Table 1 *Clinicopathological characteristics of bladder cancer patients*

Characteristics		Number of patients (n = 95)	(%)
Gender			
	Male	73	(77)
	Female	22	(33)
Age			
	Range	29–88	
	Median	71	
Stage			
	NMIBC	63	(66)
	MIBC	32	(44)
Histological grade			
	High	71	(75)
	Low	24	(25)
Carcinoma in situ			
	Negative	90	(95)
	Positive	5	(5)
Nodal status			
	N0	82	(86)
	N1, N2	13	(14)
Lymphovascular invasion			
	M0	90	(95)
	M1, M2	5	(5)

NMIBC: non-muscle-invasive bladder cancer, MIBC: muscle-invasive bladder cancer, High: bladder cancer with high grade, Low: bladder cancer with low grade.

(TCEP), 2.5% pH 3–10 pharmalyte (GE Healthcare Bio-Sciences Corp.), and one tablet of complete mini EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany) per 10 mL of solution) using an ultrasonic homogenizer (VP-050; TAITEC Co., Ltd., Saitama, Japan), and centrifuged at 20,000 × *g* for 30 min at 4°C. Finally, the protein concentration was quantified using Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Two-dimensional gel electrophoresis. 2-DE was performed according to our previous study (30). The first-dimensional agarose isoelectric focusing gel (75 mm in length and 2.5 mm in inner diameter) was made with single pharmalyte pH 3–10 (GE Healthcare Bio-Sciences Corp.). Thirty-five micrograms of each protein extracted from culture supernatants of four cell lines were equally mixed and applied to the cathodic end of the agarose isoelectric focusing gel, and loaded in stepwise voltages as follows: 100 V: 20 min, 300 V: 15 min, 500 V: 15 min, 700 V: 60 min, and 900 V: 150 min at 4°C. After fixation in 10% trichloroacetic acid and 5% sulfosalicylic acid for 3 min at R/T with mild shaking, agarose gels were placed in distilled water and washed 3 times for 15 min each at R/T. The agarose gel was equilibrated in equilibration buffer (0.06 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue (BPB)). Then, the agarose gel was placed on the top of the second-dimensional 10% polyacrylamide gel, and loaded with a constant current at 20 mA/ gel.

Immunoblotting. The separated proteins on 2-DE gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.) overnight at R/T with a constant voltage at 10 V. After blocking with 0.5% casein for 60 min, the membranes were reacted with 20-times-diluted mixed sera of four NMIBC patients each with high- or low-grade BCs with 0.05% casein/TBST for 15 h at 4°C. The membranes were washed 3 times with Tris-buffered saline containing 0.1% tween20 (TBST) and reacted with 1,000-times-diluted horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody (DAKO, Glostrup, Denmark) with 0.05% casein/TBST for 30 min at R/T. After washing another 3 times with TBST, immunoreactive spots on the membrane were visualized with stable DAB solution (Life Technologies Corp.) for 15 min at R/T. The visualized images were digitized with a high-resolution scanner (GT-9800; Seiko Epson Corp., Tokyo, Japan).

Identification of proteins recognized by autoantibodies. For the identification of proteins recognized by autoantibodies, the separated proteins on 2-DE gels were stained by coomassie brilliant blue (CBB) (PhastGel Blue R; GE Healthcare Bio-Sciences Corp.) solution, and staining images were digitized with a high-resolution scanner. In order to match the immunoreactive spots on the membrane with protein spots on the gel, both digitized images were overlayed using Adobe photoshop software (version 7.0; Adobe Systems Inc., San Jose, CA, USA). The protein spots matched with the immunoreactive spots were manually excised from the gel and destained with 50% acetonitrile/50 mM NH_4HCO_3 until they became colorless. The pieces of gel were dehydrated with 100% acetonitrile and dried under vacuum conditions. They were then rehydrated in 10 μL of trypsin solution containing 10 ng/μL trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega, Madison, WI, USA) for 45 min at 4°C and incubated for 24 h at 37°C with a minimum volume of trypsin solution and 7 μL of 25 mM NH_4HCO_3. After incubation, the digested tryptic peptide solutions were collected, and the gel was washed once with 7 μL of 5% trifluoroacetic acid/50% acetonitrile and washed solutions were collected in the same tube. They were then subjected to peptide mass fingerprint (PMF) and MS/MS analyses for protein identification. Finally, they were spotted on a Prespotted AnchorChip 96 Set for Proteomics (Bruker Daltonics, Bremen, Germany) and analyzed with MALDI-TOF/TOF-MS using Autoflex III (Bruker Daltonics) and FlexAnalysis software (version 3.0.96; Bruker Daltonics) according to the manufacturer's instructions. PMF spectra were acquired in the positive reflector mode in a mass range from 320 to 4,000 Da using the default parameters with the main parameters. The calibration of PMF spectra was carried out using the calibrant spots equipped with AnchorChip according to the manufacturer's recommendations. Irrelevant masses including matrix (855.09, 861.10, 877.10) and autodigested tryptic masses (842.51, 1,045.56, 2,211.05, 2,225.14, 2,283.20, 2,807.20) were automatically and manually excluded from the analysis. MS spectra derived from PMF analysis were further validated by MS/MS analysis. Some of the strongest peaks in each MS spectrum were selected as precursor ions, and MS/MS spectra were acquired in the positive LIFT mode using the default parameters with the main parameters. The PMF and MS/MS spectra were processed with FlexAnalysis and BioTools software (version 3.0.183; Bruker Daltonics). Furthermore, the combined spectrum data were connected with the MASCOT Server (version 2.3; Matrix Science, London, UK; www.matrixsciences.com) and database searches were run using the IPI human database (version 3.82; 92,104 sequencies; 36,547,220 residues, http://www.ebi.ac.uk/IPI/Databases.html) with the following parameters: enzyme specificity, trypsin; variable modification, oxidation with methionine, propionamide, and pyridylethyl with cysteine; maximum of one missed cleavage site; peptide mass tolerance of 100 ppm; MS/MS (fragment ion) tolerance of 0.8 Da. The Mascot score of a hit above 62 and $P > 0.05$ was set as the threshold for protein identification.

Dot-blot analysis. Based on the results of both the above proteomic approaches and database information from Uniprot (http://www.uniprot.org/), secreted proteins were selected. The recombinant proteins corresponding to identified proteins were synthesized with Gateway entry clones using an *in vitro* wheat germ cell-free protein synthesis system (15). The recombinant proteins were solubilized in lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% BPB, and 1 M phenylmethylsulfonyl fluoride) and spotted onto PVDF membranes using a micro-dot-blot array (Kakengeneqs Co., Ltd., Chiba, Japan). After blocking with Tris-buffered saline with 2% Tween20 for 60 min at R/T, the membranes were reacted with a 400-times dilution of each serum from patients with BC or healthy controls with 0.05% casein/TBST for 15 h at 4°C. The membranes were washed 3 times with TBST for 5 min each at R/T and reacted with 1,000-times diluted HRP-conjugated rabbit anti-human IgG (DAKO) with 0.05% casein/TBST for 30 min at R/T. After a further 3 washings with TBST for 5 min each, signals were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corp.). The signal intensities were analyzed using DotBlotChip System software (version 4.0; Dynacom Co., Ltd., Chiba, Japan). Each normalized signal was represented by the positive intensity minus the background intensity around the spot.

Statistical analysis. Significant differences between clinical characteristics and serum IgG levels of AAbs were tested using the Mann-Whitney *U*-test. The area under the curve (AUC) and best cut-off point were calculated employing receiver-operating characteristic curve (ROC) analysis. Cumulative survival rates of patients were determined using the Kaplan-Meier method, and the significance of survival differences between high and low serum IgG levels of

AAbs was tested using the log-rank test. Multivariate analysis was performed by employing the Cox proportional hazards regression model. A P-value of < 0.05 was used to determine the level of significance. All statistical analysis were performed using StatFlex software version 6.0 (Artech Co., Ltd., Osaka, Japan).

RESULTS

2-DE immunoblot analysis

The proteins extracted from culture supernatants of four BC cell lines were separated by 2-DE and transferred onto the PVDF membranes, and reacted with mixed sera of four patients each with high- or low-grade BCs. As the results, we detected a total of 138 immunoreactive spots, of which 25 and 32 were detected only in patients with high- or low-grade BCs, respectively. A total of 81 were detected in both groups (Fig. 1D).

Identification of proteins recognized by autoantibodies

The protein spots that matched immunoreactive spots on the membrane were excised from CBB-stained 2-DE gel (Fig. 1B, C) and underwent in-gel digestion and MALDI-TOF/TOF MS analysis. As the results, in 133 of the 138 (96%) immunoreactive spots, 61 proteins were identified. In 24 of 25 (96%) immunoreactive spots, 17 proteins were detected, and in 29 of 32 (90%) immunoreactive spots, 21 proteins were detected, in sera from high- or low-grade BC patients, respectively. Of these, 14 proteins were classified as "secreted protein" according to the Gene Ontology database (http://www.geneontology.org/) (Table 2).

Dot-blot analysis of autoantibodies against identified proteins

We synthesized 13 recombinant proteins using a wheat germ cell-free system except for a collagen alpha-1(VI) chain which we failed to synthesize (Table 2). Serum IgG levels of each AAb in 95 BC patients and 35 normal controls were investigated by dot-blot analysis with recombinant proteins. In the results of univariate analysis, the mean value (±SD) of serum IgG levels of anti-calreticulin and matrix metalloproteinase-2 (MMP2) AAbs were 14.0 ± 4.4 and 47.1 ± 9.7 in BC patients, and 9.9 ± 2.0 and 34.6 ± 5.1 in normal controls, respectively. All serum IgG levels of anti-calreticulin and MMP2 AAbs

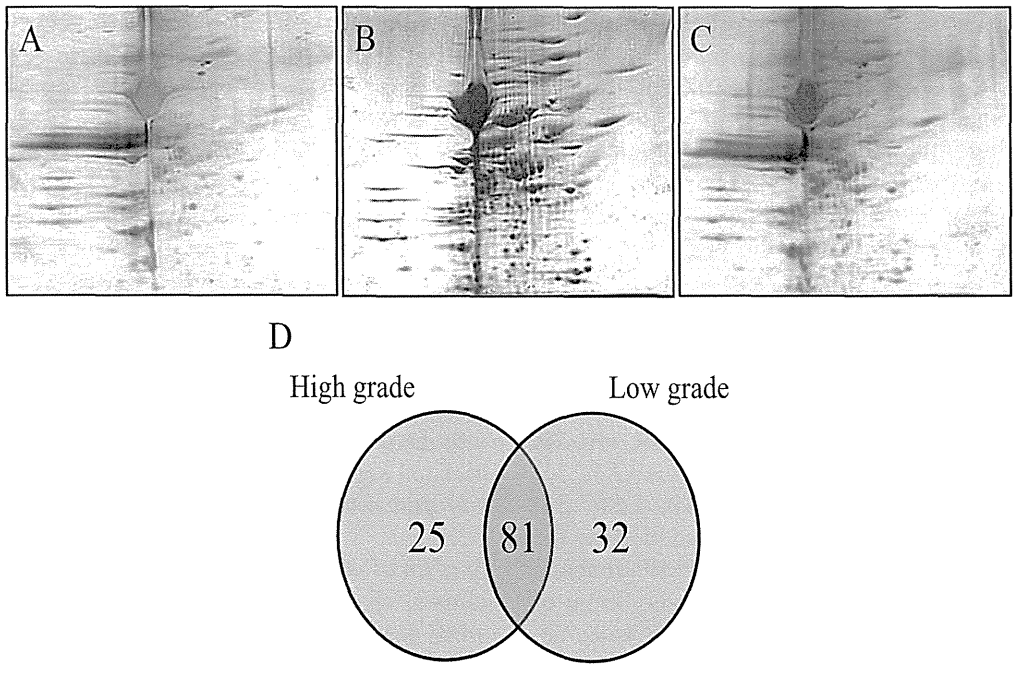

Fig. 1 Detection of autoantibodies by 2-DE immunoblot analysis in sera from BC patients. The proteins extracted from culture supernatants of the BC cell lines were separated by 2-DE and transferred to PVDF membranes. The membranes were incubated with mixed sera from BC patients (A). Protein patterns of CBB-stained 2-DE gel (B). Merged image of immunoreactive spots on the membranes and CBB-stained 2-DE gel (C). By comparing immunoreactive patterns, 25 and 32 spots were specifically detected in patients with high- and low-grade BC, respectively (D). The protein spots that matched immunoreactive ones on the 2-DE gel were excised and identified by MALDI-TOF/TOF MS.

Table 2 *Identified antigenic proteins in sera from high- or low-grade BC patients*

Gene symbol	Accession number	Protein name	Histological grade
CALR	P27797	Calreticulin	High
CTSD	P07339	Cathepsin D	High
SERPINB5	P36952	Serpin B5	High
MMP2	P08253	Matrix metalloproteinase-2	High
CPA4	Q9UI42	Carboxypeptidase A4	High
COL6A1	P12109	Collagen alpha-1(VI) chain	High
FBLN1	P23142	Fibulin-1	Low
MP10	P09238	Matrix metalloproteinase-10	Low
SPARC	P09486	SPARC	Low
MMP1	P03956	Matrix metalloproteinase-1	Low
CTSZ	Q9UBR2	Cathepsin Z	Low
SPON2	Q9BUD6	Spondin-2	Low
CTSL1	P07711	Cathepsin L1	Low
QSOX1	O00391	Sulfhydryl oxidase 1	Low

High: specifically detected in sera from high-grade BC patients.
Low: specifically detected in sera from low-grade BC patients.

were significantly higher in BC patients than in normal controls ($P < 0.01$, Fig. 2A, B). In addition, the mean value (±SD) of serum IgG levels of anti-calreticulin and MMP2 AAbs were 13.5 ± 3.1 and 40.4 ± 9.8 in the patients with low-grade BC, and 14.0 ± 4.6 and 66.3 ± 9.0 in those with high-grade BC, respectively. The serum IgG level of anti-MMP2 AAb was significantly higher in patients with high-grade than in low-grade BC ($P < 0.01$, Fig. 2D), but not for anti-calreticulin AAb (Fig. 2C). No significant difference in serum IgG levels of other AAbs between BC patients and normal controls was detected (data not shown).

Validation of anti-calreticulin and -MMP2 autoantibodies between BC patients and normal controls

Based on ROC analysis of anti-calreticulin AAb, an optimal cut-off value of 10.6 was applied, and the diagnostic sensitivity and specificity for BC patients were 64.0 and 60.0%, respectively. The AUC for anti-calreticulin AAb in BC patients compared to normal controls was 0.65 (95% confidence interval: 1.23–5.89, Fig. 3A). Regarding anti-MMP2 AAb, an optimal cut-off value of 34.6 was applied, and the diagnostic sensitivity and specificity for BC patients were 60.0 and 62.0%, respectively. The AUC for anti-MMP2 AAb in BC patients compared to normal controls was 0.59 (95% confidence interval: 0.73–3.45, Fig. 3B).

Association of serum IgG levels of anti-calreticulin and MMP2 autoantibodies with clinical outcomes

To estimate whether serum IgG levels of anti-calreticulin and MMP2 AAbs were of independent predictive value for recurrence-free survival or cancer specific-survival of BC patients, uni- and multivariate analyses were performed. At a median follow-up of 62.3 months (range: 2 to 166.4), Kaplan-Meier projection indicated that there was no significant correlation between the serum IgG level of anti-calreticulin AAb and recurrence-free or cancer-specific survival (Fig. 3C). However, the serum IgG level of anti-MMP2 AAb was significantly correlated with cancer-specific survival ($P < 0.05$; Fig. 3D). In addition, multivariate analysis with Cox proportional hazards regression analysis revealed that the serum IgG level of anti-MMP2 AAb and pathological stage were significantly correlated with cancer-specific survival ($P < 0.05$ each, Table 3). These findings suggest that an increased serum IgG level of anti-MMP2 AAb is an independent predictor of poorer survival in BC patients.

DISCUSSION

Secreted proteins reflect various states of cells in real time and under specific conditions, participate in various physiological processes, and play crucial roles in pathological processes. Thus, it has been suggested that the analysis of tumor-secreted proteins is a promising method to identify diagnostic biomarkers in cancer (6, 33, 40, 48). Actually, several studies have revealed that secreted proteins, which could be biomarker candidates, are present in the conditioned media of several tumor cells (25, 27, 47).

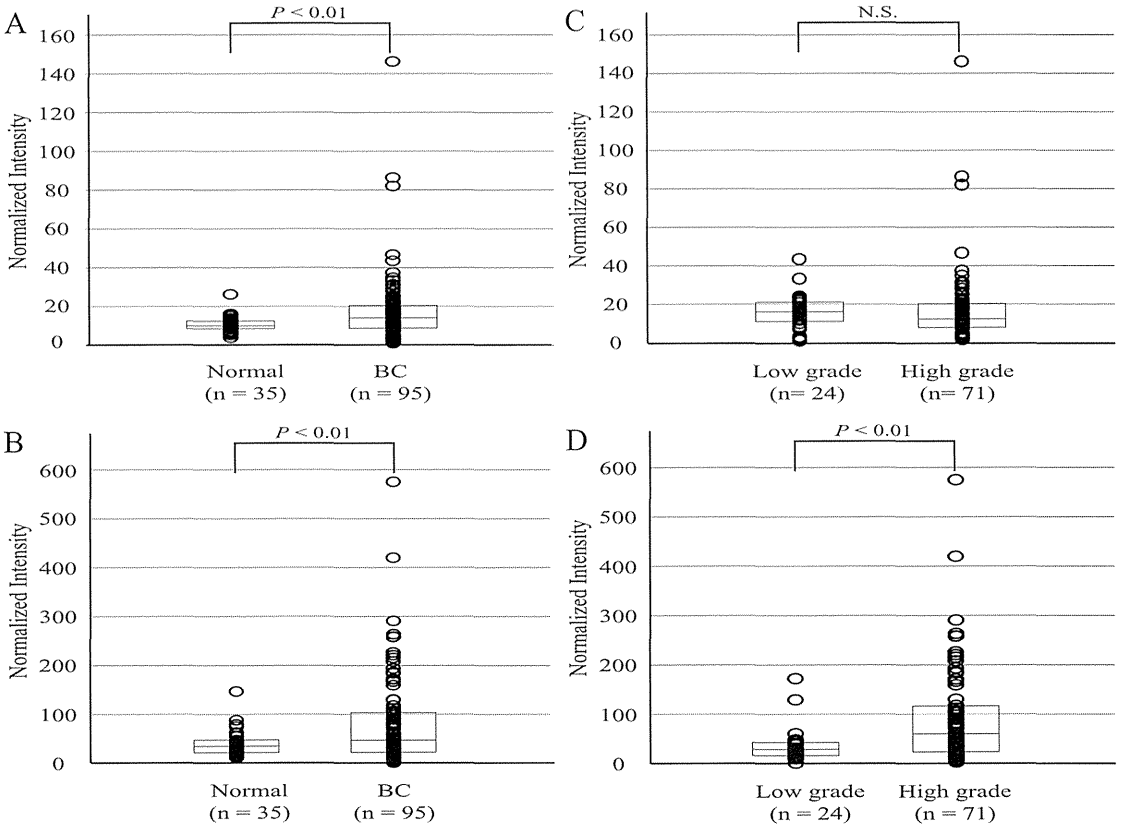

Fig. 2 Dot-blot analysis of serum IgG levels of anti-calreticulin and MMP2 AAbs. Serum IgG levels of anti-calreticulin and MMP2 AAbs in sera from BC patients and normal controls were detected by dot-blot analysis. The normalized intensity was calculated with the Mann-Whitney *U*-test. The serum IgG level of anti-calreticulin AAb was significantly higher in BC patients than in normal controls (A). No significant difference was detected between high- and low-grade tumors (C). The serum IgG level of anti-MMP2 AAb was significantly higher in BC patients than in normal controls (B) and significantly higher in high-grade than in low-grade BC patients (D). N.S.: not significant

AAbs against tumor-associated antigens have been identified in sera from patients with colon, breast, lung, ovary, and bladder cancers (4, 7, 16, 36, 50). Thus, the application of the humoral immune response for the detection of cancer biomarkers has a great potential and has been suggested as ideal screening for cancer diagnosis and their prognostic value (17, 24). Furthermore, the immune system is especially well adapted for the early detection of cancer, because AAbs can be detected before the appearance of other biomarkers or phenotypic alternations in an early stage of tumorigenesis (19).

Therefore, we performed 2-DE/immunoblot analysis to identify secreted antigenic proteins that are recognized by AAbs in the sera of BC patients. In this study, we picked up 57 immunoreactive spots that specifically differentiate the histological grade of BC. Furthermore, we confirmed the usefulness of identified AAbs as sero-diagnostic and/or -prognostic biomarkers for BC by dot-blot analysis. In the results, serum IgG levels of anti-calreticulin or MMP2 AAbs, which were identified in sera from patients with high-grade BC, were significantly higher in sera of BC patients than in normal controls. In addition, the serum IgG level of anti-MMP2 AAb was significantly correlated with the histological grade of the tumor and cancer-specific survival.

Calreticulin is diversely distributed in the cytoplasm, nucleus, plasma membrane, and extracellular spaces of cells. Because of these different localizations, it has been implicated in many cellular functions, including Ca^{2+} storage and signaling, lectin-like chaperoning, the regulation of gene expression, cell adhesion, migration, cellular proliferation, and autoimmunity (8, 26, 31). It has been reported that the overexpression of calreticulin was detected in tumor tissues and their sera of hepatocellular, colon, and lung cancers (18, 45, 49), and associated with the migration and proliferation of tumor cells and a poorer prognosis in esophageal, gastric, and breast

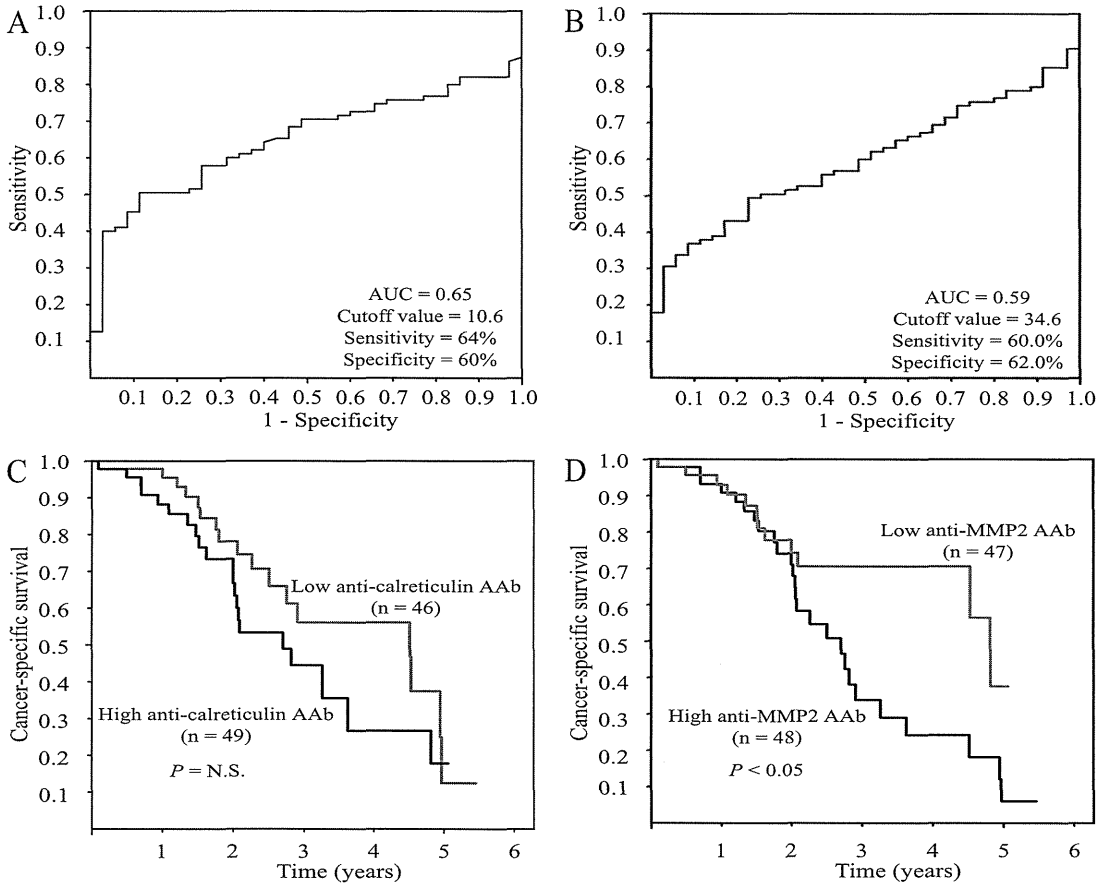

Fig. 3 Receiver-operating characteristic curve (ROC) analysis for BC and the probability of cancer-specific survival according to serum IgG levels of anti-calreticulin and MMP2 AAbs. The corresponding areas under the curves (AUC) of serum IgG levels of anti-calreticulin (A) and anti-MMP2 (B) AAbs in BC patients compared to normal controls were 0.65 and 0.59, respectively. Regarding cancer-specific survival in BC patients, a significant correlation was recognized with the IgG level of anti-MMP2 AAb (D), but not with anti-calreticulin AAb (C). N.S.: not significant

Table 3 *Multivariate Cox proportional hazards regression analyses for prediction of recurrence and survival of bladder cancer patients*

	Recurrence-free survival			Cancer-specific survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Anti-MMP2 AAb	0.77	0.33 - 1.81	N.S.	2.62	1.04 - 6.58	0.04
Gender	0.93	0.46 - 1.88	N.S.	1.41	0.66 - 3.02	N.S.
Pathological stage	1.34	0.65 - 2.79	N.S.	3.25	1.23 - 8.58	0.02
Histological grade	1.51	0.65 - 3.50	N.S.	0.83	0.31 - 2.20	N.S.
Lymphovascular invasion	0.74	0.25 - 2.22	N.S.	0.40	0.12 - 1.37	N.S.
Nodal status	1.01	0.22 - 4.55	N.S.	0.49	0.10 - 2.46	N.S.

HR: hazard ratio, CI: confidence interval, N.S.: not significant.
Multivariate Cox proportional hazards regression analysis was used to estimate HR, with the corresponding 95% CI.

cancer patients (2, 5, 9). Interestingly, it has been suggested that there is a significant correlation between calreticulin expression and the aggressiveness of BC. The BC cells with calreticulin knockdown showed the suppression of proliferation, migration, and attachment, in contrast to the overexpression of calreticulin which enhanced cell migration and attachment (28). In BC tissues, calreticulin expression was predominant in the cytoplasm of BC cells, and its expression was higher in BC cells than in normal

urothelial cells (21). Furthermore, higher levels of calreticulin were found in urine samples of patients with BC, but not in urological patients without BC, and urinary calreticulin has been proposed as a biomarker of BC (20). Although it was reported that the detection sensitivity of serum calreticulin in BC patients was 73.0% (21), this is slightly higher than that in our present anti-calreticulin AAb study. However, Heo *et al.* reported that serum tumor-associated AAbs can be detected in the early stage of BC before serum tumor-associated antigens are detectable (19). Although calreticulin overexpression in tumor cells is well known, the mechanism of this increase have not been well defined. Previous studies reported an elevated serum IgG level of anti-calreticulin AAb in patients from hepatocellular carcinoma, gallbladder adenocarcinoma, pancreatic adenocarcinoma, and breast cancer when compared to healthy controls (10, 34). These data are concordant with our present results, suggesting that humoral immunity against calreticulin may be associated with the overexpression of calreticulin.

Matrix metalloproteinases play important roles in various tumorigenic processes, such as extracellular matrix remodeling, angiogenesis, apoptosis, epithelial-to-mesenchymal transition, and cell proliferation. MMP2 (gelatinase A, 72 kDa gelatinase) is one of the enzymes of the matrix metalloproteinase family, known to be essential for the degradation of type IV collagen in tumor tissues (41). Elevation of MMP2 has been reported in cancer tissues or sera from patients with breast, lung, gastric, ovarian, and bladder cancers. An association between the expression levels and clinicopathological factors was also described (12–14, 29, 37–39, 43). In BC, it has been reported that the overexpression of MMP2 was detected in biological samples, including tissue, sera, and urine, and associated with clinicopathological factors and/or a poorer prognosis (13, 14, 43). In BC tissues, MMP2 expression was detected mostly in the cytoplasm of BC cells, and its overexpression may be an independent prognostic biomarker for BC progression (43). A majority of biomarker studies including MMP2 in patients with BC have focused on urine (35). No detailed study on the detection sensitivity of serum/plasma MMP2 levels in BC patients has been conducted. The diagnostic sensitivity of our present study for serum anti-MMP2 AAb in BC patients was 60.0%, being higher than that for urinary MMP2 (51.0%). Therefore, we expect that MMP2 and its AAb in biological samples have the potential to be tumor markers for BC. To our knowledge, this is the first report regarding the sero-diagnostic potential of AAbs to calreticulin and MMP2 in BC. Our results demonstrated that serum IgG levels of anti-calreticulin and MMP2 AAbs may be serological biomarkers for BC and, in addition, anti-MMP2 AAbs were associated with the histological grade of the tumor and cancer-specific survival of BC patients.

In conclusion, we identified several secreted proteins that were recognized by AAbs in the sera of BC patients by proteomic analysis. We also revealed that serum IgG levels of anti-calreticulin and -MMP2 AAbs were significantly higher in BC patients than in normal controls. In addition, a higher serum IgG level of anti-MMP2 AAb was associated with a high-grade tumor and poorer prognosis of BC patients. These data suggest that serum anti-calreticulin and MMP2 AAbs may be candidate sero-diagnostic and/or -prognostic markers for BC patients.

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Development of a protein array for autoantibody profiling of blood

— Comprehensive disease diagnosis using the body's defense system —

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We have developed infrastructure of the technologies and resources for post-human genome research to perform functional proteomics (the analysis of protein functions, protein-protein interactions, and human protein structures) on a large scale. A method for profiling autoantibodies in serum is developed using human protein expression resources and protein expression techniques. The human biological defense system responds to abnormalities in the body with extraordinary sensitivity. Hence, this system is an effective tool for detecting human diseases at an early stage. Health safety and security can be achieved by establishing an early diagnostic method for diseases using autoantibody profiling of blood with a protein array.

Keywords : Autoantibody, protein array, human protein, body's defense system, antigens, diagnosis, biomarker

1 Introduction

The ability to diagnose a disease before its development or at an early stage and obtaining a general diagnosis in which as much health information can be obtained in a single test is an extremely important issue in achieving a safe and secure society. In this research, we aim to achieve this objective by analyzing the types and quantities of autoantibodies in one drop of blood. Originally, the antibody is a biological defense system acquired by higher-order organisms through evolution, to defend themselves against bacteria and virus invasions. The antibody system is known to produce autoantibody against its own protein in response to the abnormal release of protein from cells or to the excessive production of proteins due to disease, as well as against external antigens. We think it is reasonable to utilize the biological defense mechanism that responds sensitively to the abnormalities of the body to detect diseases. Particularly, since the autoimmune diseases occur due to the production of antibodies that attack their own cells or tissues, the autoantibodies can be the cause of disease as well as disease markers. The detection of autoantibodies may enable presymptomatic testing for autoimmune diseases, thereby allowing early treatment. However, in practice, comprehensive tests for autoantibodies have not been established, and in most cases, one visits a hospital only after the symptoms of autoimmune diseases develop. There are also several intractable diseases in which the involvement of autoantibodies is suspected, and the development of a comprehensive detection system for autoantibodies is extremely important. There are many papers that reported the use of autoantibodies as disease markers for diseases including diabetes, cancer, Alzheimer's disease, rheumatism, and dilated cardiomyopathy.[1] We developed a comprehensive detection system of autoantibodies and have correlated the autoantibodies and diseases, by preparing antigen proteins using the world's largest human protein expression resource and a human protein synthesis technology that we have been working on for a long time. We hope to further the technological development for a comprehensive profiling of blood autoantibodies that are individually different and are closely related to health.

2 Construction of a human protein expression resource and its use

Based on the human full-length cDNA sequencing project of the Ministry of International Trade and Industry (currently Ministry of Economy, Trade and Industry) that was started in 1998, we started preparations for the following in the Protein Function Analysis Project of the New Energy and Industrial Technology Development Organization (NEDO) in 2000: (1) Human Proteome Expression (HUPEX) resource, (2) high-throughput protein synthesis technology, and (3) Human Gene and Protein Database (HGPD). At the time, the Human Genome Project to decode the genome DNA sequence was being done internationally, and taking the lead for the coming age of proteomics, Japan decided to fortify the environment for human genome research, constructed the HUPEX resource, and built a database.[2][3] The preparations of the technological foundation to carry out large-scale analyses of human protein functions, protein interactions, and protein structures were conducted as national projects. As a result, the HUPEX resource was utilized in various national research

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projects, joint research with corporations, and academic joint researches with research institutes and universities, and yielded rich results in the respective fields. One of the major results was a joint project "Special Project for Yamanaka iPS Cell" of the Japan Science and Technology Agency (JST) with Dr. Shinya Yamanaka, Director of the Center of iPS Cell Research and Application (CiRA), Kyoto University. This project led to the new discovery of Glis1, a gene for promoting the induction of iPS cells.[4] Moreover, in the joint research with the School of Medicine, Gifu University, a factor to highly efficiently induce iPS cells was discovered from the dental pulp cells in teeth. In the joint study with the School of Medicine, Keio University, a factor to promote direct reprogramming where the myocardial cells can be made from heart fibroblasts was discovered. Many results were obtained in the search for factors that may be useful in regenerative medicine. Also, major results were obtained in the development of *in vitro* visualization technology of protein interaction for a drug discovery screening system, and the development of production of standard proteins for quantitative proteomics by mass spectrometer. Such research results were expected in the initial conceptualization of the HUPEX resource uses. The HUPEX resource technology that we have constructed so far was basically to support the smooth progress of industrial proteome research. Sometimes, as we deepen our research in a discipline, new horizons that we did not initially consider begin to unfold. When we climbed the mountain of the proteome study and looked back, we realized that we could study the comprehensive antibody fields when the protein groups that comprise the proteome are considered as antigens. As shown in Fig. 1, we could study the immunome (whole immune system) from the proteome (while not all antigens are proteins, they comprise a major part). The idea of comprehensively analyzing the autoantibody in blood using the HUPEX resource and applying it in diagnosis (Fig. 2) was not considered initially. However, we were capable of using more human proteins as antigens than any other researcher in the world, and we possessed the ability to use the resource to find out whether the antibodies are present in the blood serum. Many researchers have previously reported the idea of using the autoantibodies in the blood serum.[1] However, since it was difficult to prepare the antigens to detect the antibodies, comprehensive analysis of autoantibody had not been done until now. Currently, we are able to conduct the world's most accurate profiling of the blood serum autoantibody using the HUPEX resource. To realize autoantibody profiling, in addition to the HUPEX resource, it was necessary to establish a comprehensive protein expression technology, a technology for manufacturing protein arrays, and an antibody detection method. These technologies will be explained below.

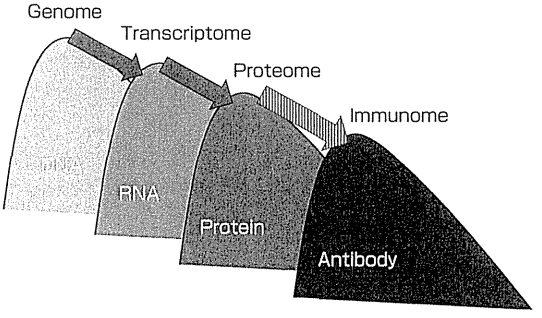


Fig. 1 From proteome to immunome

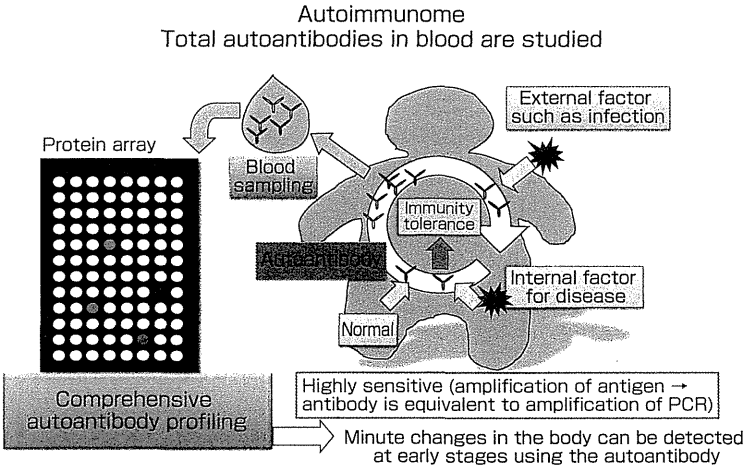


Fig. 2 Autoantibody and disease

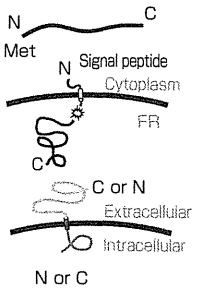
3 Development of a protein array that allows autoantibody profiling

3.1 Comprehensive human proteome expression resource and protein expression technology

We constructed a protein expression resource by introducing the Gateway cloning technology, a general-use cloning system, and created the plasmid DNA (entry clone) with site-specific recombination sequence at both ends of the open reading frame (ORF) of cDNA.[2] In the Gateway cloning technology, the expression clone can be created simply by mixing the entry clone and the destination vector, and by conducting site-specific DNA recombination using the recombination enzyme in a test tube. This is an optimal DNA recombination technology when conducting high-throughput protein expression.[5] This resource is the world's largest protein expression resource that covers about 80 % of the human genome,[2] and is named the HUPEX resource. Two types of entry clones were created: the N-type entry clone where the same amino acid sequence can be synthesized as the native protein at the C terminal per cDNA; and the F-type where the stop codon is replaced with a sense codon so a tag can be attached to the C terminal.[6] Various types of ORF type entry clones were created including the full-length ORF type with whole ORF for the gene, processing ORF type where the signal peptides were eliminated from the full-length ORF, and the domain ORF that could express the extracellular domain or intracellular domain with membrane protein with single membrane penetration domain. These resources can be freely selected according to the research objective. Also, since the protein synthesis can be conducted with all types of protein synthesis systems, the SD (Shine-Dalgarno) sequence for *E. coli* expression and Kozak sequence for eukaryotic cell expression was added to the 5' upstream of ORF. For these protein expression resources, the N-type and F-type entry clones were prepared, and about 60,000 types were created including the known and unknown clones, as well as the splicing variant clones (Table 1). To use these entry clones in a comprehensive proteome research, a clone with the longest ORF is selected for each gene as a representative clone, and they are functionally categorized according to the functions of proteins (transcription factor group, GPCR group, kinase group, unknown gene group, etc.). About 20,000 clones representing human genes were used in our research.

Table 1. Number of manufactured Gateway entry clones

Type	Determined number of entry clones	
	C terminal stop	C terminal fusion
Full-length ORF	18,744	28,386
Processing ORF	4,068	2,863
Domain ORF	2,719	-
Total	25,531	31,249
	56,780	



To conduct the comprehensive proteome research using human proteins, the technology to comprehensively synthesize the protein is necessary, as well as building the HUPEX resource. Around the year 2000 when we started constructing the protein expression resource, at the same time, Professor Yaeta Endo *et al.* of the Ehime University developed a wheat germ cell-free protein synthesis system.[7] We developed the technology for high-throughput protein synthesis using the wheat germ cell-free protein synthesis system (Fig. 3). The wheat germ cell-free protein synthesis system was superior in the points of success rate of protein synthesis, solubilization rate of the synthesized protein, and activity maintenance rate of the synthesized protein compared to other protein synthesis systems using *E. coli* or eukaryotic cells, and the protein could be synthesized at the percentage of 98 % or higher.[6] The whole reaction from DNA structuring to protein synthesis was done in an *in vitro* system (96 hole or 384 hole plate), and the reaction was optimized so only the dispensing procedure where the reacted solution was transferred to the next reaction solution was necessary. As a result, we developed a technology where the whole process of protein synthesis could be completed in

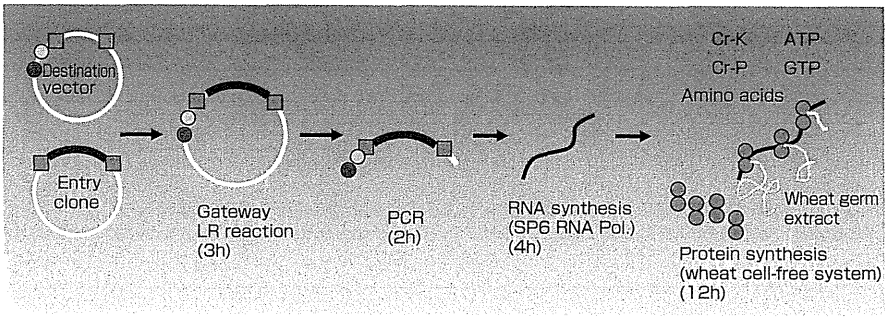


Fig. 3 Wheat germ cell-free protein synthesis system using the Gateway entry clone
Note) Modification of Fig. 6 in N. Goshima *et al.*: Constructing the foundation for comprehensive expression of human proteins, *Jpn. J. Exp. Med. (Extra no.)*, 23 (4), Chap. 3, Sec. 3, Yodosha (2005) (in Japanese).

one week. The LR product, PCR product, and mRNA that are created in the process of protein synthesis can be stored for a long period at -80 °C. In case it becomes necessary to resynthesize the same protein, it can be resynthesized in 18 hours using the stored mRNA. By combining the protein synthesis technology and a dispenser, it became possible to synthesize about 20,000 protein types at one time, and all proteins can be used in the simultaneous assay system through the array technology.

The results of the genetic information of the entry clones, the status of clones, and the results of protein expression of the wheat germ cell-free system or *E. coli* system are stored in the Human Gene and Protein Database (HGPD: http//www.HGPD.jp/), and they can be searched freely. The created entry clones are available from the National Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE) (http://www.nbrc.nite.go.jp/hgentry.html).[3][8]

3.2 Development of the protein array technology

The protein array technology is useful in comprehensively analyzing the interactions of protein-protein, protein-low molecule, protein-nucleic acid, etc., as well as for analyzing the enzyme-substrate protein. In a conventional protein array, the protein is fixed on the nitrocellulose membrane sheet, or on the surface of the slide glass coated or specially treated with nitrocellulose. Due to the nature of the fixing method, the protein is fixed in a dehydrated condition on the substrate surface, and the three-dimensional structure is not maintained. Therefore, with the conventional array, the function of the fixed protein cannot be analyzed. We aimed to recreate and analyze the biological reaction on the array using the HUPEX resource and the high-throughput protein synthesis technology that we developed. For this purpose, it was necessary to find a way to fix proteins on the array substrate in a condition where the 3D structures were maintained and the proteins could express their functions on the array.

We developed a protein array where 3D structures and functions of the proteins were maintained. First, we focused on the protein refining technology using magnetic beads. By using our protein synthesis technology, the proteins can be synthesized with various tags attached. The target protein synthesized with tags can be easily refined using the magnetic beads with ligands. First, the target protein is bonded to the magnetic beads while maintaining the 3D structure at the time of synthesis. Normally, the protein bonded to the magnetic beads are eluted, recovered, and used, but we considered a way to create an array while maintaining the bond between the magnetic beads and the protein. A well plate for bonding with the magnetic beads was developed, and by combining the high-throughput protein synthesis technology and the protein refining and array technologies using the magnetic beads, we developed the technology for creating an array while maintaining the 3D structure of the protein (Fig. 4A). The protein array, in

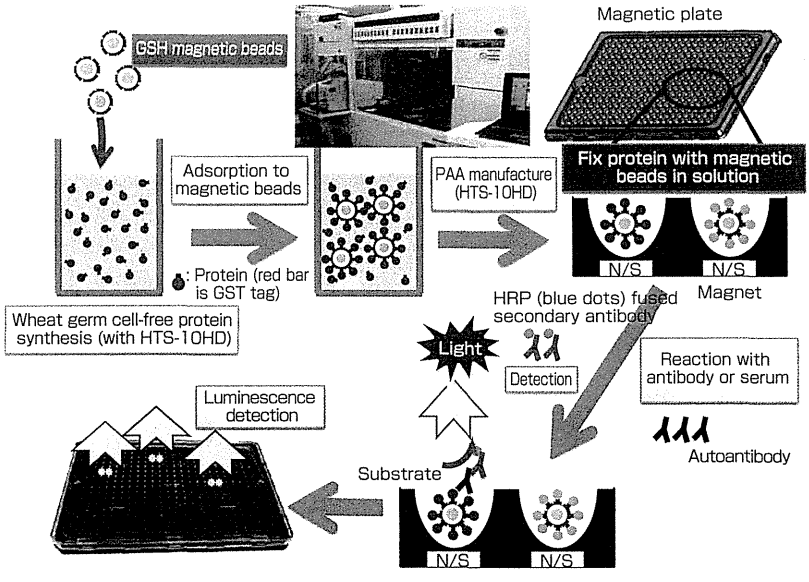


Fig. 4A Manufacture of protein active array using magnetic beads and antibody detection

The GST-fused target protein synthesized by the wheat germ cell-free synthesis system is attached to the GSH magnetic beads surface. This suspension is dispensed into the magnetic plate (originally made) equipped with magnets at the bottom of the plate, and the non-adhering fraction is removed by cleansing. The protein active array with protein fixed using the magnetic beads in the solution is manufactured. The antibody (Y) or blood serum is added to the protein active array (PAA), the protein is bonded onto the array, and the bonded antibodies are detected by chemiluminescence using the HRP-fused secondary antibody.

Note) Modification of Fig. 3 in N. Goshima: Autoantibody analysis using the array, *Handbook of Therapeutic and Diagnostic Antibodies*, Chap. 1, Sec. 5, NTS (2012) (in Japanese).

which the panoramic analysis of protein function for human proteome can be conducted, is called the "protein active array (PAA)" (Fig. 4B).

In selecting the magnetic beads used for the PAA, it is necessary to compare various magnetic beads from the following perspectives: 1) type of ligand of magnetic beads, 2) material and size of the beads, 3) amount of adsorped target protein, 4) non-specific adsorption, and 5) method for dispensing the suspension. In general magnetic beads for His tag, GST tag, or Streptavidin tag adsorption are used. For the ligand of the magnetic beads, the GST tag adsorption magnetic beads was selected because the 5' FLAG-GST tag that allows creation of active expressed protein for protein synthesis was used. From the perspectives of aforementioned 2) to 5), we selected the glutatione particles of the MagneGST Protein Purification System (Promega Corporation).

When creating the PAA, a special well plate is necessary to which the magnetic beads bonded with expressed proteins can be fixed, and which does not require dispensers or other equipment when cleansing or supplying the common reagent. We developed a well plate for the PAA, where the magnet is installed at the bottom of the well and the thickness of the well bottom is made as thin as possible to allow the magnetic beads to bind strongly to the well bottom. Normally, in assays such as ELISA, each well uses independent plates, but for the magnetic beads array, the wells are designed to be independent of each other but the reaction of the solution made of diluted serum can take place without division between the wells. We also developed a special cover plate that prevents biohazards when handling the serum samples, and that allows the reaction to be accomplished with a small amount of homogenous solution. By placing this cover plate, the target proteins on the surface of magnetic beads are covered with minute quantity of reaction liquid using a syringe, and the blood serum sample can be handled in a closed system rather than an open system.

By devising the equipment as described above, the manufacture process of the PAA can be accomplished very simply and in a short time. First, proteins synthesized by the wheat germ cell-free protein synthesis method using 96 well plates are added to the magnetic beads, and the proteins are bound to the surface of the magnetic beads. The suspension of the magnetic beads bound to proteins is dispensed to the well plate of the PAA, the proteins adhere to the well plate by magnetic force of the magnetic beads, and the array is created for the target protein. In case of long-term storage, the storage liquid is added and is stored at -80 °C. As a result of storage tests, it was confirmed that the quality could be maintained for six months in the above storage condition.

3.3 Establishment of PAA detection method

The assay by PAA can be completed in about eight hours from the initial probe (serum, low molecular compound, proteins, etc. that will be investigated for the bond with human protein) reaction to detection. The number of samples that can be processed per day is four samples per person. For detection, secondary antibodies labeled with fluorescent pigment or secondary antibodies labeled with HRP for fluorescence detection are used. For detection, devices that can obtain the western blotting image can be used, such as the chemiluminescence image detector or fluorescence image detector that are commercially available. A liquid delivery pump is used for the cleansing process of the probe and the

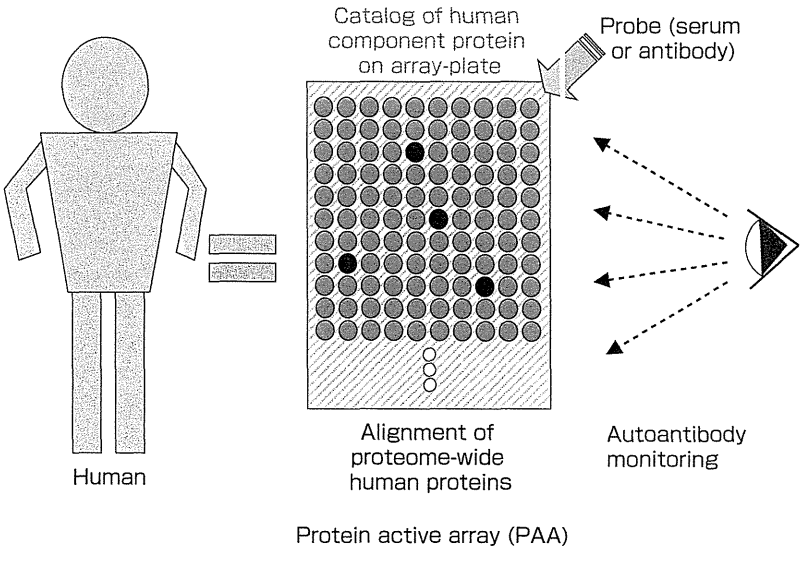


Fig. 4B Panoramic analysis by PAA
Proteome-wide proteins are arranged as arrays on a substrate, serum or antibodies that act as probes are added, bonding of antibodies to human proteins are investigated panoramically, and the autoantibodies are monitored.

reaction solution, and these can be automated in the future.

3.4 Establishment of the screening method for PAA

We are capable of synthesizing about 20,000 types of proteins in a short time by combining the protein synthesis technology and the dispenser. To screen proteins efficiently and economically using these characteristics, it is necessary to achieve higher density of PAAs or higher efficiency of screening. Cost and labor are needed to manufacture the protein arrays using the 20,000 proteins that were expressed individually and to identify the antigens, and problems may arise in the practical use and measurement of multiple samples. Therefore, we thought we could reduce the cost and labor to about one-tenth by conducting primary screening by creating an array using a compound of 10 types of proteins, and then conducting secondary screening using only the protein compounds containing the hits.

Several manufacture methods of the mixture of 10 proteins were considered. The method whereby simply synthesizing 10 proteins independently and then mixing the individual proteins would require cost and labor. Therefore, we investigated in which phase of the protein synthesis the 10 proteins could be combined for the co-expression of 10 types of proteins. In our protein synthesis system explained above (Fig. 3), the protein syntheses are conducted by mixing 10 types of entry clones, LR reaction products, PCR products, and mRNAs from each reaction step. The expressed proteins are separated by the SDS-PAGE method, and are detected by the western blotting method. In the western blotting method, anti-GST HRP-linked mouse mono Ab (NACALAI) is added to the antibody, diluted 5,000 times in 1.0 % skim milk in PBST, antibody reaction done for five minutes using ECL plus (GE Healthcare), and chemiluminescence detection is done using the Fluor-SMAX (Bio-Rad Laboratories, Inc.). As a result, for the co-expression of 10 proteins, 10 protein types were expressed efficiently in the mixture after the PCR product. Considering the cost and the complexity of the maneuver, it is concluded that the protein synthesis should be done after manufacturing the 10 mixtures using the PCR products from the uppermost stream (Fig. 5).

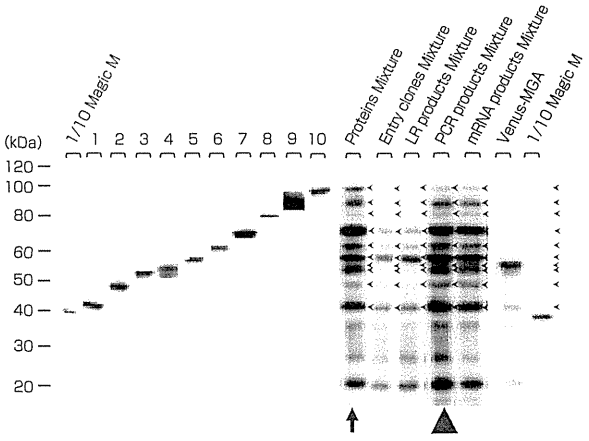
Using the PAA manufactured from the 10 types of co-expressed proteins synthesized from the mixed PCR product, we conducted an investigation of antigen-antibody reaction for the antibody to determine the antigen. As a result, the antigen could be identified using the PAA manufactured using the 10 types of co-expressed proteins.

The two-step screening method, where the 10 types of proteins obtained in the primary screening or comprehensive PAA (C-PAA) using 10 types of co-expressed protein are individually used to conduct antigen identification in the secondary screening expanded PAA (E-PAA), was employed. The screening is simplified using this two-step screening method (Fig. 6).

Using this array technology, we manufactured PAAs for about 20,000 types of proteins that are categorized by function. The manufacturing technology of PAAs was transferred to Cell Free Sciences, Co. Ltd. that is working on the product realization of the PAAs.

3.5 Analysis of the autoantibody in the blood serum of a patient with ovarian carcinoma derived paraneoplastic cerebellar degeneration

Using the PAA, the analysis of the autoantibody in the serum of a paraneoplastic cerebellar degeneration (PCD) patient was conducted. The patient visited the Department of Neurology,



Sample no.	ID	5SG(STOP)	FLJ No	ORF Len (bp)	PCR product (bp)	MW(kDa)	Molecular weight of protein expressed by a native type entry clone
1	TEST0003	test clone No.56	FLJ21903	378	3105	14.7	44.4
2	TEST0001	test clone No.5	FLJ20819	624	3351	23.5	53.2
3	TEST0011	EGFP		720	3447	26.9	56.7
4	TEST0012	Venus-MGA		762	3489	28.2	58.0
5	TEST0009	Ubiquitin	FLJ34456	858	3585	33.3	63.0
6	TEST0007	kinase	FLJ34101	966	3693	37.0	66.7
7	TEST0008	phosphatase	FLJ34434	1176	3903	43.3	73.1
8	TEST0005	Transcription factor	FLJ16264	1374	4101	53.0	82.8
9	TEST0010	Autophosphorylation	FLJ37986	1638	4365	61.4	91.2
10	TEST0002	test clone No.8	FLJ20768	1842	4569	66.9	96.7

Fig. 5 Comparison of protein co-expression

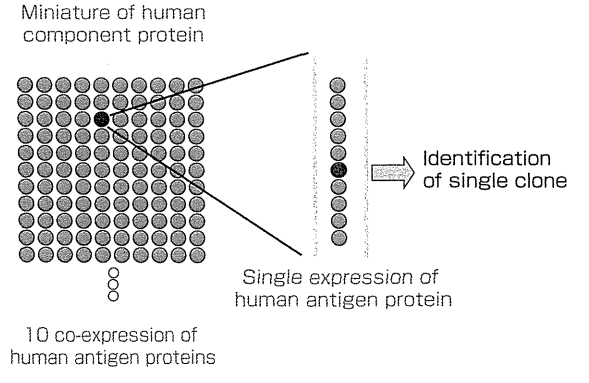


Fig. 6 Antigen identification by C-PAA and E-PAA

The University of Tokyo Hospital, with numbness in the hands as an initial symptom. The patient's blood serum was diluted 1,000 times with PBS-T, and the autoantibody detection was conducted using the chemiluminescence detector using the HRP-labeled anti-human IgG antibody. From the detection results, about 37 types of autoantibodies were found (Fig. 7). The list of detected autoantibodies are shown as antigens in Table 2. LIMS1 and TRIM21 are autoantibodies that are detected highly frequently (80 % or higher) in healthy individuals. Although it has been reported that TRIM21 may be a marker antibody for lung cancer,[9] in our research, it was detected at high percentage (65 % or higher) in healthy individuals. Recently, the relationships to cancer have been reported for the SSX family of SSX1, SSX2, SSX3, SSX4B, and SSX5,[10] cancer antigens (CTA) CTA45A4 and CTA45A5, and lipase MGLL.[11] The antigen proteins of the autoantibodies detected this time were also reported to be related to cancer. On the other hand, IRX2 is a factor related to the formation of cerebellum,[12] and CTNNB2 is a protein related to the intercellular communication in the neurological connection.[13] From these findings, it was shown that the autoantibodies in the serum could be comprehensively detected using the PAA, and the antibodies related to cancer and cerebellar degeneration were detected. Since the cancer antibody was detected by the serum antibody analysis, the patient underwent thorough tests for cancer, and an ovarian tumor was found. It is not uncommon to find cancer in patients who visit the neurology department after experiencing some neurological symptoms. First, the cancer develops and several autoantibodies are produced as a result, and some of the autoantibodies may lead to neurological diseases. Currently, we are investigating whether these detected antibodies may become new biomarkers, by employing the JST Advanced Measuring and Equipment Development Program for 2012~2015 to obtain and analyze the autoantibody data of several patients and healthy subjects. If the autoantibody profiling can be done inexpensively and quickly using the PAA, it may be an extremely effective method for increasing the precision of various diagnoses.

4 Future issues

It is said that the antibodies are produced in large amounts, almost as much as the amplification of PCR against the

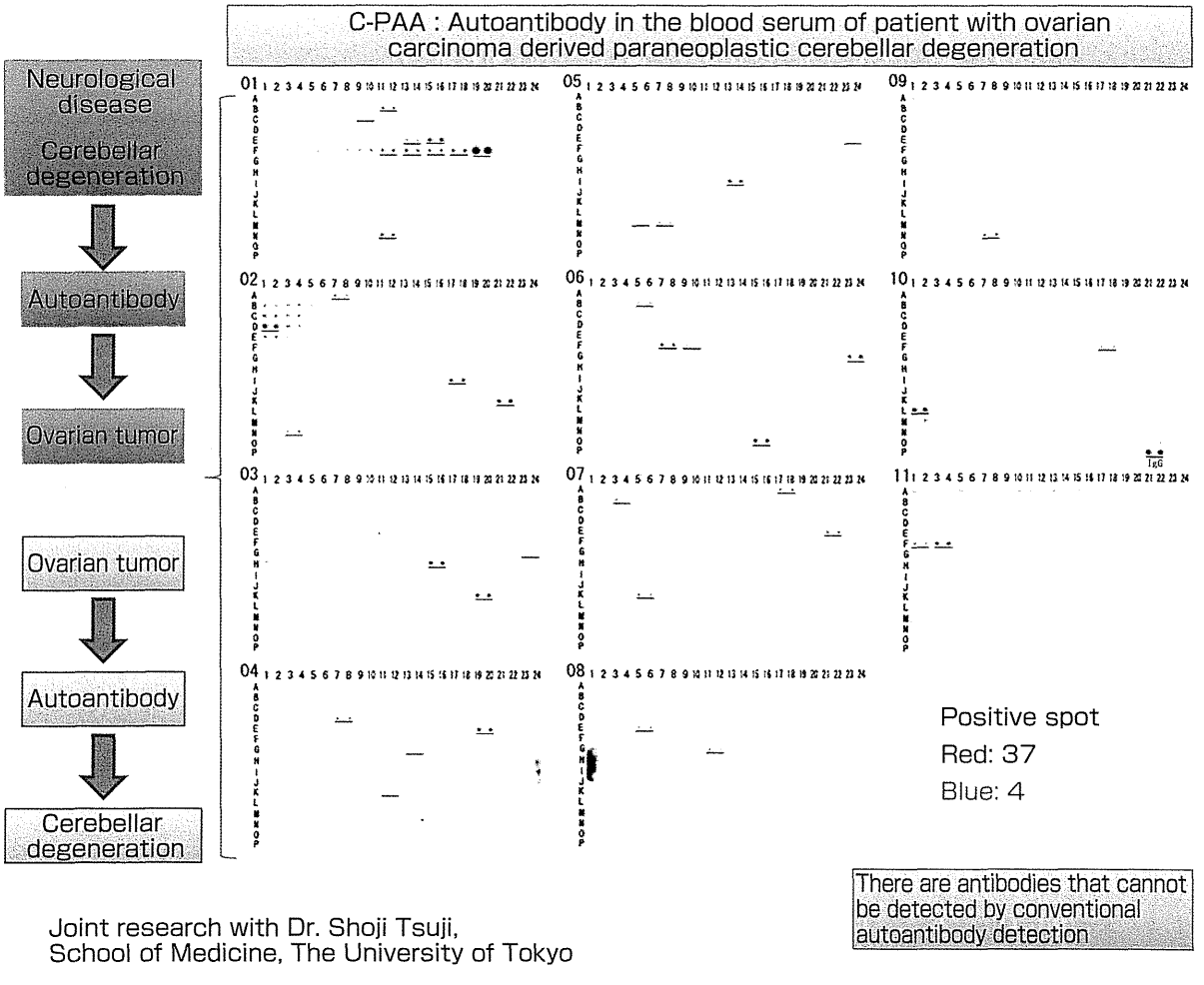


Fig. 7 Autoantibody detection from serum of patient with paraneoplastic neurological disease

Table 2. Autoantibody analysis of serum of patient with paraneoplastic neurological disease

Purified antibody no.	FLJ no.	GeneSymbol	Description
1	FLJ96281AAAF	DUSP11	dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)
2	FLJ44773AAAF	A1BG	
3	FLJ81708AAAF	SSX2	synovial sarcoma, X breakpoint 2
4	FLJ81661AAAF	SSX1	synovial sarcoma, X breakpoint 1
5	FLJ82512AAAF	SSX3	synovial sarcoma, X breakpoint 3
6	FLJ81139AAAF	SSX5	synovial sarcoma, X breakpoint 5
7	FLJ13227AAAF	NXT2	nuclear transport factor 2-like export factor 2 (NXT2)
8	FLJ25823AAAF	CT45A4	cancer/testis antigen family 45, member A4
9	FLJ44051AAAF	LOC100128002	
10	FLJ13132AAAF	BAT5	
11	FLJ45293AAAF	CTNNA2	catenin (cadherin-associated protein), alpha 2
12	FLJ94954AAAF	LIMS1	LIM and senescent cell antigen-like domains 1
13	FLJ81000AAAF	SSX4B	synovial sarcoma, X breakpoint 4B
14	FLJ81065AAAF	TRIM21	tripartite motif-containing 21
15	FLJ96747AAAF	CD320	
16	FLJ96595AAAF	MGLL	
17	FLJ83136AAAF	CT45A5	cancer/testis antigen family 45, member A5
18	FLJ31021AAAF	LOC100129917	hypothetical protein LOC100129917
19	FLJ93657AAAF	RPL3L	ribosomal protein L3-like (RPL3L)
20	FLJ93363AAAF	MLLT3	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3
21	FLJ92129AAAF	RPL6	60S ribosomal protein L6
22	FLJ92146AAAF	RGS16	regulator of G-protein signaling 16
23	FLJ96402AAAF	RGS5	regulator of G-protein signaling 5
24	FLJ96688AAAF	RGS1	regulator of G-protein signaling 1
25	FLJ30022AAAF	RGS3	regulator of G-protein signaling 3
26	FLJ20416AAAF	NXF2B	nuclear RNA export factor 2B
27	FLJ31197AAAF		
28	FLJ37690AAAF		
29	FLJ39521AAAF		
30	FLJ38906AAAF		
31	FLJ25862AAAF		
32	FLJ27182AAAF		
33	FLJ44385AAAF		
34	FLJ56587AAAF		
35	FLJ41898AAAF		
36	FLJ82376AAAF	IRX2	Iroquois homeobox 2

➡ Cancer related ➡ Cerebellum related

amount of antigens. The antibodies circulate throughout the body through blood, and therefore, it is thought that minute changes in the body can be found by studying the antibodies. In fact, it is becoming possible to profile the changes in autoantibodies of the body using PAAs, and the autoantibody detection system is entering the practical phase in terms of technology.

In the future, the comprehensive detection system for autoantibody using PAAs will allow the autoantibodies to be profiled comprehensively for various diseases such as cancer and autoimmune diseases, and we hope to accumulate the data on the relationships between several diseases and autoantibodies. By doing this, we hope it will become possible to conduct an integrated test through autoantibody profiling of the blood, to allow the evaluations of progress and treatment of disease, early detection, policy for diagnosis and treatment, and therapeutic effects. In the development of PAAs, we are working on achieving higher density of protein arrays, and on finding economic ways of conducting highly sensitive measurement using small quantities of blood. We also wish to create a system that can be used easily in general hospitals and research facilities.

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Withdrew from the doctorate course after obtaining the necessary credits from the Graduate School of Science, Osaka City University in 2008. Participated in the Regional Innovation Creation R&D Program "Development of Efficient Tumor Serum Marker by Random Immunization" and "Development of Comprehensive Diagnostic System for Specific Cancers Using Autoantibodies," as a technical staff at the Biomedicinal Information Research Center, AIST. Also worked in the "Multi-Institutional Pre-Phase II Clinical Trial for Post-Operative Esophageal Cancer Cases for CHP/NY-ESO-1 Polypeptide Cancer Vaccine" of the Ministry of Health, Labour and Welfare. Joined the Fukushima Medical Industrial Translational Research Project as a researcher of the Japan Biological Informatics Consortium from May 2013 to present. In this paper, was in charge of the creation of protein array and autoantibody measurement.

Naoki GOSHIMA

Completed the courses at the Department of Biochemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University in 1987 (Doctor of Agricultural Science). Flexible Researcher, RIKEN; Assistant, Kyoto Pharmaceutical University; Assistant Professor, Graduate School of Science, Hiroshima University; and currently, Team Leader, Quantitative Proteomics Team, Molecular Profiling Research Center for Drug Discovery, AIST. Participated in the "Protein Function Analysis Project" of the New Energy and Industrial Technology Development Organization (NEDO) from 2000, and engaged in genome-wide protein functional analysis based on the Gateway creation of human full-length cDNA and comprehensive protein expression. Utilized the human protein expression resource and obtained results for the NEDO "Chemo-Bio Project," NEDO "TR Product," and "Special Project for Yamanaka iPS Cell" of Japan Science and Technology Agency in 2006. Engaged in the development of PAA in the Kyushu Regional Consortium in 2006 and the Kanto Regional Innovation in 2008~2010. Conducted the autoantibody profiling in blood sample to search for autoantibody biomarker. In this paper, was in charge of supervising the protein array research and collaboration with the medical practices.

Discussions with Reviewers

1 Overall comments

Question and comment (Yasushi Mitsuishi, AIST Tohoku)

I read this paper with interest, on the use of the results of the national project following from the human full-length cDNA project, and on the possibility of enabling early diagnosis of disease through blood serum autoantibody profiling that was not initially considered. I understood the idea that some kind of autoantibody is produced excessively in the development of a disease and that this can be used as a disease marker, but I felt the explanation of the technological result was too brief and I was somehow left unsatisfied. The paper seems to finish with the conclusion that you created a PAA that allows the comprehensive analysis of the bonded protein in blood serum. While it is great that you can do comprehensive analysis of proteins, but in this paper, please describe in detail the types of serum autoantibodies, the amount, and the level at which the presence and degree of disease can be estimated.

Answer (Naoki Goshima)

The detailed results described in the initial manuscript will be submitted to a specialized journal, including the consideration of autoantibody as a marker. Therefore, I responded to your comment by including the analysis of the autoantibodies in the blood serum of a patient with ovarian carcinoma derived paraneoplastic cerebellar degeneration as the data of the paper that focuses on the development of protein array technology.

2 Concept of PAA

Question and comment (Noboru Yumoto, AIST)

The objective of this research is "the development of a comprehensive detection system for autoantibodies," and centering on the major breakthrough of "the construction of the world's largest protein expression resource," the scenario, in which the elemental technologies including comprehensive protein expression technology, protein array technology, antibody detection technology, and screening technology were integrated, is understandable to the readers of the bio field. However, I think it is difficult for people outside the field to understand what actually is a proteome array. Therefore, can you include a conceptual diagram of the PAA that you developed?

Answer (Naoki Goshima)

I added the conceptual diagram of the PAA to Fig. 4B, and added explanations to the diagram, Fig. 4.

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Noncanonical NOTCH Signaling Limits Self-Renewal of Human Epithelial and Induced Pluripotent Stem Cells through ROCK Activation

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NOTCH plays essential roles in cell fate specification during embryonic development and in adult tissue maintenance. In keratinocytes, it is a key inducer of differentiation. ROCK, an effector of the small GTPase Rho, is also implicated in keratinocyte differentiation, and its inhibition efficiently potentiates immortalization of human keratinocytes and greatly improves survival of dissociated human pluripotent stem cells. However, the molecular basis for ROCK activation is not fully established in these contexts. Here we provide evidence that intracellular forms of NOTCH1 trigger the immediate activation of ROCK1 independent of its transcriptional activity, promoting differentiation and resulting in decreased clonogenicity of normal human keratinocytes. Knockdown of NOTCH1 abrogated ROCK1 activation and conferred sustained clonogenicity upon differentiation stimuli. Treatment with a ROCK inhibitor, Y-27632, or ROCK1 silencing substantially rescued the growth defect induced by activated NOTCH1. Furthermore, we revealed that impaired self-renewal of human induced pluripotent stem cells upon dissociation is, at least in part, attributable to NOTCH-dependent ROCK activation. Thus, the present study unveils a novel NOTCH-ROCK pathway critical for cellular differentiation and loss of self-renewal capacity in a subset of immature cells.

Notch is an evolutionarily conserved cell surface receptor that plays essential roles in cell fate decisions as well as maintenance of self-renewing tissue organization (1–3). Notch proteins are expressed in most adult tissues, and the biological consequence of Notch activation is critically dependent on the cell type and the cellular context (4–7). In keratinocytes, Notch1 has been shown to be a key inducer of differentiation (8–11). Keratinocyte-specific conditional deletion of the *Notch1* gene results in epidermal hyperproliferation and tumor formation in mice, thus indicating a tumor-suppressive role of Notch1 in mammalian postnatal epidermis (12). The Notch receptor is generally activated by interaction with its ligands displayed on the neighboring cell surface. Cell-cell contact is a strong inducer of keratinocyte differentiation in culture, where Notch1 acts as a critical determinant in the transition from proliferation to differentiation (13, 14). Due to *cis* inhibition of Notch by its ligand when these are expressed on the same cell surface (15, 16), the relative increase in expression levels of the Notch receptor over its ligand is also shown to be a pivotal cue to activate Notch signaling and generate distinct cell fates among neighboring cells (17). We previously demonstrated that p53 and TAp63 transactivate *Notch1* gene expression and induce keratinocyte differentiation, while ΔNp63 is a transcriptional repressor of the *Notch1* gene and inhibits keratinocyte differentiation (14, 18). p63, especially ΔNp63α, is a master regulator of development and maintenance of stratified epithelia (19, 20). ΔNp63α expresses predominantly in the basal proliferating compartment, where Notch1 signaling is suppressed (21). In suprabasal layers, downregulation of ΔNp63α by miR-203 or another factor(s) (22–24) evokes activation of Notch1 signaling, which in turn further downmodulates ΔNp63α expression so as to induce differentiation (9, 21). The Notch1 precursor (~300 kDa) is processed by furin protease in the Golgi apparatus and transported to the cell surface as a mature heterodimeric complex (~120/~180 kDa) that is held by Ca^{2+}-dependent noncovalent interaction (25). Ligand binding dissociates the Notch1 extracellular domain (~180 kDa) by *trans* endocytosis. The residual transmembrane domain (~120 kDa) is sequentially cleaved by tumor necrosis factor alpha-converting enzyme/metalloprotease (TACE) and γ-secretase, resulting in release of the Notch1 intracellular domain (~110 kDa) into the cytosol (3). EDTA is reported to activate Notch signaling through disruption of the heterodimeric complex of Notch1 (25) and thus used as a tool to study Notch1 signaling (26–28). In canonical Notch1 signaling, the liberated Notch1 intracellular domain (~110 kDa) translocates into the nucleus to activate Notch-responsive genes, such as Hes1, by making a complex with CSL family members {CBF1 and RBP-Jκ in mammals, Suppressor of hairless [Su(H)] in *Drosophila*, and Lag1 in *Caenorhabditis elegans*} and its transcriptional coactivator Mastermind (MAM). Besides this canonical pathway, accumulating evidence suggests noncanonical cytoplasmic Notch functions (29–31).

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Rho-associated coiled-coil protein kinases (ROCKs) (also known as Rho kinases [ROKs]) are effectors of the small GTPase Rho and belong to a family of protein serine/threonine kinases (32–34). Activated ROCK proteins regulate actomyosin cytoskeletal dynamics and contractility through phosphorylation of multiple downstream targets, such as myosin phosphatase (MYPT1), to drive cell motility. In keratinocytes, ROCK proteins play a role in differentiation (35, 36), and their selective inhibitor, Y-27632, completely inhibits differentiation as well as stratification of keratinocytes in organotypic raft culture (37). Y-27632 also enables efficient immortalization of not only human primary keratinocytes but also several other primary human epithelial cells in the presence of fibroblast feeders (37, 38), although molecular details supporting immortalization remain elusive.

In addition, Y-27632 has been shown to increase the survival rate and cloning efficiency of human embryonic stem cells (hESCs) dissociated with EDTA (39) through blocking the Rho-ROCK-myosin light chain signaling cascade (40, 41). However, the precise mechanisms by which EDTA activates ROCK have not been elucidated (41, 42).

These results let us hypothesize a possible link between NOTCH1 and ROCK activation. Here we show a novel function of NOTCH1 as a critical upstream regulator of ROCK1 and its relevance to loss of self-renewal capacity in human keratinocytes as well as human induced pluripotent stem (hiPS) cells.

MATERIALS AND METHODS

Cell culture. Normal human cervical keratinocytes (HCKs) were obtained with written consent from a patient who underwent abdominal surgery for a gynecological disease other than cervical cancer and were retrovirally transduced with the catalytic subunit of human telomerase reverse transcriptase (hTERT) for immortalization (HCK1Ts) (14). HCK1Ts were cultured in serum-free keratinocyte-SF medium supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 μg/ml of bovine pituitary extract (Invitrogen, Life Technologies, Saint Aubin, France). Primary human dermal keratinocytes (HDKs) were purchased from Cell Applications Inc. (San Diego, CA). Primary human foreskin keratinocytes (HFKs) were obtained from Denise A. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). HDKs and HFKs were cultured in serum-free keratinocyte-SF medium supplemented with 5 ng/ml EGF and 50 μg/ml of bovine pituitary extract (Invitrogen, Life Technologies). Human endometrium cells were collected by scraping tissues from surgical specimens, with signed informed consent and with ethical approval of the Institutional Review Board of the National Institute for Child Health and Development, Japan. All experiments involving human cells and tissues were performed in line with Tenets of the Declaration of Helsinki. Human iPS cell lines, MRC-hiPSCs and UtE-hiPSCs, were established from MRC-5 fetal lung fibroblasts (43) and UtE1104 endometrium-derived cells (44), respectively, via procedures described by Takahashi et al. (45) with slight modification (46, 47). Human iPS cells were maintained in iPSellon medium (Cardio Incorporated, Osaka, Japan) supplemented with 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in the presence of irradiated mouse embryonic fibroblast (MEF) feeders.

Retroviral vector construction and transduction. Retroviral vector plasmids were constructed using the Gateway system according to the manufacturer's instructions (Invitrogen). Segments of the intracellular domain of human NOTCH1 (ICN1), a truncated form of MAML1 corresponding to amino acids 13 to 74 fused to N-terminal hemagglutinin (HA) tag (MAML61-3HA), and c-MYC were cloned and recombined into retroviral expression vectors to generate pCLXSN-ICN1 (14), pCLXSN-MAML61-3HA (48, 49), and pCMSCVpuro-c-MYC (50). Human ROCK1, ROCK1ΔC241, ROCK1-D1113A, ROCK1-K105A, ICN1-ERT, ICN2-ERT2, RhoA, and enhanced green fluorescent protein (EGFP) were cloned into a lentiviral vector, CSII-TRE-Tight-RfA, in which the elongation factor promoter in CSII-EF-RfA (a gift from Hiroyuki Miyoshi, RIKEN, BioResource Center) was replaced with the tetracycline-responsive promoter from pTRE-Tight (Clontech, Mountain View, CA). The Notch1 short hairpin RNA (shRNA) vectors were described previously (14, 18). To generate ROCK1- or ROCK2-specific shRNA expression vectors pCL-SI-MSCVpuro-ROCK1Ri-1,-2,-3 and pCL-SI-MSCVpuro-ROCK2Ri-1,-2,-3, the following sequences were chosen as the targeted sites: 5′-GTACTTGTATGAAGATGA-3′ (51), 5′-GGTATATGCTATGAAGCTT-3′, and 5′-GCGAAATGGTGTAGAAGAA-3′ for ROCK1 and 5′-GAAACTAATAGGACACTAAC-3′ (52), 5′-GGTTTATGCTATGAAGCTT-3′, and 5′-GGATAAACATGGACATCTA-3′ for ROCK2. The retroviral vector and packaging constructs pCL-GagPol and pEF6/env (10A1) or the lentiviral vector and packaging constructs pCAG-HIVgp and pCMV-VSV-G-RSV-Rev were cotransfected into 293FT cells (Invitrogen) using TransIT-293 (Mirus Co., Madison, WI) according to the manufacturer's instructions, and the culture fluid was harvested at 60 to 72 h posttransfection. Titers of the recombinant viruses were determined by drug resistance with HeLa cells or a real-time PCR method (TaKaRa, Otsu, Japan) to detect the viral RNA genome, yielding titers equivalent to greater than 1×10^6 CFU/ml. Following addition of the recombinant viral fluid to cells in the presence of 4 μg/ml Polybrene, infected cells were selected in the presence of 0.5 μg/ml puromycin or 50 μg/ml G418, and promptly after drug selection, pooled cell populations were used for most subsequent experiments.

Tet-On keratinocytes. HCK1T cells were stably transduced with Tet-On ADV and tTS expression vectors, encoding the rtTA-Advanced transactivator and transcriptional silencer, respectively (Clontech). The resultant HCK1T Tet-On cells were then introduced with CSII-TRE-Tight-ROCK1, ROCK1ΔC241, ROCK1-D1113A, ROCK1-K105A, ICN1-ERT, ICN2-ERT2, RhoA (constitutive active and dominant negative forms), and EGFP by retroviral gene transfer. Induction of these transgenes was routinely achieved by treatment with 1 μg/ml doxycycline (DOX) for 72 h.

Inhibitors. The following pharmacological inhibitors were used: cycloheximide (CHX) (239764; Calbiochem, Darmstadt, Germany), z-VAD-fmk (caspase inhibitor IV) (219007; Calbiochem), γ-secretase inhibitor IX (DAPT) (565784; Calbiochem), Y-27632 (08945-84; Nacalai Tesque, Kyoto, Japan), C3 ADP-ribosyltransferase (Rho inhibitor) (CT04; Cytoskeleton, Inc., Denver, CO), and blebbistatin (sc-203532; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were pretreated with inhibitors for 2.5 h. For DAPT, in addition to pretreatment, cells were incubated with this inhibitor during and after exposure to EDTA or differentiation stimuli for up to 48 h.

Induction of keratinocyte differentiation. At 48 h after plating, HCK1T cells were treated with 2.5 mM EDTA in phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] for 10 min or exposed to 0.7% and 5% bovine serum albumin (BSA) or 10% serum-containing medium in the presence of 10 μg/ml of bovine pituitary extract. To induce ligand-dependent NOTCH activation, HCK1T cells were harvested in subconfluent and 7-day-postconfluent states. HCK1T cells were also introduced with ICN1 by retroviral gene transfer to induce differentiation.

Dissociation of human iPS cells. First, hiPSC colonies were treated with collagenase IV solution at 37°C for 10 min. The detached hiPSC clumps were recovered, incubated with 0.005% trypsin–2.5 mM EDTA solution at 37°C for 5 min, and dissociated into single cells by pipetting. The dissociated cells were counted with Vi-CELL (Beckman Coulter, Brea, CA) and seeded onto MEF feeders.

Immunoblotting. Whole-cell protein extracts were used for analysis, and immunoblotting was conducted as described previously (14). Primary antibodies against Notch1 (sc-6014; Santa Cruz Biotechnology), activated Notch1 (cleaved Notch1 Val1744 2421; Cell Signaling Technology, Danvers, MA), Notch2 (clone C651.6DbHN; Developmental Studies Hybridoma Bank, University of Iowa), Hes1 (Toray Industries, Inc., To-