Protein profiles of peripheral blood mononuclear cells as a candidate biomarker for Behçet's disease

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ABSTRACT

Objective. *To investigate the pathophysiology of Behçet's disease (BD) and find biomarkers for the disease, we analysed protein profiles of peripheral blood mononuclear cells (PBMCs).*

Methods. *Proteins, extracted from PBMCs, were comprehensively analysed in 16 patients with BD, 16 patients with rheumatoid arthritis (RA), 12 patients with Crohn's disease (CD), and 16 healthy control subjects (HC) by 2-dimensional differential gel electrophoresis (2D-DIGE). Differently expressed proteins were identified by mass spectrometry.*

Results. *563 protein spots were detected. We completely discriminated between the BD and HC groups, between the BD and RA groups, and between the BD and CD groups by multivariate analysis of intensity of 23, 35, and 1 spots, respectively. The spots contributing to the differences included proteins related to cytoskeleton, transcription/translation, T cell activation, bone turnover, regulating apoptosis, and microbial infection. Intensity of 3 spots (tyrosine-protein phosphatase non-receptor type 4, threonine synthase-like 2, and β-actin) provided area under the receiver operating characteristic curves (AUROC) of 0.889 for discrimination between the BD group and the non-BD groups. Informatively, intensity of the above 1 spot completely discriminated the CD group from the other groups (AUROC 1.000). This spot, identified as β-actin, had different pI from the above β-actin-spot probably due to different post-translational modification.*

Conclusion. *PBMC protein profiles, especially the profile of the 3 spots, would be candidate biomarkers for BD. The latter β-actin subtype would be useful for discriminating inflammatory bowel diseases from BD and other diseases. The identified proteins may play important roles in the pathophysiology of BD.*

Introduction

Behçet's disease (BD) is a systemic vasculitis characterised by recurrent attacks of acute inflammation (1, 2). Although the prognosis has been improved recently, the eye lesions still need intensive treatment such as anti-TNF-α therapies (3). The involvement of gastrointestinal tract, large vessels, and central nervous system remains life-threatening (1, 2, 4).

The etiology of BD remains unclear, however, pathogenic roles of cross-reactive immunity to microbial- and autoantigens have been proposed (2, 5, 6). BD patients possess γδT cells and autoantibodies reactive to both microbial heat shock protein (HSP) 65 and human HSP60 (5, 6). Since HSP60 is a ligand for toll like receptor (TLR) 2 and TLR4 expressed on monocyte/macrophages, it would activate transcription factors to produce inflammatory cytokines and induce Th1-type immune responses (7-9). Thereby, T cells in BD would be activated, recognising autoantigens presented by macrophages differentiated from monocytes (5, 7-9). A part of such T cells would drive B cells to produce autoantibodies (5, 7, 10). Thus, investigation of protein expression in peripheral blood mononuclear cells (PBMCs) would be useful to understand the pathophysiology of BD.

Diagnostically, biomarkers specific for BD have not been established yet. The strong association of HLA-B51 with BD (11) has been confirmed in the recent genome-wide association studies (GWAS) (12, 13). However, its prevalence in BD patients is less than 60% (11, 12). The GWAS also indicated BD-susceptible single nucleotide polymorphisms in IL-10 and IL-23R/

Table I. Clinical information of the BD patients.

Pt. no.	Sex/Age (y.o.)	Disease duration (years)	Main symptoms: Oral ulcers	Skin lesions	Eye lesions	Genital ulcers	Activity*	Laboratory data: ESR (mm/h)	CRP (mg/dl)	HLA-B51	Treatment: PSL (mg/d)	COL (mg/d)	Others
1	F/60	22	+	+	+	+	I	5	<0.03	n.e.	6	1	None
2	M/56	21	+	+	+	+	I	8	0.16	n.e.	3	1	SASP 1.0g/d
3	M/44	9	+	+	+	+	A	n.e.	0.14	n.e.	0	0.5	None
4	M/36	9	+	+	-	+	A	1	0.45	n.e.	9	1	5-ASA 1.5g/d
5	M/59	10	+	-	+	-	I	3	0.04	n.e.	0	1	LOX 180mg/d
6	F/26	2	+	+	+	+	I	7	0.04	n.e.	1	0	LOX 180mg/d
7	F/34	14	+	+	-	-	I	9	0.03	-	15	1	LOX 180mg/d
8	F/37	12	+	+	-	+	A	10	<0.03	n.e.	0	1.5	None
9	F/51	8	+	+	+	+	A	16	0.07	n.e.	5	1	CYA 125mg/d, 5-ASA 1.5g/d
10	F/29	2	+	+	+	+	I	16	<0.03	-	0	0	IFX 270mg/2m
11	F/44	10	+	+	+	+	A	5	<0.03	n.e.	0	0.5	CYA 75mg/d
12	M/53	34	+	+	+	+	I	35	0.12	n.e.	0	0.5	None
13	M/68	34	+	+	+	+	A	76	4.02	n.e.	0	0	None
14	F/68	8	+	-	-	-	I	4	0.03	n.e.	6	0.5	None
15	F/56	17	+	+	-	-	A	n.e.	<0.03	n.e.	4	0	None
16	F/17	3	+	+	-	-	A	48	1.9	+	25	1	None

Six men and 10 women, mean age 46 years, were enrolled.
*Disease activity of BD was assessed by the classification proposed by the BD Research Committee of Japan (Ref.2).
Pt.: patients; A: active; I: inactive; PSL: prednisolone; COL: colchicine; SASP: salazosulfapyridine; 5-ASA: 5-aminosalicylic acid; LOX: loxoprofen sodium; CYA: cyclosporine; IFX: infliximab; None: None of non-steroidal anti-inflammatory drugs, immunosuppressants, or biological drugs was used; n.e., not examined.

IL-12RB2 loci with weaker association (12, 13). Several proteomic studies have tried to establish BD biomarkers (14-19). Serum haptoglobin and amyloid A levels were higher in BD than in Vogt-Koyanagi-Harada syndrome (VKH) and healthy condition (15). The profile of 6 unidentified serum proteins discriminated BD from VKH and healthy condition fairly well (14). Autoantibodies against several proteins such as α-enolase have been detected in BD patients, which would be the candidate biomarkers (16-19). However, these biomarkers did not completely discriminate BD from other diseases.

We here found that PBMC protein profiles of BD patients were different from those of healthy control (HC) subjects and patients with rheumatoid arthritis (RA) and Crohn's disease (CD). Our study would provide a new insight into the pathophysiology and diagnosis of BD.

Materials and methods

Patients

PBMCs were obtained from 16 BD patients, 16 RA patients, and 16 HC subjects (6 men and 10 women, mean age 46 years [range 25–62 years]) (Table I, II). PBMC-derived protein samples obtained from 12 CD patients in the previous study (20) (except CD12) were also used. Disease activity of CD was assessed using a score proposed by international organisation for the study of inflammatory bowel disease (IOIBD) (active, IOIBD score ≥2; inactive, IOIBD score <2) (20). BD was diagnosed according to the criteria proposed by International Study Group for

Table II. Clinical information of the RA patients.

Pt. no.	Sex / Age (y.o.)	Disease duration (years)	DAS28-CRP*	Stages**	Laboratory data: ESR (mm/h)	CRP (mg/dl)	Treatment: PSL (mg/d)	MTX (mg/w)	Others
1	F/29	2	1.03	II	11	<0.03	0	8	IFX 150mg/2m
2	F/69	14	3.27	II	43	0.88	5	13	SASP 1.0g/d
3	M/60	3	1.95	I	15	0.03	0	6	None
4	F/61	36	1.61	II	n.e.	<0.03	8	8	None
5	F/47	4	1.81	I	11	<0.03	2	7.5	ETN 25mg/w
6	F/27	14	0.99	II	14	0.09	15	10	ETN 25mg/w
7	F/44	6	2.31	I	47	0.71	5	8	TAC 1.5mg/d, TCZ 370mg/m
8	M/68	7	1.04	II	6	0.16	3	6	None
9	F/53	2	1.06	II	20	0.32	0	8	ETN 25mg/w
10	F/33	7	2.17	I	19	0.36	6	0	None
11	M/52	5	4.39	I	9	0.60	0	10	None
12	F/37	7	3.17	I	32	1.83	0	0	SASP 1.0g/d
13	F/34	5	3.22	II	35	1.77	0	0	ETN 50mg/w
14	M/61	5	1.90	II	9	0.09	0	6	LOX 180mg/d
15	M/46	7	1.15	II	5	0.16	4	0	LOX 60mg/d
16	M/46	4	1.61	I	2	0.27	5	6	BUC 200mg/d, LOX 120mg/d

Six men and 10 women, mean age 47 years, were enrolled.
*Disease activity of RA was assessed using disease activity score (DAS) 28-CRP (active, DAS28-CRP ≥3.2; inactive, DAS28-CRP<3.2). **Steinbrocker stages of the patients are described.
Pt.: patients; PSL: prednisolone; MTX: methotrexate; IFX: infliximab; SASP: salazosulfapyridine; ETN: etanercept; TAC: tacrolimus; TCZ: tocilizumab; LOX: loxoprofen sodium; BUC: bucillamine; None: No non-steroidal anti-inflammatory drugs, immunosuppressants, or biological drugs were used; n.e.: not examined.

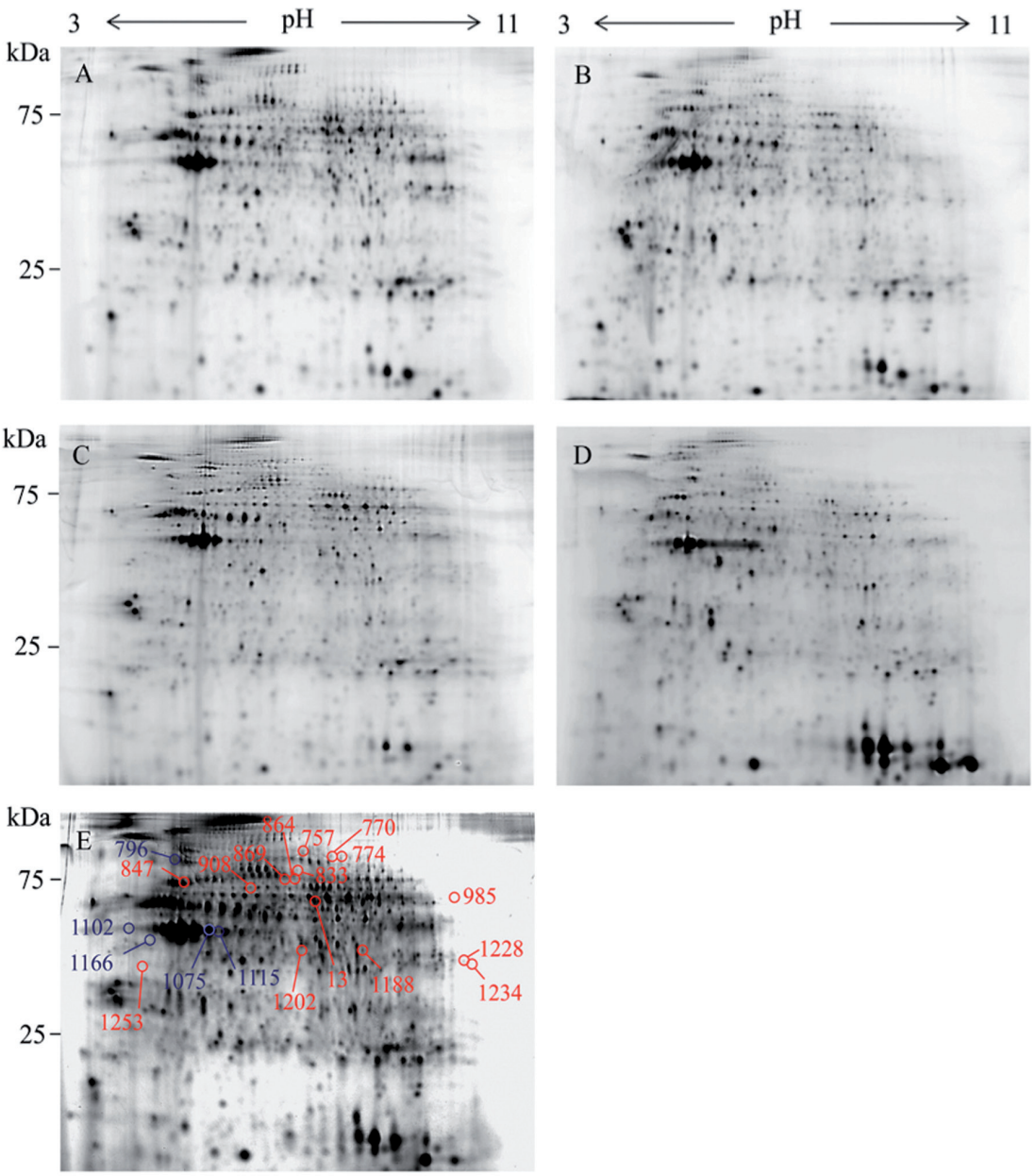

Fig. 1. 2D-DIGE of PBMC-derived proteins. Proteins were extracted from PBMCs obtained from 16 BD patients, 16 HC subjects, 16 RA patients, and 12 CD patients, and subjected to 2D-DIGE. Each protein sample labelled with Cy5 and an internal control standard sample labelled with Cy3 were electrophoresed on a gel by 2D-DIGE system. Representative results from a BD patient (Cy5, **A**), a HC subject (Cy5, **B**), an RA patient (Cy5, **C**), and a CD patient (Cy5, **D**) are shown. Location of the identified protein spots are indicated in the result of the standard sample (Cy3, **E**). The spots, intensity of which was significantly higher in the BD group than in the HC, RA, or CD group, are indicated by red circles. Conversely, the spots, intensity of which was significantly lower in the BD group than in the HC, RA, or CD group, are indicated by blue circles.

BD (21). RA was diagnosed according to the American Rheumatism Association 1987 revised criteria (22). The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, and was approved by the ethics committee in St. Marianna University School of Medicine.

Two dimensional-differential image gel electrophoresis (2D-DIGE) and identification of PBMC proteins

PBMCs were isolated by cell density-based centrifugation using Ficoll-Paque (GE Healthcare, Buckinghamshire, UK). To avoid platelet contamination, we re-

Table III. Numbers of protein spots with significantly different intensity between the BD and non-BD groups.

Spot intensity	Differences (folds)	Numbers of protein spots		
		BD/HC	BD/RA	BD/CD
Increased	5.0≤	0	0	9
	2.0≤	1	12	113
	1.5≤	5	33	191
	1.2≤	14	98	227
Decreased	- 1.2≥	9	17	141
	- 1.5≥	2	3	115
	- 2.0≥	0	0	73
	- 5.0≥	0	0	12

Numbers of protein spots, intensity of which was significantly altered in the BD group compared to that in the HC, RA, or CD group, are shown ($p<0.05$).

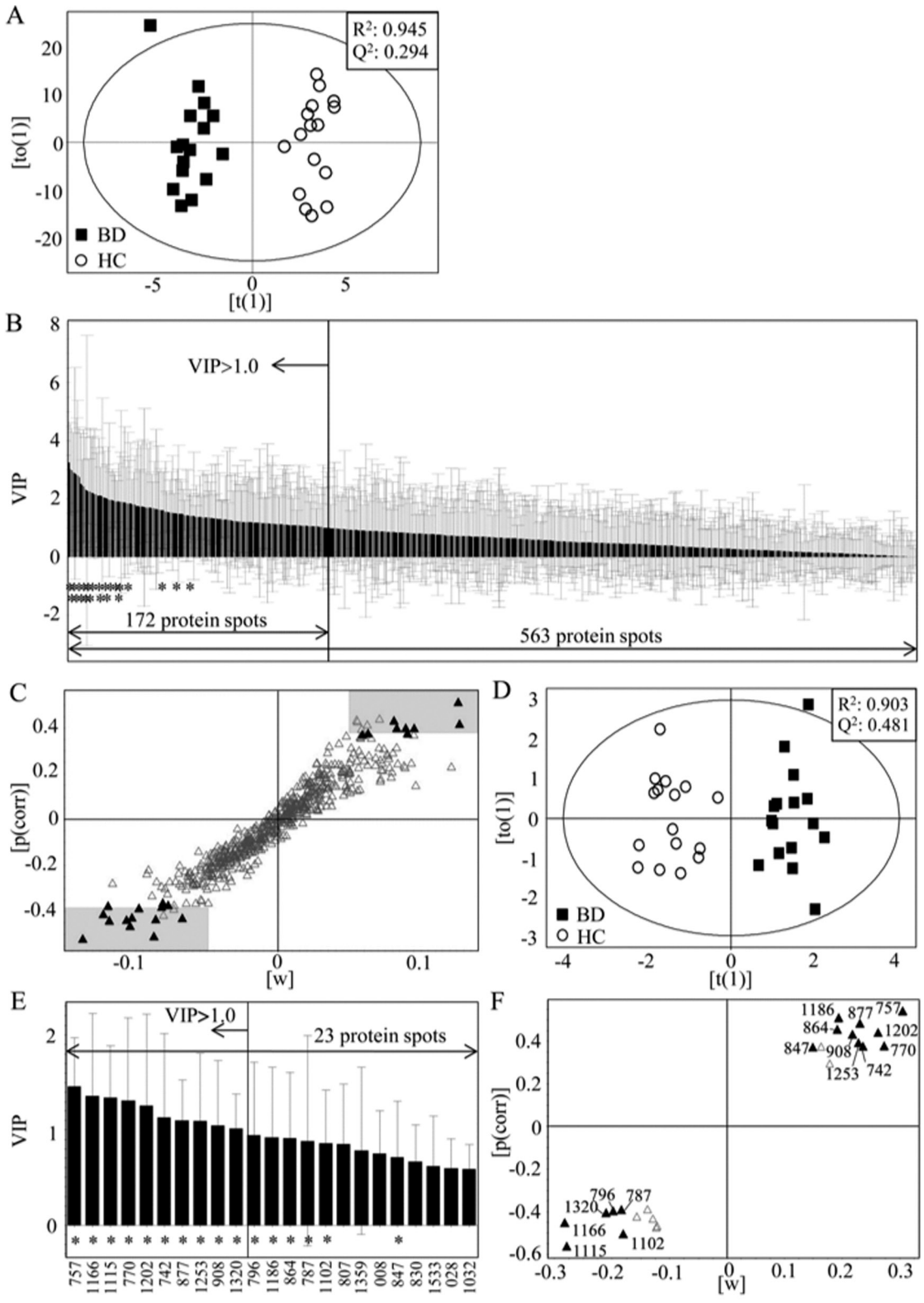

Fig. 2. Discriminant models between the BD and HC groups.
A. Intensity of all the 563 spots from 16 BD patients and 16 HC subjects was subjected to OPLS-DA (BD/HC-563 model). The x axis indicates the first principal component for the discrimination.
B. VIP of the 563 spots. Twenty-three out of the 172 spots (VIP>1.0), which highly contributed to the generation of this BD/HC-563 model, were used to generate the second discriminant model (BD/HC-23 model).
C. S-plot of the 563 spots. Magnitude (x axis) and reliability (y axis) of all the spots are visualised. Twenty-three spots selected from the spots with relatively high magnitude (|w|>0.05) and reliability (|p(corr)|>0.38) in this BD/HC-563 model (the upper right- and lower left-shaded zones) were used to generate the second discriminant model (BD/HC-23 model).
D. Intensity of the 23 spots with VIP>1.0, |w|>0.05, and |p(corr)| >0.38 in the BD/HC-563 model was subjected to OPLS-DA (BD/HC-23 model). The x axis indicates the first principal component.
E. VIP of the 23 spots.
F. S-plot of the 23 spots.
Asterisks (B, E) and closed triangles (C, F) indicate the spots intensity of which was at least 1.2-fold different between the BD and HC groups (p<0.05).

moved the plasma layer and washed the isolated PBMCs in calcium-free phosphate buffer saline. PBMC proteins were extracted and analysed by 2D-DIGE as previously described (20). In this study, an equal weight of proteins taken from all the BD, HC, and RA samples were mixed and labelled with Cyanine dye 3 (Cy3, Cy Dye DIGE Saturation dye, GE Healthcare) for preparation of an internal control "standard sample". 2.5 μg each of the BD, HC, RA, and CD protein samples was labelled with Cyanine dye 5 (Cy5, GE Healthcare). An isoelectric focusing gel, Immobiline Drystrip (pH 3-11, NL 24 cm, GE Healthcare), was used. Proteins were identified as described previously (20, 23).

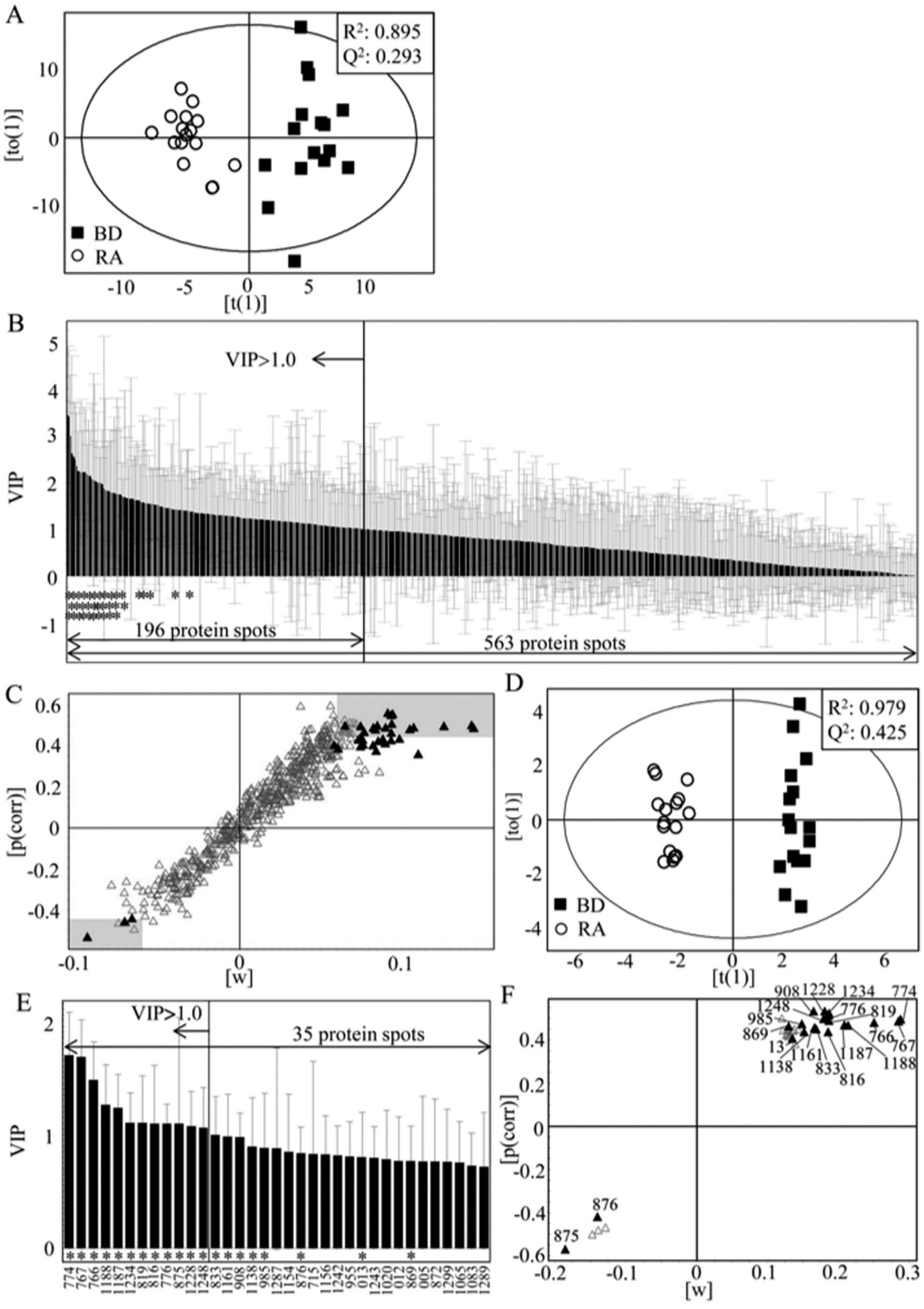

Fig. 3. Discriminant models between the BD and RA groups.
A. Intensity of all the 563 spots from 16 BD patients and 16 RA patients was subjected to OPLS-DA (BD/RA-563 model). The x axis indicates the first principal component for the discrimination.
B. VIP of the 563 spots. Thirty-five out of the 196 spots (VIP>1.0), which highly contributed to the generation of this BD/RA-563 model, were used to generate the second discriminant model (BD/RA-35 model).
C. S-plot of the 563 spots. Thirty-five spots selected from the spots with relatively high magnitude (|w|>0.06) and reliability (|p(corr)| >0.45) in this BD/RA-563 model (the upper right- and lower left-shaded zones) were used to generate the second discriminant model (BD/RA-35 model).
D. Intensity of the 35 spots with VIP>1.0, |w|>0.06, and |p(corr)| >0.45 in the BD/RA-563 model was subjected to OPLS-DA (BD/RA-35 model). The x axis indicates the first principal component.
E. VIP of the 35 spots.
F. S-plot of the 35 spots.
Asterisks (B, C) and closed triangles (E, F) indicate the spots intensity of which was at least 1.5-fold different between the BD and RA groups (p<0.05).

Statistical analysis

Significance of protein spot intensity difference in 2D-DIGE results was calculated by Student's *t*-test. Discriminant models were generated using orthogonal partial least square-discriminant analysis (OPLS-DA, SIMCA-P+, Umetrics, Umea, Sweden) (20). Receiver operating characteristic (ROC) analysis was conducted by JMP statistical software (SAS Institute, Cary, NC).

Results

Comparison of PBMC protein spot intensity between the BD and non-BD groups

To detect PBMC proteins differently expressed in BD, we analysed PBMC

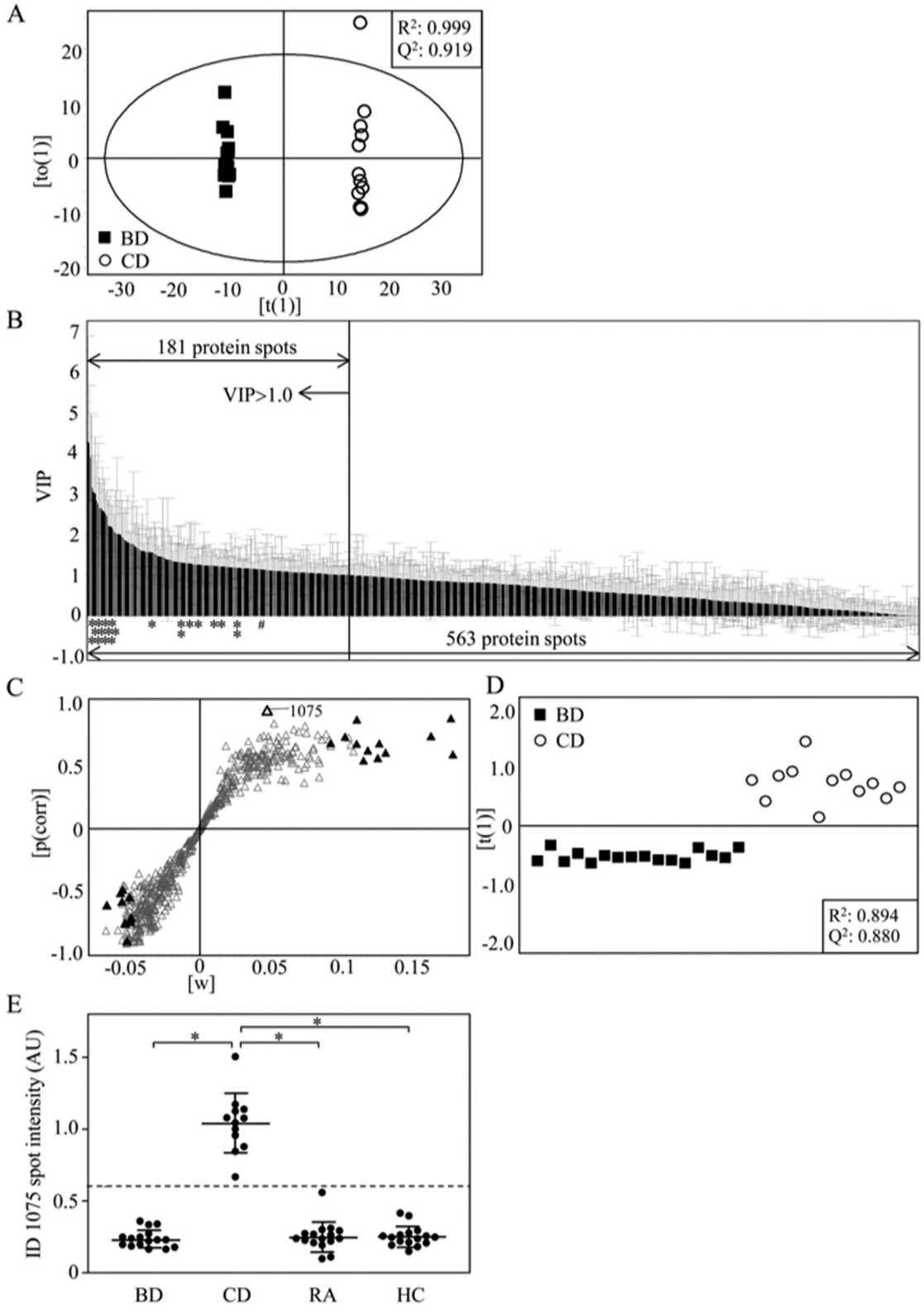

Fig. 4. Discriminant models between the BD and CD groups.
A. Intensity of all the 563 protein spots from 16 BD patients and 12 CD patients was subjected to OPLS-DA (BD/CD-563 model). The x axis indicates the first principal component for the discrimination.
B. VIP of the 563 protein spots. Asterisks indicate protein spots intensity of which was at least 5.0-fold different between the BD and CD groups (p<0.05). A single protein spot of ID 1075 (#) was used to generate the second discriminant model.
C. S-plot of the 563 protein spots. Closed triangles indicate the protein spots intensity of which was at least 5.0-fold different between the BD and CD groups (p<0.05). Intensity of ID 1075, which showed extremely high reliability (|p(corr)|=0.946), was used to generate the second discriminant model.
D. Intensity of ID 1075 was subjected to OPLS-DA. The y axis indicates the first principal component.
E. Intensity of ID 1075 (BD/CD-1 model) was significantly higher in the CD group than the BD, RA, and HC groups (*p<0.05). The intensity completely discriminated the CD group from the other groups. The broken line shows a cut-off value (0.60).

protein profiles of patients with BD, RA, and CD, and HC subjects by 2D-DIGE (Fig. 1). We selected 563 definite protein spots for quantitative analysis, and found that intensity of 14 spots was at least 1.2-fold higher in the BD group than in the HC group (p<0.05, Table III). Conversely, intensity of 9 spots was at least 1/1.2 (-1.2)-fold lower in the BD group than in the HC group.
The difference between the BD and RA groups was more distinct than that between the BD and HC groups. Intensity of 98 and 33 spots was at least 1.2-fold and 1.5-fold higher in the BD group than in the RA group, respectively (p<0.05, Table III). Conversely, intensity of 17 and 3 spots was at least -1.2-fold and

-1.5-fold lower in the BD group than in the RA group, respectively.
Interestingly, the difference between the BD and CD groups was much greater than those of the above 2 comparisons. Intensity of 227 and 9 spots was at least 1.2-fold and 5.0-fold higher in the BD group than in the CD group, respectively ($p<0.05$, Table III). Conversely, intensity of 141 and 12 spots was at least -1.2-fold and -5.0-fold lower in the BD group than in the CD group, respectively.

Discriminant models between the BD and HC groups

We tried to discriminate between the BD and HC groups by OPLS-DA, using the intensity of protein spots. A model generated using all the 563 spots accurately distinguished the 16 BD patients from the 16 HC subjects (BD/HC-563 model) (Fig. 2A). Variable of importance (VIP) scores of 172 out of the 563 spots were more than 1.0, indicating that these 172 spots highly contributed to the discrimination of the BD group from the HC group (Fig. 2B). The above-mentioned 14 and 9 spots with at least 1.2-fold intensity difference between the BD and HC groups (Table III) showed VIP scores of more than 1.0 in the BD/HC-563 model (Fig. 2B). Those spots were located at the left lower and right upper areas of the S-plot panel (Fig. 2C), which indicated their high potential as discriminants for BD and HC, respectively.
Next, we tried to find a minimal number of spots for the discrimination between the BD and HC groups. To achieve this, we gradually decreased number of the spots, retaining spots with high scores of VIP, reliability (p(corr)), and magnitude (w) in the BD/HC-563 model. We successfully discriminated between the BD and HC groups, using 23 protein spots with VIP>1.0, |p(corr)|>0.38, and |w|>0.05 in the BD/HC-563 model (BD/HC-23 model) (Fig. 2D). The explanatory variable (R^2) and the predictive variable (Q^2) of this model were 0.903 and 0.481, which indicated its high potential for the discrimination and the prediction, respectively (20). Sixteen out of the 23 spots in the BD/HC-23 model were the spots with at least 1.2-fold significant difference between the BD and HC groups (Fig. 2E-F, Table III).

Discriminant models between the BD and RA groups

Similarly, we tried to discriminate between the BD and RA groups. A discriminant model generated using the 563 spots (BD/RA-563 model) accurately distinguished the 16 BD patients from the 16 RA patients (Fig. 3A). The above-mentioned 33 and 3 spots with at least 1.5-fold intensity difference between the BD and RA groups (Table III) showed VIP scores of more than 1.0 in the BD/RA-563 model (Fig. 3B). The 33 and 3 spots were located at the right upper and left lower areas of the S-plot, indicating their high potential as discriminants for BD and RA, respectively (Fig. 3C).
Gradually decreasing the number of spots, we successfully discriminated between these 2 groups, using 35 spots with VIP>1.0, |p(corr)|>0.45, and |w|>0.06 in the BD/RA-563 model (BD/RA-35 model) (Fig. 3D). R^2 and Q^2 of this model was 0.979 and 0.425, which indicated its high potentials for the discrimination and the prediction, respectively. Twenty out of the 35 spots used in the BD/RA-35 model were the spots with at least 1.5-fold significant difference between the BD and RA groups (Fig. 3E - F, Table III).

Discriminant models between the BD and CD groups

A discriminant model generated using the 563 spots (BD/CD-563 model) accurately distinguished the 16 BD patients from the 12 CD patients (Fig. 4A). R^2 and Q^2 of this model were 0.999 and 0.919, which indicated its extremely high potential for the discrimination and the prediction, respectively. The above-mentioned 9 and 12 spots with at least 5.0-fold intensity difference between the BD and CD groups (Table III) showed VIP scores of more than 1.0 in the BD/CD-563 model (Fig. 4B). The 9 and 12 spots were located at the left lower and right upper areas of the S-plot, indicating their high potential as discriminants for BD and CD, respectively (Fig. 4C).
Surprisingly, we found that intensity of a single spot (ID 1075), which showed the highest reliability in the BD/CD-563 model (p(corr)=0.946), completely discriminated between the BD and CD groups (Fig. 4D). The intensity of ID 1075 (BD/CD-1 model) also completely discriminated the CD group from the RA and HC groups (Fig. 4E, Table IV).

Identification of the protein spots that contributed to the difference between the BD and non-BD groups

We tried to identify proteins contained in spots that greatly contributed to the difference between the BD and non-BD groups. For this aim, in the comparison of the BD and HC groups, we selected the 16 spots for protein identification, which showed at least 1.2-fold significant intensity difference and were also included in the BD/HC-23 model (Fig. 2EF). Similarly, in the comparison of the BD and RA groups, we selected the 20 spots which showed at least 1.5-fold significant intensity difference and were also included in the BD/RA-35 model (Fig. 3EF). In the comparison of the BD and CD groups, we tried to identify the spot of ID 1075 of the BD/CD-1 model (Fig. 4E). As a result, we identified 11, 9, and 1 protein spots in the BD *versus* HC comparison, in the BD *versus* RA comparison, and in the BD *versus* CD comparison, respectively (Table V).
Interestingly, in both of the BD *versus* HC and BD *versus* RA comparisons, 2 cytoskeleton-related proteins of radixin (ID833 and 864) and moesin (ID864 and 869) showed higher intensity in the BD group than in the HC and RA groups (Table V). Similarly, a phospho-lyase of threonine synthase-like 2 (THNS2, ID908) showed higher intensity in the BD group in both comparisons.
In the BD *versus* HC comparison, an apoptosis-regulating protein of dynamin-like 120kDa protein (ID770), a cytoskeleton-related protein of tyrosine-protein phosphatase non-receptor type 4 (PTN4, ID757), a cytokine of gremlin-2 (ID1253), and a protein involved in T cell activation of plastin-2 (ID847) showed higher intensity in the BD group (Table V). In contrast, a cytoskeletal protein of actin, cytoplasmic 1 (β-actin, ID1102, 1115, and 1166) and

an actin-binding protein of alpha-actinin-1 (ID796) showed lower intensity in the BD group than in the HC group.
In the BD *versus* RA comparison, a cytoskeleton-related protein of microtubule-associated protein 6 (MAP6) showed greatly higher intensity in the BD group (4.87-fold, ID774) (Table V). Three nucleoproteins of 39S ribosomal protein L3, mitochondrial (RM03, ID1188), heterogeneous nuclear ribonucleoprotein A1 (ROA1, ID1234), and non-POU domain-containing octamer-binding protein (NONO, ID985), and a protein involved in endocytosis of ras-related protein Rab-7a (ID1228) also showed higher intensity in the BD group than in the RA group.
In the BD *versus* CD comparison, spot ID 1075 was identified as β-actin (Table V).

Discrimination of the BD group from the non-BD groups, using a small number of protein spots

To serve a candidate combination of proteins for clinical laboratory examination, we tried to select a small number of spots that discriminated between the BD and non-BD groups by analysis of area under the ROC curve (AUROC). To achieve this, we generated OPLS-DA models using intensity of the identified protein spots. Specifically, the prediction values of BD and non-BD were defined as 1 and 0, respectively. The calculated values of individual patients and subjects were used to draw the ROC curve. As a result, the combination of 3 spots, ID757 (PTN4), ID908 (THNS2), and ID1102 (β-actin), showed the highest AUROC of 0.889, and provided sensitivity of 81.3% and specificity of 93.2% (BD/nonBD-3 model) (Table IV).

PBMC protein spots involved in the disease activity and the disease subtypes of BD

To find protein spots involved in the disease activity, we divided the 16 BD patients into 2 subgroups, the active (n=8) and inactive (n=8) groups (Table I). We found that intensity of 5 spots was at least 1.2-fold higher, and that of 18 spots was at least -1.2-fold lower, in the active group than in the inactive group (p<0.05, Table VI). Among the identified spots (Table V), ID 869 (moesin) and ID 985 (NONO) was included in the 18 spots lower in the active group (both, -1.65 fold, p<0.05). An OPLS-DA model using ID 869 and ID 985 (Active/Inactive model) showed AUROC of 0.891, sensitivity of 87.5%, and specificity of 100.0% (Table IV).
To find protein spots involved in the difference of disease subtypes of BD, we focused on the eye lesions (eye-lesion-present patients, n=10; eye-lesion-absent patients, n=6; Table I). We found that intensity of 5 spots was at least 1.2-fold higher, and that of 7 spots was at least -1.2-fold lower, in the eye-lesion-present group than in the eye-lesion-absent group (p<0.05, Table VI). Among those spots, ID 833 (radixin), the only identified spot (Table V), showed the greatest increase (2.46-fold) in the eye-lesion-presence to eye-lesion-absence comparison. An OPLS-DA model using ID 833 (Eye+/Eye- model) showed AUROC of 0.733, sensitivity of 70.0%, and specificity of 83.3% (Table IV).

Discussion

We comprehensively investigated protein expression in PBMCs of BD patients by 2D-DIGE (Fig. 1). In BD patients with severe arthralgia, it is needed to exclude RA. Approximately 10% of BD patients are associated with similar intestinal lesions to those of CD. Therefore, we compared PBMC protein profiles between BD and these diseases.
To exclude the possibility that difference of PBMC protein profiles simply reflects difference of the cell subpopulation, we investigated the proportion of lymphocytes to monocytes in all the groups. We found no significant difference between the BD group (mean±standard deviation, 4.43±2.03) and the non-BD groups (HC, 4.48±1.76, p=0.735; RA, 3.41±1.86, p=0.083; CD, 3.46±1.86, p=0.164). We also found no skew of the proportion by the PBMC isolation procedure (before, 4.58±0.25; after, 4.25±1.39; p=0.70; n=3).
PBMC protein profiles would be also affected by disease activity, disease subtypes, and treatment. Furthermore, the

Table IV. Discriminative ability of the OPLS-DA models generated with intensity of a small number of protein spots.

Models		Patient numbers			Sensitivity	Specificity	PPV	NPV	Accuracy	AUROC
			Observed							
			CD	nonCD						
BD/CD-1	Predicted	CD	12	0	100.0%	100.0%	100.0%	100.0%	100.0%	1.000
		nonCD	0	48						
			BD	nonBD						
BD/nonBD-3	Predicted	BD	13	3	81.3%	93.2%	81.3%	93.2%	90.0%	0.889
		nonBD	3	41						
			Active	Inactive						
Active/Inactive	Predicted	Active	7	0	87.5%	100.0%	100.0%	88.9%	93.8%	0.891
		Inactive	1	8						
			Eye+	Eye-						
Eye+/Eye-	Predicted	Eye+	7	1	70.0%	83.3%	87.5%	62.5%	75.0%	0.733
		Eye-	3	5						

nonCD, the BD, HC, and RA groups; nonBD, the HC, RA, and CD groups; Active, Inactive, subgroups in BD; Eye+, eye-lesion-present subgroup in BD; Eye-, eye-lesion-absent subgroup in BD; PPV, positive predictive value; NPV, negative predictive value.

Table V. Identification of protein spots that contributed to the difference between the BD and non-BD groups.

*Spot ID	Difference (BD/HC)	VIP (BD/HC-23)	MW (kDa)/pI (Observed)	Protein	Accession ID	MW (kDa)/pI (Theoretical)	Matched / Searched	Seq.cov. (%)	Mascot score	Sequence confirmed by LID (Mascot ion score)
770	2.20	1.31	101.4 / 7.10	Dynamin-like 120 kDa protein, mitochondrial	OPA1_HUMAN (gil215274226)	111.6 / 7.88	11 / 15	13	57	-
757	1.76	1.46	107.0 / 6.75	Tyrosine-protein phosphatase non-receptor type 4	PTN4_HUMAN (gil131531)	105.8 / 7.15	13 / 15	16	69	^{281}ETLLGFNMVNYRACK^{295} (4)
1202	1.46	1.26	33.6 / 6.67	ADP-ribosylation factor 4	ARF4_HUMAN (gil114123)	20.5 / 6.59	8 / 12	44	80	-
864	1.40	0.92	77.4 / 6.62	Radixin	RADI_HUMAN (gil464541)	68.5 / 6.03	8 / 11	9	103	^{238}IGFPWSEIR^{246}(12) ^{263}KAPDFVFYAPR^{273}(9) ^{264}APDFVFYAPR^{273}(30)
				Moesin	MOES_HUMAN (gil127234)	67.8 / 6.08	7 / 11	8	90	^{238}IGFPWSEIR^{246}(12) ^{263}KAPDFVFYAPR^{273}(9) ^{264}APDFVFYAPR^{273}(30)
1253	1.38	1.09	30.8 / 4.53	Gremlin-2	GREM2_HUMAN (gil62510699)	19.3 / 9.36	6 / 11	27	63	^{51}EVLASSQEALVVTER^{65}(20)
908	1.38	1.05	68.1 / 6.05	Threonine synthase-like 2	THNS2_HUMAN (gil313104279)	54.1 / 6.07	8 / 11	18	88	^{1}MWYVSTRGVAPR^{12}(3)
847	1.30	0.72	73.1 / 5.15	Plastin-2	PLSL_HUMAN (gil308153685)	70.2 / 5.29	15 / 23	25	126	-
1102	-1.29	0.87	41.7 / 4.4	Actin, cytoplasmic 1	ACTB_HUMAN (gil46397333)	41.7 / 5.29	8 / 10	25	323	^{29}AVFPSIVGRPR^{39}(25) ^{85}IWHHTFYNELR^{95}(45) ^{96}VAPEEHPVLLTEAPLNPK^{113}(61) ^{239}SYELPDGQVITIGNER^{254}(88) ^{360}QEYDESGPSIVHR^{372}(18)
796	-1.41	0.95	95.3 / 5.07	Alpha-actinin-1	ACTN1_HUMAN (gil46397817)	103.0 / 5.25	15 / 17	19	129	-
1115	-1.75	1.34	41.4 / 5.60	Actin, cytoplasmic 1	ACTB_HUMAN (gil46397333)	41.7 / 5.29	10 / 20	30	110	-
1166	-1.76	1.36	37.8 / 6.78	Actin, cytoplasmic 1	ACTB_HUMAN (gil46397333)	41.7 / 5.29	7 / 11	24	178	^{85}IWHHTFYNELR^{95}(43) ^{96}VAPEEHPVLLTEAPLNPK^{113}(28) ^{360}QEYDESGPSIVHR^{372}(16)

**Spot ID	Difference (BD/RA)	VIP (BD/RA-35)	MW (kDa)/pI (Observed)	Protein	Accession ID	MW (kDa)/pI (Theoretical)	Matched / Searched	Seq.cov. (%)	Mascot score	Sequence confirmed by LID (Mascot ion score)
774	4.87	1.70	101.1 / 7.24	Microtubule-associated protein 6	MAP6_HUMAN (gil 205830862)	86.5 / 9.20	15 / 19	26	85	-
833	2.50	1.00	84.7 / 6.66	Radixin	RADI_HUMAN (gil121137)	68.5 / 6.03	10 / 14	16	69	-
1188	2.37	1.26	34.6 / 7.51	39S ribosomal protein L3, mitochondrial	RM03_HUMAN (gil133116)	38.6 / 9.52	11 / 14	27	86	-
1234	1.91	1.11	31.4 / 8.98	Heterogeneous nuclear ribonucleoprotein A1	ROA1_HUMAN (gil288558857)	38.7 / 9.17	6 / 9	17	114	^{337}SSGPYGGGGQYFAKPR^{352}(16)
1228	1.84	1.07	31.8 / 8.90	Ras-related protein Rab-7a	RAB7A_HUMAN (gil1709999)	23.5 / 6.40	10 / 14	34	71	-
985	1.61	0.88	58.5 / 8.75	Non-POU domain-containing octamer-binding protein	NONO_HUMAN (gil67469924)	54.2 / 9.01	8 / 10	18	78	-
e13	1.58	0.80	59.3 / 6.88	Phosphatidylinositol 3-kinase regulatory subunit gamma	P55G_HUMAN (gil317373310)	54.4 / 9.01	11 / 17	21	84	-
908	1.55	0.98	68.1 / 6.05	Threonine synthase-like 2	THNS2_HUMAN (gil313104279)	54.1 / 6.07	8 / 11	18	88	^{1}MWYVSTRGVAPR^{12}(3)
869	1.53	0.76	77.6 / 6.54	Moesin	MOES_HUMAN (gil127234)	67.8 / 6.08	6 / 9	9	70	^{264}APDFVFYAPR^{273}(2)

**Spot ID	Difference (BD/CD)	VIP (BD/CD-1)	MW (kDa)/pI (Observed)	Protein	Accession ID	MW (kDa)/pI (Theoretical)	Matched / Searched	Seq.cov. (%)	Mascot score	Sequence confirmed by LID (Mascot ion score)
1075	-4.37	1.0	47.0 / 5.50	Actin, cytoplasmic 1	ACTB_HUMAN (gil46397333)	41.7 / 5.29	14 / 43	35	225	^{29}AVFPSIVGRPR^{39}(26) ^{85}IWHHTFYNELR^{95}(34) ^{239}SYELPDGQVITIGNER^{254}(95)

*The protein spots that showed at least 1.2-fold difference between the BD and HC groups ($p<0.05$) and were also included in the BD/HC-23 model were identified; **The protein spots that showed at least 1.5-fold difference between the BD and RA groups ($p<0.05$) and were also included in the BD/RA-35 model were identified; ***The protein spot included in the BD/CD-1 model was identified.
MW: molecular weight; Seq. cov.: sequence coverage; LID: laser-induced dissociation.

profile would change even in an identical patient during the clinical course. In our study, numbers of the patients in the active and inactive subgroups were identical in the BD group (each, n=8) (Table I) and in the CD group (each, n=6) (20). However, the ratio of patients in the active and inactive subgroups was approximately 1:4 in the RA group (active, n=3; inactive, n=13) (Table II). To exclude the possibility that the difference of protein profiles between the BD and RA groups was due to difference of disease activity, we compared the profiles between the BD-inactive and RA-inactive groups. We found significant difference of the profiles between the 2 inactive groups (128 spots showed at least 1.2-fold difference, $p<0.05$), suggesting that the difference was due to that of the pathophysiology of 2 diseases. In addition, almost all the enrolled patients were treated with various drugs (Tables I-II) (20), which may affect the protein profiles.

Comparing 563 protein spot intensity between the BD group and each of the HC, RA, and CD groups, we found that 23, 115, and 368 spots showed at least 1.2-fold intensity differences, respectively (Table III). The greater number of spots with intensity differences in the BD/RA and the BD/CD comparisons than in the BD/HC comparison suggested the protein profile deviations specific for the respective diseases. Most of the spots with different intensity showed higher intensity in the BD group than in the HC, RA, and CD groups (Table III). The proteins in the higher-intensity spots in the BD group may be up-regulated to increase the disease activity or to ameliorate the disease.

Among the identified proteins, 6 related to cytoskeleton. Moesin, radixin, and PTN4 are involved in the connections between major cytoskeletal proteins such as β-actin and plasma membrane (24-26). Their spot intensity was higher in the BD group compared to the HC and/or RA groups (Table V). MAP6, involved in microtubule stabilisation, was also increased in the BD group compared to the RA group. In contrast, β-actin and α-actinin-1 showed lower expression in the BD group compared to the HC group (Table V). The heterogeneous changes of these cytoskeleton-related proteins would reflect the activated states of PBMCs in BD. Autoimmunity to β-actin reported previously (18, 19) may be also involved in the down-regulation of β-actin in BD. Moesin, radixin, PTN4, and MAP6 would be up-regulated to compensate the decrease of β-actin.

Table VI. Numbers of protein spots with significantly different intensity between the subgroups of BD.

Spot intensity	Differences (folds)	Numbers of protein spots	
		Active / Inactive	Eye + / Eye –
Increased	2.0≤	0	3
	1.5≤	0	4
	1.2≤	5	5
	-1.2≥	18	7
Decreased	-1.5≥	7	1
	-2.0≥	0	1

The 16 BD patients were divided into the active group (n=8) and the inactive group (n=8). Similarly, the 16 BD patients were divided into the eye-lesion-present group (Eye+, n=10) and the eye-lesion-absent group (Eye-, n=6). Numbers of protein spots, intensity of which was significantly altered in the active group compared to the inactive group, and in the eye-lesion-present group compared to the eye-lesion-absent group, are shown ($p<0.05$).

Three nucleoproteins, NONO, ROA1 and RM03, showed higher intensity in the BD group than in the RA group. NONO is involved in transcriptional regulation, RNA processing, and DNA repair (27). ROA1 is also involved in RNA processing (28, 29). RM03, a mitochondrial ribosomal protein, is suggested to be involved in translation (30, 31). These proteins may be associated with the different PBMC protein expression between BD and RA by altering transcription and translation (Table III).

Plastin-2, THNS2, gremlin-2, and Rab-7a were increased in the BD group compared to the HC and/or RA groups. Since phosphorylation of plastin-2, which increases its F-actin binding activity (32), is involved in the activation of T cells (33), the up-regulation of plastin-2 may reflect the actin cytoskeleton rearrangement during the activation of T cells in BD. An isoform of THNS2, called secreted osteoclastogenic factor of activated T cells (SOFAT), is an inducer of both osteoblastic IL-6 production and osteoclast formation so that SOFAT is considered to exacerbate inflammation (34). THNS2 may also present those functions in PBMCs of BD. Gremlin-2 inhibits the activity of bone morphogenetic proteins (35), which may play a role in arthritis in BD. The up-regulation of Rab-7a may be related to the infection of microbes in BD, since some intracellular microbial pathogens survive by suppressing Rab-7a activity (36). The products of BD-susceptible genes such as IL-10 and IL-23R/IL-12RB2 were not identified in our study. It would be because the expression levels of cytokines and their receptors are generally under the detection levels of 2D-DIGE.

In the comparison between the active and inactive subgroups and between the eye-lesion-present and eye-lesion-absent subgroups in BD, a relatively small number of protein spots showed different intensity, suggesting the limited differences between the subgroups (Table VI). Usefulness of the active/inactive model and the Eye+/Eye - model should be validated with more numbers of BD patients (Table IV). Intensity of the radixin spot (ID833), increased in the eye-lesion-present group compared to the eye-lesion-absent group (2.46-fold), was also increased in the BD group compared to the RA group (2.50-fold, Table V). Radixin may be involved in the BD-specific inflammation, associated with the eye lesions that are not observed in RA.

Considering that no specific biomarker for BD has been established to date, it is noteworthy that complete discriminations of BD from other diseases and from healthy condition were achieved in this study (Fig. 2-4). The spots used for the BD/HC-23 model and the BD/RA-35 model would include important proteins for the diagnosis of BD. The BD/nonBD-3 model showed sensitivity

and specificity high enough for the clinical use. This model was not useful for discriminating the RA group or the HC group from the other groups (RA *vs*. non-RA, 0.492; HC *vs*. non-HC, 0.634), however, it showed relatively high AUROC for discriminating the CD group from the BD, RA, and HC groups (CD *vs*. non-CD, 0.822). The BD/nonBD-3 model should be validated with more numbers of patients in future.

In contrast, the BD/CD-1 model showed complete discrimination of the CD group from the other groups (Fig. 4E, Table IV). In the previous study, in which PBMC protein profiles of CD and ulcerative colitis (UC) were compared by 2D-DIGE (20), ID 1075 showed nearly equal intensity levels (CD, 1.462±0.523 AU; UC, 1.467±0.377 AU; p=0.976). Therefore, ID 1075, identified as β-actin (Table V), may be useful for discrimination of inflammatory bowel diseases (IBD) from other diseases including BD. β-actin was detected from multiple spots (Table V), suggesting different post-transcriptional modifications of this protein. Since β-actin was also detected from multiple spots useful for the discrimination of CD and UC (20), analysis of the β-actin subtypes with different post-transcriptional modifications may provide higher differential diagnostic ability among BD, CD, and UC.

In conclusion, we here identified novel PBMC proteins altered in BD. Those proteins may orchestrate the inflammatory state in BD. Investigation of the protein expression in each of T cells, B cells, NK cells, and monocytes may elucidate more precise disease-mechanisms of BD. Detailed studies of the identified proteins would lead to establishment of biomarkers for BD.

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