Proteomic Identification of Dihydrolipoamide Dehydrogenase as a Target of Autoantibodies in Patients with Endometrial Cancer

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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 endometrial cancer patients as novel diagnostic markers for the disease. Materials and Methods: The reactivity of individual sera from patients was examined by 2-dimensional (2-D) immunoblotting using HeLa cell lysates as antigens to identify autoantigens. ELISA was established to quantitatively measure autoantibody titer of patients' sera . Results: A mitochondrial protein, dihydrolipoamide dehydrogenase (DLD), was identified as an autoantigen specific to endometrial cancer patients. The levels of immunoglobulin (Ig)A but not IgG autoantibody to DLD were significantly increased in the sera of endometrial cancer patients. Conclusion: IgA autoantibody against DLD could be a novel diagnostic marker for endometrial cancer.*

The incidence of endometrial and cervical cancer in young women has recently increased in developed countries. In the year 2009, 7,713 and 3,909 women died from endometrial and cervical cancer, respectively, which in total ranked fourth among many types of cancer in the United States (1). In Japan, biennial cervical smear cytology is strongly recommended as a cervical cancer screening test for women aged over 20. However, the rate of participation in the screening program is extremely low, about 20% of the subjects (2), which is far less than that in the United States. Cytological screening is less effective for the diagnosis of endometrial cancer (3). Under such circumstances, serum diagnostic markers are potentially useful.

Currently available serum markers for the diagnosis of cancer are mostly cancer antigens, such as prostate-specific antigen (PSA) for prostate cancer (4), carbohydrate antigen (CA)125 for ovarian cancer (5) and CA19-9 for breast cancer (6). However, there is no reliable marker for cervical or endometrial cancer. Although cancer embryonic antigen (CEA) (7), squamous cell carcinoma-related antigen (SCCA) (8), CA125 and CA19-9 have been used, the sensitivity and specificity of these markers are not sufficient for the screening of these cancers, especially at early stages (9, 10).

Accumulating evidence shows that various types of cancer induce a specific immune response, resulting in the production of antibodies against self-components (autoantibodies) (reviewed in 11). During the development of cancer, mutations occur that either activate proto-oncogenes or inactivate the function of tumor suppressor gene products. For instance, the *p53* gene undergoes missense mutations that inactivate its gene product in most types of cancer. Such missense mutations may alter the structure of the gene product and trigger an immunological response in the host. Indeed, the anti-p53 antibody titer is increased among patients suffering from many types of cancer and the proportion of antibody-positive patients in each cancer type correlates well with the mutation frequency of *p53* in the corresponding cancer (12). Antibody titer to p53 is clinically utilized in hospitals in Japan for the diagnosis of esophageal, breast and colorectal cancer.

Besides missense mutations, the cell biological context may change in cancer cells, which affects their protein expression profiles. Furthermore, cancer cells often undergo apoptotic or necrotic cell death, which may release proteins

Abbreviations: DLD, dihydrolipoamide dehydrogenase.

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usually not present outside the cells. These events may also elicit a disease-specific immunological response. Proteomic techniques have been used to systematically identify the antigens of these disease-specific autoantibodies. Anti-annexin, anti-gene product 9.5 and anti-14-3-3 antibodies were, thus, identified in sera from lung cancer patients (13-15). These autoantibodies are also candidate serum markers for cancer diagnosis. With regard to endometrial and cervical cancer, several studies have reported the presence of autoantibodies in patient sera (16, 17); however, proteomic approaches have not been applied for the global identification of patient-specific autoantibodies.

Previously, 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 the sera of systemic lupus erythematosus (SLE) patients and identified 11 antigens recognized by the autoantibodies (18, 19). Interestingly, we showed that the level of one of the autoantibodies correlated with psychiatric syndromes (19).

In the present study, we applied the same strategy to identify autoantibodies specific to endometrial and cervical cancer. We first screened for autoantigens by 2-D immunoblotting using sera from 27 gynecological cancer patients and identified candidate autoantigens. We then examined the reactivity of sera from cervical and endometrial cancer patients to these candidate antigens. We found that the level of immunoglobulin (Ig) A autoantibody to a mitochondrial protein, dihydrolipoamide dehydrogenase (DLD), was significantly increased in the sera of endometrial cancer patients, suggesting that this autoantibody could be a novel diagnostic marker for endometrial cancer.

Materials and Methods

Sera. Sera from patients with endometrial cancer (68 sera, mean age 59.4 years) and patients with cervical cancer (42 sera, mean age 51.8 years) were obtained at the Department of Obstetrics and Gynecology, Nippon Medical School Hospital. Normal control sera were taken at the Kawagoe Research and Development Center, BML Inc. (36 sera, mean age 45.1 years). All sera were taken with informed consent and anonymized. This project was performed with the approval of the appropriate Institutional Research Ethics Committees.

Cell culture. HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium, high-glucose (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37˚C under 5% CO_2.

Two-dimensional (2-D) immunoblotting and identification of antigens detected by autoantibodies. 2-D immunoblotting was carried out as described previously (18). Briefly, HeLa cells were lysed with a rehydration solution (18) and the cell lysates (100 μg) were applied onto an Immobiline DryStrip gel (13 cm long, pH 3-10 NL) (GE Healthcare, Milwaukee, WI, USA) and isoelectric focusing was carried out using Ettan IPGphor II (GE Healthcare) as described (18). Protein concentration was determined by the Bradford method (20). The second dimension of electrophoresis was 9% SDS-PAGE. After 2-D gel electrophoresis, the separated proteins were transferred onto Immobilon-P (Millipore, Billerica, MA, USA) and stained *in situ* with the Cy5 fluorescent dye (GE Healthcare) (18). Immunoblot was performed using serum from an individual patient diluted 1:500 as described (18). Either horseradish peroxidase (HRP)-conjugated goat anti-human IgG (heavy and light chain; H+L) diluted 1:20,000 or anti-human Ig(G+A+M) (H+L) diluted 1:10,000 (Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody. Acquisition of ECL-plus (GE Healthcare) and Cy5 signals was carried out using a Typhoon 9400 scanner (GE Healthcare) (18). Identification of autoantigens was done with increased amount of HeLa cell lysates (300 μg) (18). Patient sera-specific spots were excised and proteins in the spots were subjected to peptide mass fingerprinting (18). Peptide mass fingerprinting data were compared with 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 expression of DLD. A cDNA of *DLD* was amplified by polymerase chain reaction (PCR) using a cDNA library prepared from HEK293T cells. The primers used for the PCR were DLD-F: 5'-GAGGGATCCATGCAGAGCTGGAGTCGTG-3' and DLD-R: 5'-GTGGTCGACTCAAAAGTTGATTGATTTGCC-3'. The amplified cDNA was digested with BamHI and SalI and cloned into a BglII/SalI-digested pEF-BOS-FLAG vector (21). The structure of the resulting plasmid (pEF-BOS-FLAG-DLD) was confirmed by sequencing.

To obtain FLAG-tagged DLD protein expressed in mammalian cells, HEK293T cells were transfected with pEF-BOS-FLAG-DLD using Lipofectamine 2000 (Invitrogen) and incubated for 24 h. The cells were then lysed with a buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 10 mM NaF, 2 mM EGTA, 25 mM β-glycerophosphate, 2 mM DTT, 1% (v/v) NP-40, 10% (v/v) glycerol, protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA) and 0.2% (v/v) benzonase (Novagen, Madison, WI, USA). The lysates were centrifuged at 10,000 × g and the supernatants were saved. Lysates of cells from 10 dishes (10-cm diameter) were immunoprecipitated with 200 μl of anti-FLAG M2 agarose (Sigma, St. Louis, MO, USA) for 2 h at 4˚C. After washing the beads with PBS three times, immunoprecipitated FLAG-DLD was eluted three times with 600 μl of PBS containing 1 mM FLAG peptide. The eluates were combined and used as purified antigen for immunoblotting and ELISA.

Slot-blot analyses. Purified DLD (2.5 μg/gel) was applied to 9% SDS-PAGE and subjected to slot blot analyses using a screener blotter (25 lanes; Sanplatec, Kita-ku, Osaka, Japan). The conditions for immunoblotting were the same as those described for 2-D immunoblotting.

Quantification of anti-DLD autoantibody titers. Anti-DLD autoantibody titers in the sera of patients and healthy controls were quantified by ELISA using a commercially available antibody against DLD (Sigma) as a standard. Briefly, the recombinant FLAG-DLD protein purified from HEK293T cells as above was loaded into

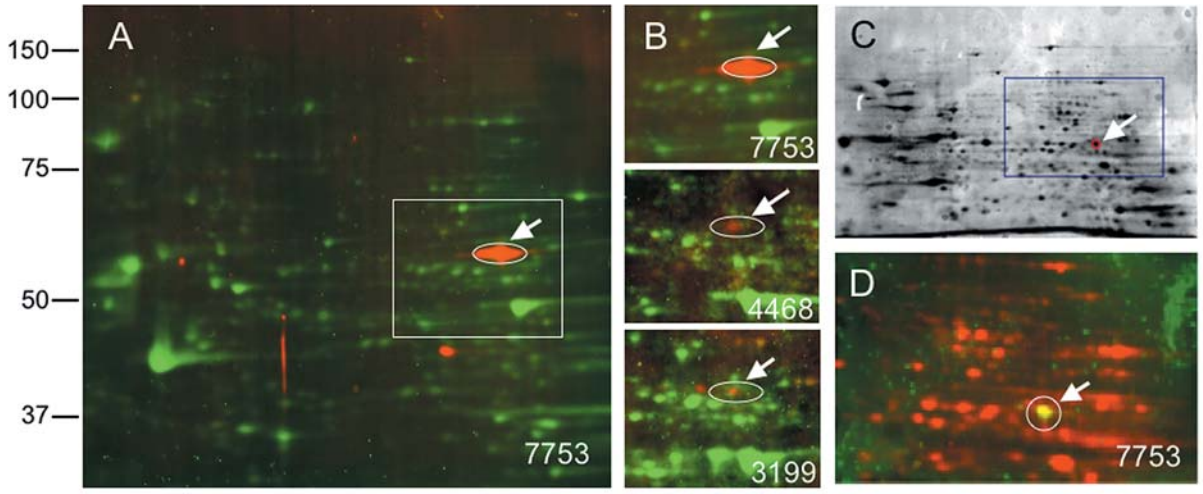

Figure 1. *Identification of dihydrolipoamide dehydrogenase (DLD) as an antigen of autoantibodies induced in endometrial cancer patients. Two-dimensional western blot analyses were performed as described in the Materials and Methods and a typical result obtained with serum from a cervical cancer patient (#7753) is shown. In A, western blot signals detected by ECL-plus are shown in pseudocolored red and the total protein profile detected by Cy5 is shown in pseudocolored green; the two signals have been merged. Circles indicate spots that reacted with patient's serum. The relative molecular mass of commercial pre-stained markers (in kDa) is indicated on the left. The gel images are displayed with the acidic region to the left and the basic pI region to the right. (B) shows a magnified view of the area containing spot #1 (shown as a white box in A) for three patients (#3199, #4468 and #7753). IgG autoantibody (#7753) and Ig(G+A+M) response (#3199, #4468) were examined. In C and D, the total protein profile of the organelle fraction (C) and 2-D Western blotting around spot 1 (D; corresponding to the boxed area in C), are shown to identify the antigen in spot #1.*

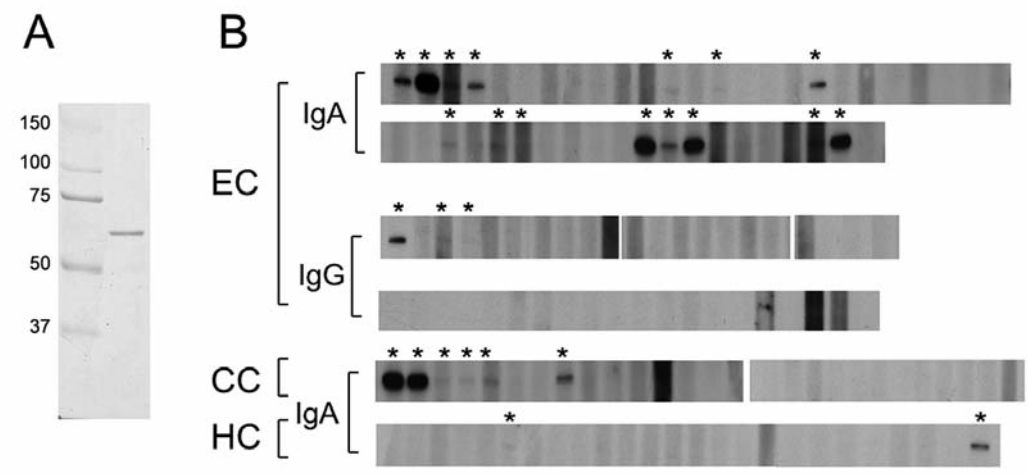

Figure 2. *Slot-blot analysis of patient sera using purified DLD as an antigen. A. FLAG-tagged DLD was expressed in HEK293T cells, immunopurified using anti-FLAG M2 beads and competitively eluted by a solution containing FLAG peptide. An aliquot of the purified sample was analyzed by 9% SDS-PAGE followed by staining with Coomassie brilliant blue. Molecular weight markers and their size in kDa are shown on the left. B. Slot blot analyses of anti-DLD IgG or IgA antibody in sera from patients with endometrial cancer (EC, n=45), cervical cancer (CC, n=27) or healthy controls (HC, n=25). Sera were diluted 500-fold and reacted antibodies were detected by HRP-labeled anti-human IgG antibody (IgG) or anti-human IgA (IgA) antibody using ECL-plus. Asterisks mark positive samples.*

the wells of a microtiter plate (400 ng/well; Nunc, Roskilde, Denmark) and incubated overnight at 4˚C. The wells were blocked for 18 h with PBS containing 1% bovine serum albumin (BSA). The diluted serum (1:100 in PBS-T containing 0.3% BSA) was incubated in each well at room temperature for 2 h. After the plate was washed, 100 μl of diluted HRP-conjugated anti-human IgG or IgA (diluted 1:10,000 in PBS-T containing 1% BSA) was added to each well and the plate was incubated at room temperature for 1 h. After the plate was washed, 100 μl of TMB (3,3', 5,5"-tetramethyl benzidine) substrate reagent (OptEIA, BD Biosciences, Bedford, MA, USA)

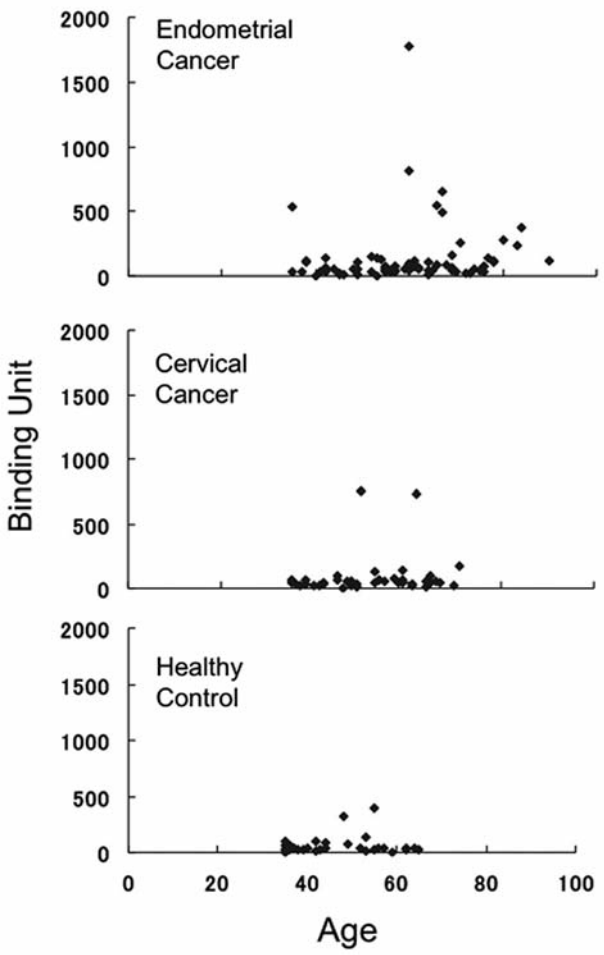

Figure 3. *Quantitation of autoantibodies against DLD in patients with endometrial cancer, cervical cancer and healthy controls. The binding units of each serum are defined as (A405/mean A405 of control sera + 2 SD) ×100. Data are presented as scattergrams of binding units versus age.*

were added. After a 30-min incubation at room temperature, the reaction was stopped by the addition of 100 μl of 2 N H_2SO_4 and the absorbance was measured at 450 nm using a microplate reader. Autoantibody titers were expressed as the mean absorbance values of the duplicate wells. As a negative control, ELISA was performed with BSA as an antigen and the values were subtracted from those obtained by ELISA with FLAG-DLD as an antigen. The titer of each serum was expressed in arbitrary "binding units" as follows:
binding units=(A_{405}/mean A_{405} of control sera + 2 × standard deviation (SD)) ×100. For the calculation of SD, sera with a titer higher than the mean + 2 SD were excluded. The cut-off value was set as 100, namely, the mean A_{405} of control sera + 2 SD.

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

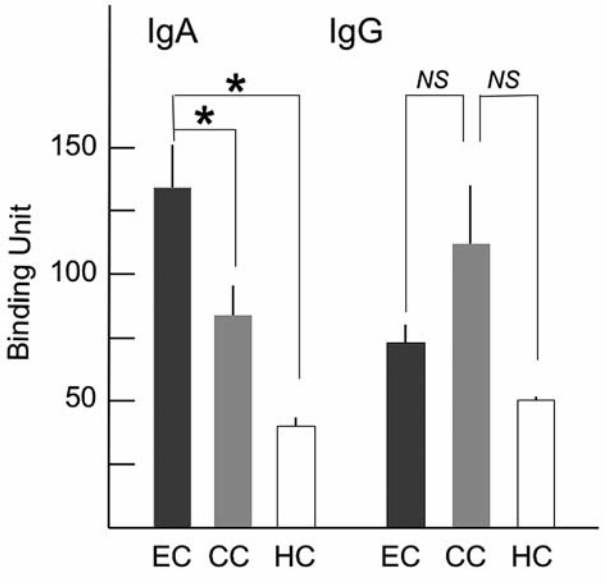

Figure 4. *Increased titer of IgA anti-DLD antibody in sera from endometrial cancer (EC, n=68) patients compared with cervical cancer (CC, n=42) patients or healthy controls (HC, n=36). Mean values±SD for each group are shown as bar graphs. Asterisks show statistically significant differences (p<0.05).*

Results

As a first screening, 27 serum samples from patients with cervical cancer were individually analyzed by 2-D immunoblotting using whole-cell lysates from HeLa cells. As control experiments, five sera from healthy women were individually analyzed and a pool of eight sera from healthy women was also used. Antibody responses that were not observed with sera from healthy donors were selected. Figure 1 shows a representative result with serum from a cervical cancer patient (#7753). Proteins on the membrane were labeled *in situ* with the fluorescent dye Cy5 (shown pseudocolored green), the ECL reaction was monitored by its fluorescence (pseudocolored red) and the two patterns were merged. A protein spot indicated by an arrow in the Figure was detected with sera from multiple patients but not with those from healthy donors. Figure 1B shows that the sera from three patients (#3199, #4468, #7753) reacted with a protein in the same spot.

There were too many spots around the reactive spot to identify the protein that was detected by patients' sera. In cell fractionation experiments, the antigen in the spot was recovered in the membrane/organelle fraction (Figure 1C, D). Using this fraction (300 μg), we then identified the antigen as DLD, a mitochondrial enzyme that oxidizes dihydrolipoamide to lipoamide (DLD) (GI:181575; molecular weight, 54,242; isoelectric point (pI), 8.20; sequence coverage, 9%; score,

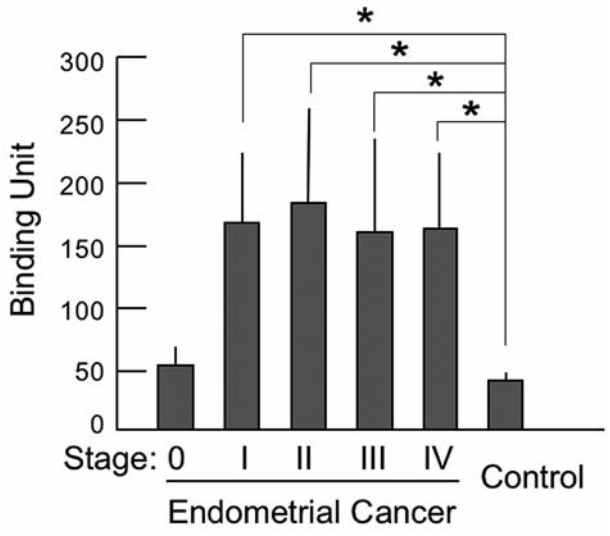

Figure 5. *Patients with endometrial cancer were grouped according to their clinical stage, and mean binding units ±SD are shown. Stage 0 means atypical endometrial hyperplasia. Asterisks show statistically significant differences ($p<0.05$).*

112). The molecular weight and isoelectric point of DLD were consistent with its location on the gel.

We generated *Escherichia coli*, insect and mammalian expression vectors for DLD and assessed the reactivity observed in 2-D immunoblotting with patients' sera. However, only the DLD protein produced in mammalian cells reproduced the results (data not shown). Therefore, we further examined the reactivity of patients' sera to purified DLD from mammalian cells. FLAG-tagged DLD was expressed in HEK293T cells and purified by immunoprecipitation with anti-FLAG M2 antibody (Figure 2A). FLAG-DLD was competitively eluted by the addition of FLAG peptide and the eluates were subjected to SDS-PAGE (Figure 2A), which demonstrated that FLAG-DLD was purified to near homogeneity. Using the purified DLD, we performed slot-blot analyses (Figure 2B). At the initial stage of the screening we used anti-human IgG or anti-human Ig(G+A+M) antibody as a secondary antibody and noticed that they gave different results. In some studies, the titer of IgA but not IgG autoantibody is increased in cancer patients' sera (22-25). Taking these observations into consideration, we separately performed slot-blot analyses using anti-human IgA or IgG antibody as a secondary antibody (Figure 2B).

The slot-blot analyses revealed that autoantibody reactivities in sera from endometrial and cervical cancer patients are mainly due to IgA antibody rather than IgG antibody (Figure 2B). Therefore, we measured the titer of IgA antibody to DLD quantitatively by ELISA, employing additional patient and control sera. The IgG antibody titer was also measured by the same ELISA. Figure 3 shows scattergram plots of binding units (as defined in the Materials and Methods) *versus* age. The titer of anti-DLD IgA antibody in sera from the endometrial cancer patients was significantly higher than those from the healthy donors ($p=0.002$; Mann-Whitney *U*-test) (Figure 4). In contrast, the titer of anti-DLD IgG antibody was not significantly different between the two groups ($p=0.237$). Although the initial screening was carried-out using sera from cervical cancer patients, the IgA antibody titer of the endometrial cancer patients was significantly higher than that of the cervical cancer patients ($p=0.024$), whereas the titer of IgG antibody did not differ significantly between the two groups ($p=0.556$). The titer of anti-DLD IgA antibody was not different between cervical cancer patients and healthy controls ($p=0.281$). These results suggest that anti-DLD IgA antibody may be a good biomarker for the diagnosis of endometrial cancer. When endometrial cancer patients were grouped according to cancer stages I to IV, the sera from all the groups showed a significantly higher anti-DLD titer than the healthy controls did (Figure 5). Patients with atypical endometrial hyperplasia did not show a statistically significant difference (stage 0 in Figure 5). The sera from any cancer stage of the cervical cancer patients did not show this difference (data not shown).

We then evaluated the positivity rate for anti-DLD IgA antibody in the endometrial cancer patients. When the cut-off value was set as the mean + 2SD, the positivity rate for stage I to IV patients was 26% (10/38), 60% (4/7), 38% (3/8) and 67% (2/3), respectively. Overall, 34% (19/56) were positive, whereas only 6% (2/36) of healthy donors were positive. Although the number of cases is small, this value is superior to that of known tumor markers for endometrial cancer: CA19-9 (11%, n=9) or CA125 (11%, n=9) (data not shown). CA19-9 or CA125-positive patients were not included among the anti-DLD antibody-positive patients, suggesting that simultaneous examination of these markers would improve diagnosis of endometrial cancer.

Discussion

In this study, we showed that sera from endometrial cancer patients showed higher titers of anti-DLD antibody than sera from cervical cancer patients or healthy donors did. When the titer of anti-DLD IgA and IgG antibody was measured separately by ELISA, the titer of IgA but not IgG antibody was significantly higher in patients with endometrial cancer. The endometrial cancer patients at all stages showed statistically higher IgA antibody titers than the healthy donors did. Generally, the titer of cancer biomarkers is very low in the early stages of cancer. For example, 98% of patients (57/58) with clinical and surgical stage I or II endometrial cancer had normal preoperative serum CA125

levels; in contrast, all eight patients with clinically-advanced endometrial cancer had elevated CA125 levels before surgery (9). In this study, the positivity rate of anti-DLD IgA antibody was 26% for stage I patients and even higher for stage II to IV patients, suggesting the usefulness of this autoantibody as a diagnostic biomarker.

In the initial phase of this study, we employed sera from cervical cancer patients to carry-out 2-D western blotting to identify autoantibodies. Although the sera from several cervical cancer patients showed a high antibody titer to DLD (Figure 2B), detailed analyses employing slot-blotting and ELISA revealed that a higher titer of anti-DLD IgA antibody was present in sera from endometrial cancer rather than cervical cancer patients.

The induction of IgA but not IgG autoantibody is described in sera from patients with gastrointestinal cancer (22), melanoma (23), breast tumor (24) and esophageal squamous cell carcinoma (25). IgA autoantibodies against melanin and tyrosinase might function as blocking antibodies that suppress antibody-dependent cytotoxicity (23). IgA autoantibody level to ATP-binding cassette 3 (ABCC3) is significantly higher in male patients, suggesting some gender difference (25). However, the 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). The use of protein arrays dramatically accelerates the identification of autoantibodies (11). Identification of multiple autoantigens may improve the accuracy of diagnosis using autoantigen panels. The effectiveness of an antigen panel consisting of six antigens (p53, c-Myc, Her2, nYeso1, BRCA2 and MuC1) was examined using sera from 97 patients with breast cancer, 40 with ductal carcinoma *in situ* and 94 healthy controls (26). 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 (26). Other combination of autoantigens was also shown to be effective in the diagnosis of breast cancer (27). The positivity rate of IgA antibody to DLD (34%) is equally high with that of the most sensitive autoantigen (26). Therefore, DLD, a novel autoantigen that induces a specific IgA autoantibody in endometrial cancer, may contribute to better diagnosis of endometrial cancer.

Conflicts of Interest

The Authors have no financial conflicts of interest.

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