Proteomic Identification of Autoantibodies in Sera from Patients with Ovarian Cancer as Possible Diagnostic Biomarkers

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Abstract. *Background/Aim: Accumulating evidence shows that various types of cancers induce a specific immune response resulting in the production of antibodies against self-components (autoantibodies). The aim of the present study was to identify antigens for autoantibodies in sera from patients with ovarian cancer, especially clear cell carcinoma (CCC), as novel diagnostic markers for the disease. Materials and Methods: The reactivity of individual sera from patients was examined by two-dimensional (2-D) immunoblotting using lysates of CCC cell lines, ES-2 and RMG-1, as antigens to identify autoantigens. ELISA was established to quantitatively measure autoantibody titer of patients' sera. Results: Autoantibodies against RhoGDI were induced in sera of ovarian cancer patients. Elevated levels of autoantibodies against heterogeneous nuclear ribonucleoprotein L (hnRNPL) and a mitochondrial protein, dihydrolipoamide dehydrogenase (DLD), were detected in patients with CCC. Conclusion: Autoantibodies against RhoGDI and hnRNPL and DLD may serve as novel diagnostic markers for ovarian cancer and CCC, respectively.*

Ovarian cancer is the leading cause of mortality among gynecological malignancies in developed countries with 238,700 new cases and 151,900 estimated deaths worldwide in 2012 (1). Epithelial ovarian cancer is heterogeneous and primarily classified into serous, mucinous, endometrioid, clear cell, transitional and squamous cell tumors, based on histological grade, molecular phenotype, and genotype (2, 3). World Health Organization (WHO) classification further sub-divides these tumors into benign, borderline and malignant (carcinoma) forms (4). Among them, clear cell carcinoma (CCC) is a type with a highly malignant potential. CCC can be distinguished from other types of epithelial ovarian cancer by its high recurrence rate, significantly poor prognosis and extreme chemo-resistance to cytotoxic anti-cancer agents (2, 5).

It is widely recognized that diagnosis of ovarian cancer at early stages is particularly important for successful clinical treatment. Cancer antigen 125 (CA125/MUC16) has been used for the diagnosis of ovarian cancer (6-8). However, prospective studies indicated the inadequate sensitivity of CA-125 in the setting of ovarian cancer screening in asymptomatic populations. CA125-based diagnosis often provides false positives, because serum CA125 level increases in patients with other cancers or in women suffering from inflammation, benign tumors or even in healthy women in pregnancy or menstruation (6). Furthermore, the level of CA125 is significantly lower in the sera of clear cell and mucinous cancer patients than patients with other cell types. Indeed, 13% of CCC patients (13/101) had a prechemotherapy CA125 level of <35 U/ml (9). There is, thus, an urgent need for the discovery and development of alternative biomarkers for the diagnosis of CCC.

There are lines of evidence that various types of cancers induce a specific immune response, resulting in the production of antibodies against self-components (autoantibodies) (10-13). These autoantibodies may result from the aberrant context of cancer cells including over-production of various proteins, release of proteins from apoptotic cells and/or abnormal modification of proteins. Furthermore, various mutations that either activate proto-oncogenes or inactivate the function of

Abbreviations: CCC, Clear cell carcinoma; 2-D, two-dimensional; hnRNPL, heterogenous nuclear ribonucleoprotein L; DLD, dihydrolipoamide dehydrogenase.

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tumor suppressor gene products take place during the development of cancer. For instance, the *p53* gene undergoes missense mutations that inactivate the gene product in most types of cancer. In 42 CCC cases, mutations in the *PI3KCA* (14), *KRAS, ARID1A* (15, 16) and *PPP2R1A* (17) genes are observed in 40%, 4.7%, 57% and 7.1% of the cases, respectively (15). Such missense mutations may alter the structure of the gene product and trigger immunological responses of the hosts. Indeed, anti-p53 antibody is induced among patients suffering from many types of cancer and the ratio of antibody-positive patients in each cancer type correlates well with the mutation frequency of *p53* gene in the corresponding cancer (18, 19).

We carried out two-dimensional (2-D) western blot analyses to systematically identify disease-specific autoantibodies and their antigens. Using this strategy, we analyzed autoantibodies in sera of patients with systemic lupus erythematosus and identified 11 antigens that are the targets of autoantibodies (20, 21). Interestingly, the level of one of the autoantibodies correlates with psychiatric syndromes (21). Furthermore, we demonstrated that an IgA autoantibody against a mitochondrial protein, dihydrolipoamide dehydrogenase (DLD), is induced in patients with endometrial cancer (22).

In the present study, we applied this strategy to identify autoantibodies specific to CCC patients. We identified 24 proteins as candidate targets of autoantigens of autoantibodies induced in sera of CCC patients. Slot-blot analyses revealed that patients with ovarian cancer showed higher autoantibody response to RhoGDI (a modulator of rho family G proteins). Furthermore, quantification of serum autoantibody titer by ELISA demonstrated that IgA antibodies against heterogeneous nuclear ribonucleoprotein L (hnRNPL) and DLD are induced in sera of patients with CCC. These results suggest that these autoantibodies may serve as diagnostic biomarkers for ovarian cancer and CCC.

Materials and Methods

Sera. Sera from patients with ovarian cancer (n=49; mean age, 53.3 years), borderline tumor (n=15; mean age, 62.0 years) and benign tumor (n=43; mean age, 47.2 years) were obtained at the Department of Obstetrics and Gynecology, Nippon Medical School Main Hospital, Tokyo, Japan. The subtypes of the ovarian cancer included serous (n=16; mean age, 56.4 years), mucinous (n=14; mean age, 51.8 years), clear cell (n=14; mean age, 50.5 years) and endometrioid (n=5; mean age, 65.0 years). Normal control sera were taken at the Research and Development Center, BML, Inc. Kawagoe, Japan (n=54;, mean age, 40.8 years). All sera were obtained from donors who provided written informed consent. This project was performed with the approval of the appropriate institutional research ethics committees.

Cell culture. CCC cell lines, ES-2 and RMG-1, were obtained from the American Type Culture Collection (Manassas, VA, USA) and the RIKEN Bioresource Center, respectively (Tsukuba, Ibaraki, Japan). ES-2 and RMG-1 cells were cultured in McCoy's 5 (Invitrogen, Carlsbad, CA, USA) and Ham's F12 (Sigma, St. Louis, MO, USA) media, respectively, supplemented with 10% (v/v) fetal bovine serum. HeLa cells and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, USA) supplemented with 10% (v/v) fetal bovine serum. All cell lines were cultivated at 37˚C under 5% CO_2.

2-D immunoblotting. To prepare total cell lysates, ES-2 and RMG-1 cells grown to saturation density were washed with cold phosphate-buffered saline (PBS) twice and mixed at 1:1. The cells were then lysed by sonication (Sonifier 250D; Branson Ultrasonics Corp., Danbury, CT, USA) on ice in a lysis buffer containing 8 M urea, 4% CHAPS, 40 mM Tris-HCl, pH 7.0, and complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA) and the supernatants were saved as total cell lysates after centrifugation at 10.000 × *g* for 15 min. The membrane fractions were prepared from ES-2 cells with a 2-D Fractionation Kit (GE Healthcare, Milwaukee, WI, USA) according to the manufacturer's instructions. To obtain conditioned media, ES-2 cells were cultured in serum-free Opti-MEM medium (Invitrogen, Carlsbad) for 2 days and the supernatants were harvested after centrifugation at 10,000 × *g* for 20 min. The supernatants were concentrated by ultrafiltration before use. Protein concentration was determined by the BCA Kit (Pierce, Rockford, IL, USA). All protein samples were desalted with 2-D Clean-Up Kit (GE Healthcare) and the precipitated proteins were dissolved in DeStreak Rehydration Solution (GE Healthcare) containing 50 mM DTT and 0.5% IPG buffer 3-10 (GE Healthcare).

2-D gel electrophoresis was carried out as described (20). The sample was applied onto an Immobiline DryStrip gel (GE Healthcare, 7 or 11 cm long, pH 3-11 NL) and isoelectric focusing was carried out using Ettan IPGphor II (GE Healthcare) according to the manufacturer's instructions. The second dimension was 9% SDS-PAGE. After 2D-PAGE, the separated proteins were transferred onto Immobilon-P (Millipore, Billerica, MA, USA) and the proteins on the membrane were stained with 0.4 μg/ml Cy5 Mono-Reactive Dye (GE Healthcare) (20). After blocking the membrane with phosphate-buffered saline-0.1% Tween 20 (PBS-T) containing 5% dry fat milk, immunoblotting was carried out using serum from an individual patient or pooled sera diluted 1:200 to 1:500 in the blocking solution as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-human IgG and/or anti-human IgA (Binding Site, Birmingham, UK) as a secondary antibody (20). After washing, the membrane was reacted with ECL-plus (GE Healthcare). The fluorescent signals of Cy5 and ECL-plus were obtained using a Typhoon 9400 scanner (GE Healthcare) with appropriate excitation and emission wavelengths and emission filters (Cy5, excitation at 633 nm, detection at 670 nm, emission filter 670BP30; ECL-plus, excitation at 457 nm, detection at 520 nm, emission filter 520BP40).

Identification of antigens of autoantibody detected by patients' sera. For the identification of autoantigens, protein samples (300 μg) was separated using 2D-PAGE as described above, transferred onto a ProBlott membrane (Applied Biosystems, Foster City, CA, USA) and stained with Coomassie brilliant blue (CBB) R-350 (PhastGel Blue R-350; GE Healthcare). Patients' sera-specific spots were excised and proteins in the spots were subjected to peptide mass fingerprinting as described by Iwamatsu (23). The resulting peptides

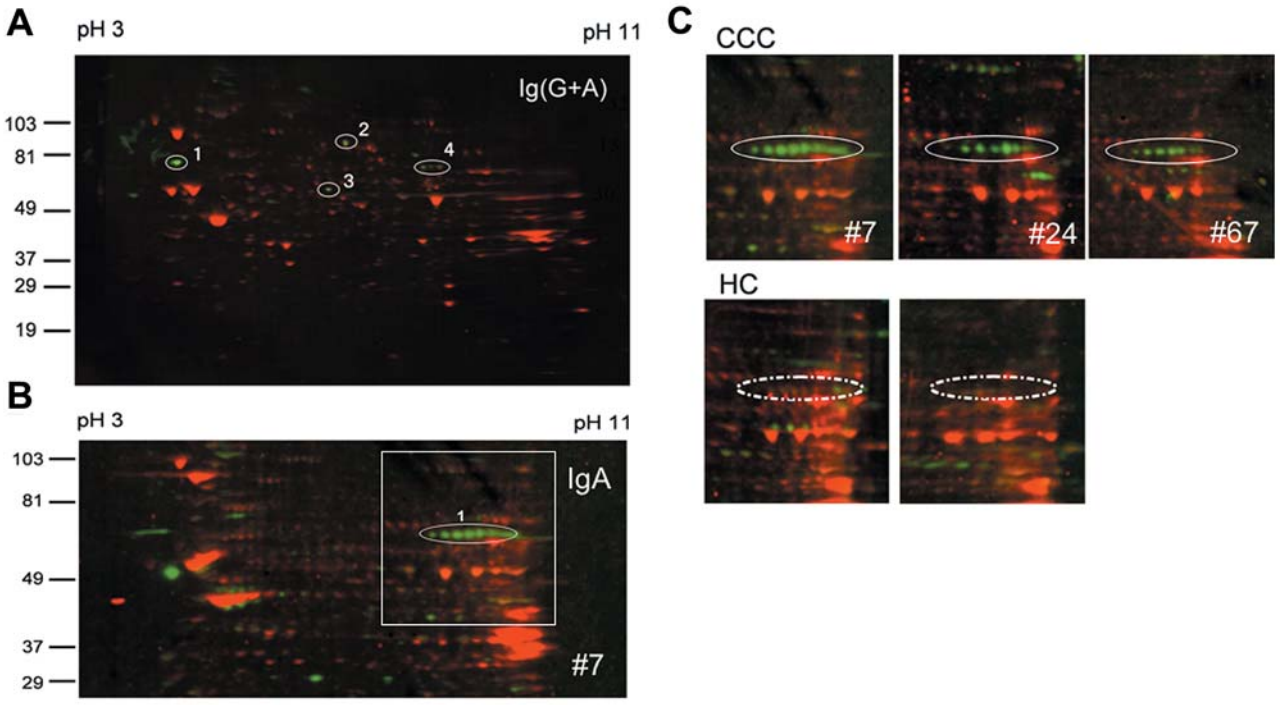

Figure 1. *Detection of autoantibody-reactive protein spots on 2-D immunoblotting. A. An equal mixture of total cell lysates from ES-2 and RMG-1 cells was subjected to 2-D immunoblotting using pooled sera of CCC patients (#7, #11, #24, #67, #115) and HRP-conjugated anti-human Ig(G+A). Protein spots 1 to 4 in the figure were identified as PP2a, ezrin, hnRNPH and hnRNPL, respectively. B. A mixture of ES-2 and RMG-1 total cell lysates was subjected to 2-D immunoblotting using a CCC patient's serum (#7) and HRP-conjugated anti-human IgA. Proteins from spots in circle 1 were identified as hnRNPL. In C, magnified view of IgA response of the area containing circle #1 (shown as a white box in B) for three patients (#7, #24 and #67) and two pools of sera from healthy controls (#201, #204, #205; and #206, #208, #209).*

were purified using NuTip NT1HIL.96 solid-phase extraction cartridges (Glygen, Columbia, MD, USA), mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix and analyzed using a MALDI-TOF mass spectrometer (Voyeger™; Applied Biosystems). Peptide mass fingerprinting data were compared to the NCBInr databases (human, 233173 sequences; date 2010/12/24) using the Mascot Search engine (Peptide Mass Fingerprint, version 2.2; Matrix Science, Boston, MA, USA).

cDNA cloning and generation of recombinant proteins. cDNAs of the identified antigens were amplified by polymerase chain reaction (PCR) using cDNA libraries prepared from ES-2 and RMG-1 cells. The attB1 and attB2 sequences were included in the sense and antisense primers, respectively, to insert the product into the GATEWAY system (Life Technologies, Carlsbad). Histidine hexamer was added at the carboxy terminal of the recombinant proteins for purification. The amplified cDNAs were integrated into mammalian vector pcDNA DRST40, baculovirus vector pDEST8 or *Escherichia coli* vector pDEST14 by the GATEWAY system (Life Technologies). The recombinant His-tag fusion proteins were transiently expressed in COS-7 cells, insect High Five cells or *E. coli* BL21 and purified using sequentially TALON metal affinity resin (Clontech Laboratories, Mountain View, CA, USA), Ni Sepharose, Hi Trap Q and/or Hi Trap S (GE Healthcare). FLAG-DLD was purified as described previously (22).

Slot blot analyses. Purified recombinant proteins (2.5 μg/gel) were applied to SDS-PAGE (5-20% gradient gel, WAKO Pure Chemicals, Tokyo, Japan) and subjected to slot blot analysis using a screener blotter (Sanplatec, Tokyo, Japan) (22).

Quantification of autoantibody titers by ELISA. Antibody titers against recombinant proteins in the sera of patients with ovarian cancer and controls were quantified by ELISA (20, 22).

Statistical analyses. The results of the ELISA were correlated with the final clinical diagnosis for each patient. Two-group comparisons were analyzed using the Mann–Whitney *U*-test for continuous variables and the Fisher' exact test for categorical variables. Values of $p<0.05$ were considered statistically significant.

Results

Detection of autoantibody-reactive protein spots by 2-D immunoblotting. A pool of five sera from CCC patients (#7, 11, 24, 67, 115) was analyzed by 2-D immunoblotting using an equal mixture of total cell lysates from CCC cell lines, ES-2 and RMG-1. As control experiments, pooled or individual sera from healthy women were used. Figure 1A shows a representative result. The ECL signal showing

Table I. *Candidate autoantigens identified by peptide mass fingerprinting.*

Antigen (cell line)	Protein name	Accession No.	Coverage (%)	Score
Total cell lysates	alpha isoform of regulatory subunit A, protein phosphatase 2	NP_055040	16	96
(ES-2 + RMG-1)	ezrin	P15311	11	90
	heterogeneous nuclear ribonucleoprotein H2	CAB55879	14	72
	αactinin 4	BAA24447	33	285
	alanyl-tRNA synthetase	NP_001596	7	62
	major vault protein	AAH15623	9	65
	FARSLB protein	AAH06502	20	149
	heterogeneous nuclear ribonucleoprotein L	BAB18649	18	86
	calponin 3	AAH25372	19	67
	isocitrate dehydrogenase 3 (NAD+) alpha precursor	NP_005521	19	60
	eukaryotic translation initiation factor 2 beta	AAH00934	15	60
	rho GDP dissociation inhibitor	CAA45344	25	100
	proteasome activator subunit 1 isoform 2	NP_788955	14	57
	chain B, human heart L-lactate dehydrogenase H chain	1I0Z_A	45	155
	dimethylarginine dimethylaminohydrolase 1	NP_036269	31	102
	protein disulfide isomerase-associated 6	AAH01312	15	72
	karyopherin beta 1	NP_002256	17	121
	superoxide dismutase (Mn-SOD)	1JA8_A	52	138
	pyrroline-5-carboxylate reductase 1 isoform 1	NP_008838	21	99
Culture supernatants	enolase 1	NP_001419	18	82
(ES-2)	eukaryotic translation elongation factor 2	NP_001952	9	93
	glutathione S-transferase omega 1	NP_004823	14	59
	F-actin capping protein alpha-1 subunit	NP_006126	29	115
	ubiquitin specific protease 14 isoform b	NP_001032411	20	106
Membrane fractions	F-actin capping protein alpha-1 subunit	NP_006126	36	130
(ES-2)	calponin 3	NP_001830	21	95
	ezrin	P15311	27	214

Candidate autoantigens identified by 2-D immunoblotting are shown with their accession numbers, and their sequence coverages and scores, as identified by peptide mass fingerprinting.

Ig(G+A) reactivity was monitored by its fluorescence (expressed by a green pseudocolor), and the proteins on the membrane labeled with a fluorescent dye Cy5 *in situ,* were monitored by a different fluorescence signal (shown in red pseudocolor), and two patterns were merged.

Protein spots numbered in the figure were commonly detected with sera from multiple CCC patients but not with those from healthy donors. We also performed 2-D immunoblot to analyze IgA autoantibodies (Figure 1B, patient #7) since IgA but not IgG autoantibody response is induced in certain types of cancer (22, 25-28). Notably, the spots in the circle 1 in Figure 1B corresponded to the spots in the circle 5 in Figure 1A. IgA response to the spot 1 proteins was also observed with sera from other two patients (#24, 67) but not with those from healthy donors (Figure 1C).

To facilitate the identification of antigens of the autoantibodies, subcellular fractions (membrane fractions and culture conditioned media) were also employed. Through these experiments, we picked-up 37 protein spots as candidate autoantigens.

Identification of autoantigens. The proteins in the reactive spots were subjected to peptide mass fingerprinting. Among 37 protein spots analyzed, we could identify 24 proteins from 27 spots with high confidence, which are listed in Table I. From the spots 1-4 in Figure 1A, alpha isoform of protein phosphatase 2 (PP2A), ezrin, heterogeneous nuclear ribonucleoprotein H2 (hnRNPH) and hnRNPL were identified, respectively. It is of note that hnRNPL was also identified as IgA-reactive spots (Figure 1B, spots in circle 1) and that ezrin, calponin 4 and F-actin capping protein α1 subunit were repeatedly identified in total cell lysates, membrane fractions and/or culture conditioned media (Table I).

Slot-blot analyses of reactivity of CCC patient sera with recombinant autoantigens. We next generated His-tagged recombinant proteins for the candidate autoantigens to assess the reactivity of sera from CCC patients and control donors with the autoantigens. The recombinant proteins were made in mammalian, insect and/or *Escherichia coli* cells based on their expression level and solubility. The recombinant proteins were purified to near homogeneity through multiple chromatography

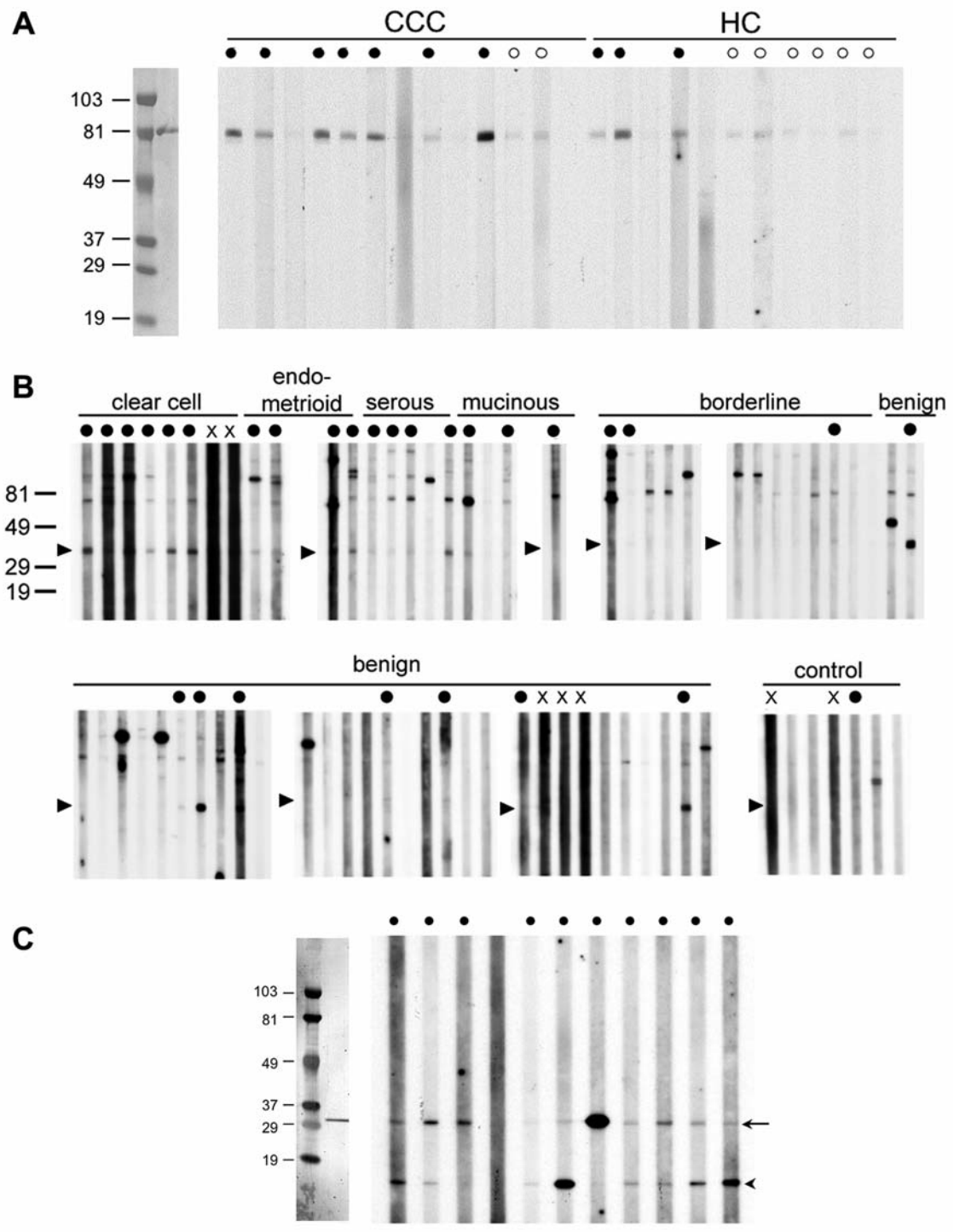

Figure 2. *Slot-blot analyses of patients' autoantibodies using recombinant autoantigens. A. His-tagged hnRNPL protein generated in High Five cells (2.5 μg/gel) was subjected to slot-blot analysis using sera from CCC patients and healthy controls (HC). SDS-PAGE (5-20% gradient gel) of recombinant hnRNPL sample (2 μg, Coomassie blue staining) is shown with molecular weight markers with their molecular weights (in kDa) (left panel). Slot blot analysis of anti-hnRNPL IgA antibody in sera from CCC patients (n=13) and controls (n=11) (right). The autoantibody was detected with HRP-labeled anti-human IgA. Closed and open circles indicate strong and mild responders, respectively. B. Slot-blot analysis of anti-RhoGDI antibody in sera from malignant (clear cell, endometrioid, serous and mucinous), borderline and benign ovarian tumors and healthy controls. The autoantibody was detected with HRP-labeled anti-human Ig(G+A). Closed circles indicate positive reactions. The reactivity of samples marked by crosses could not be evaluated due to the high background noises. C. SDS-PAGE of recombinant Mn-SOD and the results of slot blot analysis using sera from CCC patients. The autoantibody response was detected with HRP-labeled anti-human IgG. Closed circles indicate positive reactions.*

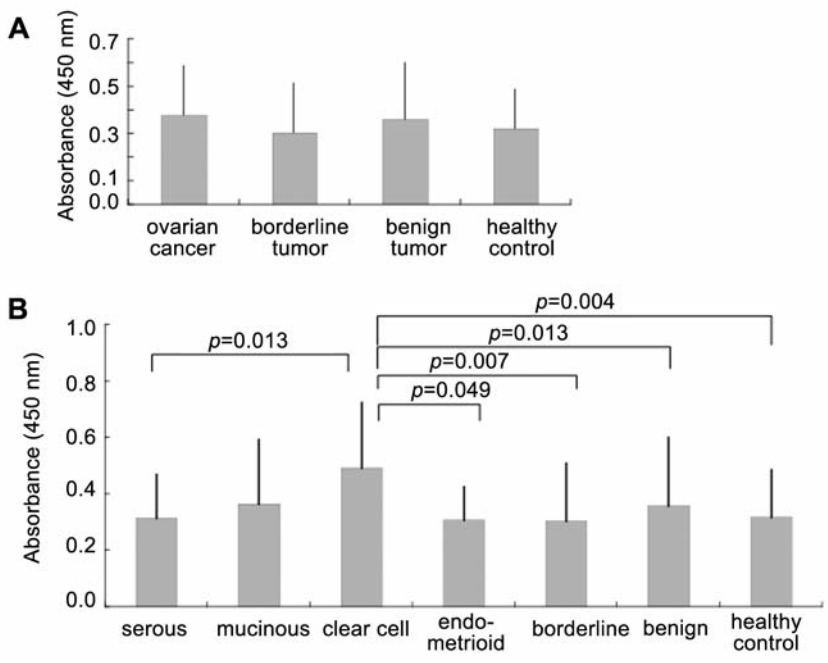

Figure 3. *Quantitation of autoantibodies against hnRNPL in patients with ovarian cancer by ELISA. A. Comparison of anti-hnRNPL IgA titers in sera from patients with ovarian cancer (n=46; mean age, 54.2), borderline tumor (n=15; mean age, 62.0) and benign tumor (n=39; mean age, 40.0) and healthy controls (n=21; mean age, 39.4). B. Comparison of anti-hnRNPL IgA titers in sera from patients with serous (n=15; mean age, 57.6), mucinous (n=13; mean age, 50.2), clear cell (n=13; mean age, 52.8) and endometrioid (n=5; mean age, 65.0) carcinoma; patients with borderline tumor, patients with benign tumor; and healthy controls. p-Values were calculated using the Mann-Whitney U test.*

steps and used as antigens in slot-blot analyses. Figure 2A shows a result with hnRNPL. hnRNPL was expressed in an insect cell line (High Five) and purified by successive chromatographies on TALON metal affinity resin and Hi Trap Q to near-homogeneity (Figure 2A, left panel). Using purified hnRNPL as an antigen, slot blot analysis was carried out (Figure 2A, right panel). Intense IgA reactivity was observed with about half of CCC patients (7/13, marked by closed circles), whereas less than a third of healthy controls exhibited similar reactivity (3/11). Other healthy donors showed no or weaker reactivity (marked by open circles).

Interestingly, sera from malignant ovarian cancer patients including serous, mucinous, endometrioid and clear cell carcinoma, collectively, showed preferential reactivity to RhoGDI (17/19, 89%) over those from borderline tumors (3/13, 23%), benign tumors (8/29, 29%) or healthy controls (1/5, 20%) (Figure 2B), which was shown to be statistically significant the by Fischer's exact test (*vs*. borderline tumor, $p=0.0002$; *vs*. benign tumor, $p<0.0001$; *vs*. healthy control, $p=0.0065$). Although the number of cases was rather small, the positivity rate of CCC patients was also statistically higher than that of patients with borderline (Fischer's exact test, $p=0.0031$) or benign tumor ($p=0.0019$) or healthy controls ($p=0.0152$).

However, sera from both patients and control donors also reacted with proteins other than RhoGDI in the slot-blot analysis, which prevented correct estimation of the antibody titers by ELISA. Similarly, irrespective of the high purity of recombinant Mn-SOD (manganese superoxide dismutase), sera from CCC patients reacted with both target Mn-SOD (indicated by an arrow) and with a protein with lower molecular weight (an arrowhead) (Figure 2C). Such protein samples were considered not suitable for ELISA because of the background signals. Therefore, we further examined the reactivity to hnRNPL quantitatively by ELISA. We also carried-out slot-blot analyses for other candidate autoantigens; however, positivity rates of CCC patient sera for these candidate autoantigens, including Mn-SOD, were similar to those of sera from healthy controls or patients with benign and borderline tumors.

Quantitative analysis of IgA titers to hnRNPL by ELISA. For the assessment of IgA titers to hnRNPL in CCC patients by ELISA, we employed sera from 46 patients with ovarian cancer consisting of four typical subtypes (serous, mucinous, clear cell and endometrioid carcinomas), 15 patients with borderline ovarian tumor, 39 patients with benign ovarian tumor and 21 healthy women. The sera of CCC patients used in the screening by 2-D immunoblotting were included in

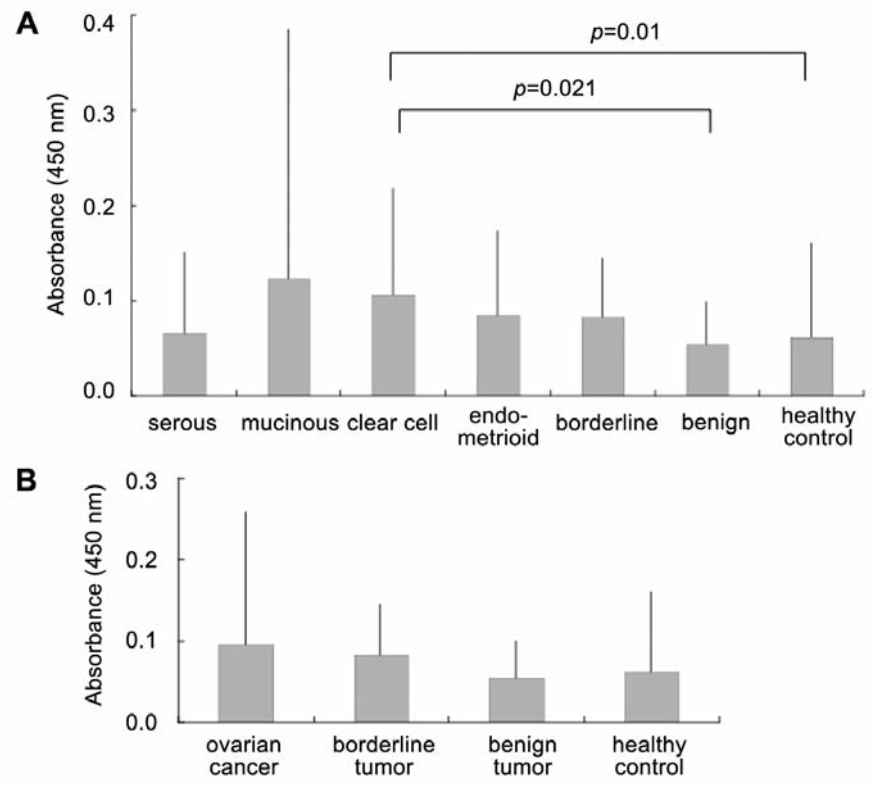

Figure 4. *Quantitation of autoantibodies against DLD in patients with ovarian cancer by ELISA. A. Comparison of anti-DLD IgA titers in sera from patients with serous (n=16; mean age, 56.4), mucinous (n=14; mean age, 51.8), clear cell (n=14; mean age, 50.5) and endometrioid (n=3; mean age, 54.3) ovarian cancers, patients with borderline tumor, patients with benign tumor and healthy controls. p-Values were calculated using the Mann-Whitney U-test. B. Comparison of anti-DLD IgA titers in sera from patients with ovarian cancer (n=47; mean age, 53.1), borderline tumor (n=14; mean age, 56.7), benign tumor (n=43; mean age, 47.2) and healthy controls (n=54; mean age, 40.8).*

this assay. Overall, the titers of anti-hnRNPL IgA antibody in sera from ovarian cancer patients were not significantly different from those of sera from patients with borderline or benign tumor or from healthy women (Figure 3A). However, when the patients were grouped into the aforementioned four subtypes, CCC patients showed significantly higher anti-hnRNPL IgA titers (Fig. 3B). The Mann-Whitney U test revealed that CCC patients showed statistically higher anti-hnRNPL IgA responses compared with patients with serous (p=0.013) and endometrioid cancer (p=0.049), borderline tumor (p=0.007), benign tumor (p=0.013) and healthy women (p=0.004). The difference was not significant between patients with CCC and mucinous cancer (p=0.086).

IgA titers to DLD by ELISA. We recently found that IgA but not IgG autoantibody responses to a mitochondrial protein, dihydrolipoamide dehydrogenase (DLD), were significantly increased in patients with endometrial cancer (22). Although DLD was not detected in the present screening, analogy between the two IgA autoantibodies prompted us to examine whether anti-DLD IgA antibody is increased in CCC patients. FLAG-tagged DLD was produced in HEK293T cells and immunopurified using anti-FLAG-conjugated M2 agarose beads (22), which was used as an antigen in ELISA. As shown in Figure 4A, the IgA titers against DLD in CCC patients were significantly higher than those in patients with benign diseases (p=0.021) and healthy controls (p=0.010). However, there was no significant difference in the IgA titers among patients with ovarian cancer as a whole, borderline tumor, benign tumor, and healthy controls (Figure 4B).

Discussion

In this study we carried out 2-D Western blotting using sera from ovarian cancer patients to identify antigens of cancer-specific autoantibodies. The sera from patients with ovarian cancer, including CCC, reacted with RhoGDI at a significantly higher positive ratio than those from patients with borderline and benign tumors and from healthy controls. We also observed that titers of IgA autoantibody

against hnRNPL in sera from patients with CCC were statistically higher than those from other types of malignant ovarian tumor patients, benign tumors patients or healthy donors. Titers of anti-DLD IgA autoantibody were also higher in CCC patients than in benign tumor patients or control donors. These results suggest that RhoGDI, as well as hnRNPL and DLD may serve as diagnostic biomarkers for malignant ovarian cancer and CCC, respectively. Up-regulation of RhoGDI gene expression has been described for oral squamous cell carcinoma (29).

Although CA125 is widely used for the diagnosis of ovarian cancer, the sensitivity of CA125 screening in asymptomatic populations has been shown to be inadequate (6-8). CA125-based diagnosis often provides false positives (6). Furthermore, the level of CA125 is significantly lower in the sera of CCC patients than patients with other cell types (9). Therefore, it should be important to develop an alternative biomarker for the diagnosis of CCC.

The induction of IgA but not IgG autoantibody is described in sera from patients with gastrointestinal cancer (25), melanoma (26), breast tumor (27) and esophageal squamous cell carcinoma (28). IgA autoantibodies to melanin and tyrosinase might function as blocking antibodies that suppress antibody-dependent cytotoxicity (26). IgA autoantibody level to ATP-binding cassette 3 (ABCC3) is significantly higher in male patients suggesting some gender difference (28). However, a detailed mechanism for the specific induction of IgA autoantibody response remains to be clarified.

Autoantibodies have been identified in sera from people with various types of cancers (11-14). The use of protein arrays dramatically accelerates the identification of autoantibodies (13, 14). Identification of multiple autoantigens may improve the accuracy of diagnosis since a combined analysis of autoantibodies has been shown to be effective. The effectiveness of an antigen panel consisting of six autoantigens (p53, c-Myc, Her2, nYeso1, BRCA2 and MuC1) was examined using sera from 97 patients with breast cancer (30). Although the sensitivity of individual autoantibodies ranged from 8% to 34%, the sensitivity and specificity of the panel was 64% and 85%, respectively, clearly demonstrating the superiority of the panel over individual screenings (30). Other combination of autoantigens was also shown to be effective in the diagnosis of lung cancer (31), pancreatic cancer (32) and colon cancer (33). Recently, Arakawa *et al*. described a novel biomarker, TFPI2, that seems to be superior to CA125 for the diagnosis of CCC (34). Therefore, autoantibodies identified in this study may be useful in combination with such biomarkers for more precise diagnosis of ovarian cancer including CCC.

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Conflicts of Interest

The Authors have no financial conflicts of interest.

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