguing because keratin and vimentin have been demonstrated to be posttranslationally modified (citrullination

Table 1. List of proteins different in synovial fluid (SF) from rheumatoid arthritis (RA) and non-RA patients.

	Spot number	NCBI Accession Number	Protien name	Coverage (%)	Mascot score[a]	Matched peptides
RA-specific proteins	1	gi|119577202	Ryanodine receptor 1 (skeletal), isoform CRA_d	7	69	18
	2	gi|119577297	Ribosomal protein S16	39	74	6
	3	gi|112700829	Immunoglobulin heavy-chain variable region	79	48	4
	4	gi|435476	Cytokeratin 9	21	71	9
	5	gi|47156647	Immunoglobulin heavy-chain variable region	82	49	4
	6	gi|435476	Cytokeratin 9	20	61	8
	7	gi|112700829	Immunoglobulin heavy-chain variable region	79	74	5
	8	gi|71051561	ZNF169 protein	62	60	5
	9	gi|119580263	HORMA domain-containing 2, isoform CRA_a	31	70	8
	10	gi|62871242	Immunoglobulin-alpha heavy-chain variable region	63	49	5
	11	gi|18848326	Rab GDP dissociation inhibitor beta	38	66	6
	12	gi|21961605	Keratin 10	25	111	15
	13	gi|435476	Cytokeratin 9	26	61	8
	14	gi|31982909	Zinc finger protein 394	18	54	9
	15	gi|435476	Cytokeratin 9	26	66	9
	16	gi|110624781	Myosin-13	9	52	14
	17	gi|119602291	Zinc finger and BTB domain-containing protein 42	17	62	11
	18	gi|119604675	Zinc finger protein 563, isoform CRA_b	56	53	6
	19	gi|47132620	Keratin, type II cytoskeletal 2 epidermal	28	70	11
	20	gi|47124562	Haptoglobin	33	69	9
	21	gi|119597845	Guanine nucleotide-binding protein (G protein), beta-5	44	67	8
	22	gi|5911871	Solute carrier family 12 (potassium/chloride transporters), member 7	39	57	8
	23	gi|239812496	MHC class I antigen	60	67	6
	24	gi|435476	Cytokeratin 9	43	155	18
	25	gi|119613847	TRAF3 interacting protein 3	17	53	8
	26	gi|435476	Cytokeratin 9	24	69	10
	27	gi|189054178	Keratin 1	30	96	13
	28	gi|34366439	Cytoplasmic dynein 1 light intermediate chain 1	22	61	8
	29	gi|119597313	STEAP family member 4	25	67	8
non-RA-specific proteins	30	gi|119395750	Keratin, type II cytoskeletal 1	26	66	9
	31	gi|20260702	MHC class II antigen	56	55	5
	32	gi|18874468	Partitioning-defective 3-like protein splice	13	48	11

[a]The respective protein scores have confidence region, and displayed along with the numbers with a 95% significance.
Here, that protein scores greater than 66 are significant.

or deamination) in RA patients. Citrullination (or deimination) is a posttranslational modification of the arginine residues in proteins by the enzyme peptidyl arginine deiminase (PADs), and citrullinated proteins within the rheumatoid joint would act as autoantigens in RA progression [21, 25, 26]. These results suggest that citrullinated vimentin or citrullinated keratin may be captured and aggregated by autoantibodies raised in RA patients. The 11 spots in the normal individuals were identified as apolipoprotein A-I, complement component C3, laminin receptor 1, and enolase 1. The six spots in the early RA patients were identified as calcium uptake protein 1, zinc finger protein

Table 2. List of proteins changed during RA progression.

	Spot number	NCBI Accession Number	Protein name	Coverage (%)	Mascot score[a]	Matched peptides
Disappeared proteins during RA progression	22	gi|119620272	Rho GTPase-activating protein 25	21	54	12
	28	gi|545257	Cytokeratin 9	37	71	14
	31	gi|239938886	Keratin, type I cytoskeletal 9	39	99	16
	34	gi|85566950	Family with sequence similarity 83, member B (Protein FAM83B)	21	72	16
	36	gi|545257	Cytokeratin 9	39	75	13
	40	gi|74752614	Transcription elongation factor A protein-like 2	36	56	7
	47	gi|119581139	Keratin, hair, acidic, 2	33	74	12
	48	gi|435476	Cytokeratin 9	40	112	17
	51	gi|83318444	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	35	71	15
	52	gi|435476	Cytokeratin 9	36	73	12
	55	gi|62414289	Vimentin	49	155	22
	62	gi|17426164	Macrophin 1 isoform 2	10	73	43
	64	gi|119610330	A kinase (PRKA) anchor protein 4, isoform CRA_a	30	53	13
	73	gi|186772	Keratin 1	42	93	16
	75	gi|1195531	Type I keratin 16	35	71	12
Normal specific proteins	5	gi|347447518	Human apolipoprotein A-I	51	72	10
	8	gi|55958061	Inter-alpha (globulin) inhibitor H2	43	93	13
	9	gi|347447518	Human apolipoprotein A-I	60	121	13
	12	gi|5531813	Dynein light chain-A	34	75	13
	17	gi|221039556	Tubulin alpha-1C chain	42	93	16
	19	gi|161761214	Laminin receptor 1 (ribosomal protein SA, 67 kDa)	45	89	13
	27	gi|239938886	Keratin, type I cytoskeletal 9	38	87	14
	44	gi|203282370	Human enolase 1	40	91	15
	45	gi|435476	Cytokeratin 9	41	93	16
	65	gi|5107637	Karyopherin beta2-Ran Gppnhp nuclear transport complex	35	66	9
	87	gi|78101272	Human complement component C3c	28	89	17
Early RA-specific proteins	13	gi|194390950	Calcium uptake protein 1, mitochondrial isoform 3	41	50	11
	14	gi|39644707	ZNF672 protein, partial	45	71	7
	15	gi|1195531	Type I keratin 16	49	137	20
	23	gi|119569562	Antigen KI-67 isoform 2	14	71	29
	26	gi|55665688	Vasohibin 2	29	64	9
	30	gi|42716289	Erythrocyte membrane protein band 4.1 isoform 2	20	53	12
Active RA-specific proteins	2	gi|134035376	Zinc finger protein 658	21	73	15
	32	gi|194391080	Serum albumin	41	141	22

[a]The respective protein scores have confidence region, and displayed along with the numbers with a 95% significance.
Here, protein scores greater than 70-71 are significant.

672, keratin 16, and vasohibin 2, which has been demonstrated as a positive regulator of angiogenesis [16, 17]. Interestingly, a correlation exists between the expression of vasohibin 1 and the histological inflammation score in RA synovial tissue [14]. Together, these findings and the literature highlight the feasibility of vasohibin 2 as a novel early diagnostic marker for RA. The two proteins identified in active RA were zinc finger protein 658 and serum albumin. Notably, zinc finger protein 658 was identified only in active RA, suggesting the possibility that it could be used

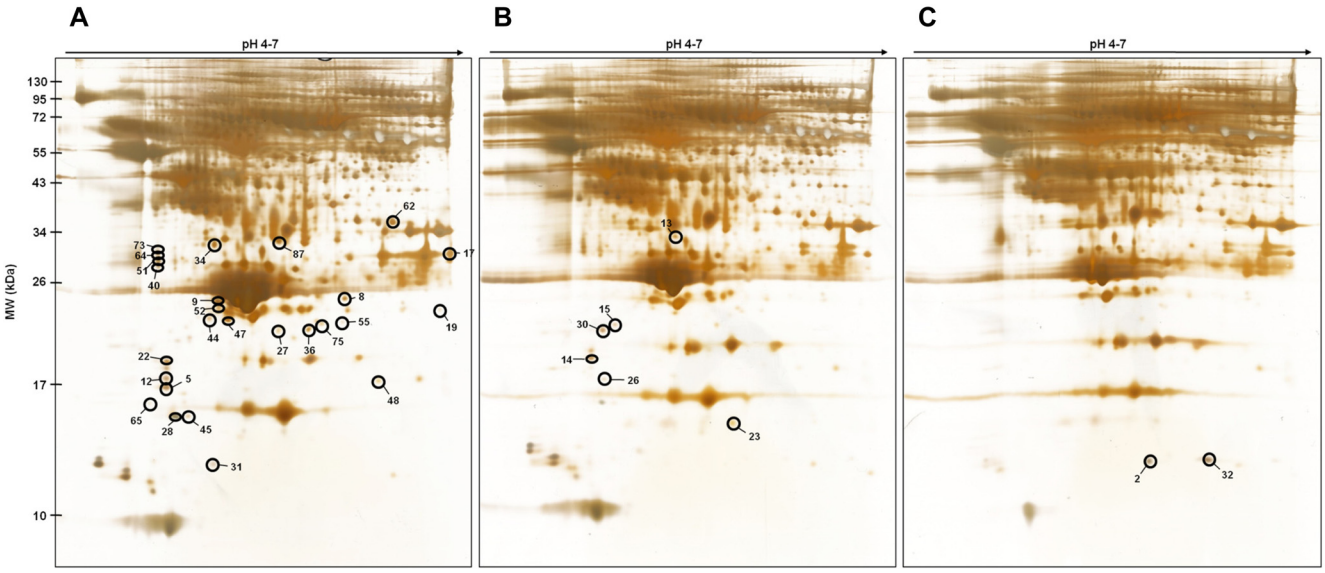

Fig. 3. Two-dimensional gel image of serums from RA and non-RA patients.
Immunodepleted proteins (300 μg) from normal patients, early RA patients, and active RA patients were separated by 2DE and stained with silver staining. A total of 34 protein spots were visualized. (**A**) Fifteen spots disappeared during RA progression and 11 spots were found in normal serum. (**B**) Six spots were found in early RA serum. (**C**) Two spots were found in active RA serum. These protein spots identified using MALDI-TOF/TOF MS are indicated by numbers at their spot positions in the gel (Table 2).

as a prognostic marker. The immune response protein complement component C3 was identified in the normal serum and not in RA serum, suggesting that the RA immune response is different from non-RA immune responses.

Validation of two RA-Specific Markers, STEAP4 and ZNF 658

In order to confirm the proteomic screening results, we carried out immunoblotting using commercially available antibodies. We tried immunoblotting with several antibodies, but most of them did not recognize proteins in the serum or SF. This might be due to insufficient quantities of the proteins in human samples. Among the candidate proteins, we observed that STEAP4 was detected in RA SF but not in non-RA SF, which is consistent with the mass spectrometry results (Fig. 4A; left panel). However, the level of STEAP4 did not change in the serums (Fig. 4A; right panel), indicating that it is specific for SF. Moroever, we also examined the protein level of ZNF 648 in the serum samples. In concordance with the previous mass spectrometry results, ZNF 658 levels increased during the progression of RA (Fig. 4B). These results suggest that at least two

Fig. 4. Validation of two RA-specific markers, STEAP4 and ZNF 658.
Immunodepleted proteins from SFs and serums were analyzed by immunoblotting. (**A**) Proteins prepared from SFs of RA or non-RA patients and serums of normal, early RA, or active RA patients were analyzed by immunoblotting using anti-STEAP4 antibody. (**B**) Proteins prepared from serums of normal, early RA, or active RA patients were analyzed by immunoblotting using anti-ZNF 658 antibody.

proteins identified in this study, STEAP4 and ZNF 648, could be applicable to the early diagnosis of RA using SF and serums.

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