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Autoantibody to Tumor Antigen, Alpha 2-HS Glycoprotein: A Novel Biomarker of Breast Cancer Screening and Diagnosis

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Abstract

We sought to identify a new serum biomarker for breast cancer screening and diagnosis using stepwise proteomic analysis of sera from breast cancer patients to detect the presence of autoantibodies that react with urinary protein. Two-dimensional immunoblotting was done for screening autoimmunogenic tumor antigens in the urine of breast cancer patients. Reactive spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Among urinary proteins separated by two-dimensional electrophoresis, 13 spots showed strong reactivity with pooled sera from breast cancer patients or control sera. By mass spectrometry, we identified α 2-HS glycoprotein (AHSG) as a tumor antigen. Peripheral blood was obtained from 81 women diagnosed with breast cancer before surgery and 73 female donors without evidence of any malignancy for the individual analysis. In one-dimensional Western blot analysis, AHSG autoantibody was detected in 64 of 81 breast cancer patients (79.1%) and in 7 of 73 controls (9.6%). The sensitivity of this test in breast cancer patients was 79.0%. Our results suggest that AHSG and anti-AHSG autoantibody may be useful serum biomarkers for breast cancer screening and diagnosis. (Cancer Epidemiol Biomarkers Prev 2009;18(5):1357–64)

Introduction

Breast cancer is a leading cause of cancer death worldwide (1), with the total number of breast cancer patients seeming to have increased in response to exposure to several risk factors including abnormal levels of estrogen (2). Small breast cancer lesions frequently remain undetected upon examination and may not be visible by mammography, particularly in young women with dense breast tissue (3). It is clear that the successful prevention of breast cancer will depend upon the reduction of risk factors along with improved and more specific methods of screening using accurate biomarkers (4). For the discovery of biomarkers, body fluids such as blood, saliva, and urine were considered as ideal sources in which to assess the presence of cancer biomarkers (5-7). In particular, serum contains various circulating antigens and antibodies related to cancer progression and development (8).

Autoantibodies in serum against several tumor antigens such as p53, embryonic neural proteins, and antineural/antinuclear antigens also have been evaluated (9, 10). Recently, autoantibodies against annexin-I, annexin-II (11), peroxiredoxin-I (12), peroxiredoxin-VI (13), and calreticulin (14) were found in the sera of lung, pancreatic, and liver cancer patients using a proteomics approach.

In breast cancer, humoral responses against many tumor-associated proteins such as c-*erb*B-2/HER2/*neu* (15), RS/DJ-1 (16), and mucin-related antigens (17) have been detected. Specifically, the proto-oncogene c-*erb*B-2/HER2/*neu*, which encodes a growth factor receptor, is overexpressed in 20% to 30% of patients with breast cancer (18). The presence of c-*erb*B-2/HER2/*neu* autoantibodies has been observed in 11% of breast cancer cases and has been found to correlate with the overexpression of the protein in tumor tissues (19). In addition, elevated c-*erb*B-2/HER2/*neu* protein levels have been found in the serum of 29% of patients with breast carcinoma and are associated with poor prognosis (20). Although many candidate biomarkers have been identified and evaluated, efforts continue for the discovery of novel biomarkers. One frequent problem is that biomarker detection assays may not be sufficiently sensitive or specific enough to be reliable for early diagnosis. Presently, great effort is being made toward the discovery of better biomarkers for the early detection of breast cancer (21). For this purpose, new biological samples of various cancers and powerful technology platforms should be established.

The analysis of urinary proteome is emerging as a promising noninvasive tool for the diagnosis and monitoring of human cancers, because urinary proteome is available in almost all patients and its procedure of collection is easy and does not require any invasive procedures (22).

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J.K. Yi and J.W. Chang contributed equally to this work.

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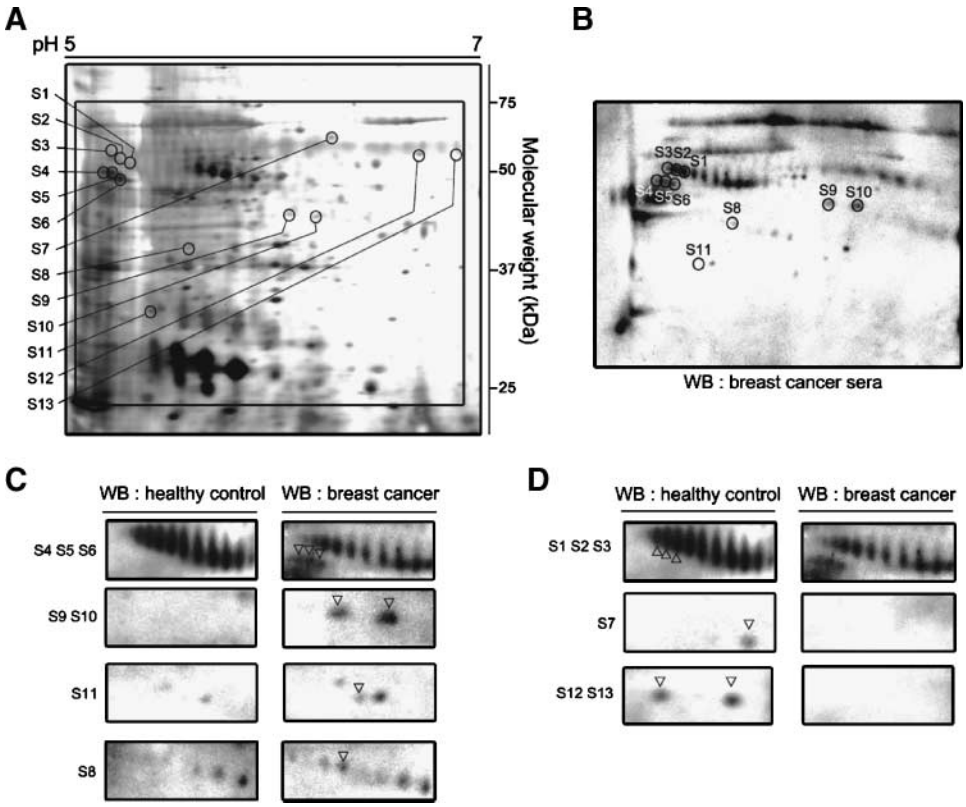

Figure 1. Screening of autoantibodies against urinary proteins. **A.** Silver staining of urinary proteins separated by two-dimensional electrophoresis (pH4-7). The immunoreactive spots (S1-S13) recognized by pooled serum are marked by the circle. **B.** Boxed area, representative result of two-dimensional immunoblot done with breast cancer patient serum. **C.** Increased immuoreactive spots on immunoblotting with sera from cancer patients. **D.** Decreased reactive spots in immunoblotting with sera from cancer patients.

Therefore, urine has been considered as the most ideal source for discovery of biomarkers nowadays.

In the present study, we used two-dimensional immunoblot analysis to screen sera from breast cancer patients for autoantibodies that might serve as potential biomarkers that react against tumor-associated proteins in urine and investigated the expression levels of a particular candidate in breast cancer patients. This study was also designed to evaluate the sensitivity of the molecule as a useful biomarker of breast cancer screening and diagnosis.

Materials and Methods

Patients and Clinical Samples. Peripheral blood (BD) was obtained, centrifuged (4°C, 1,000 *g*), filtered by microfilter (0.2 μm pore), and stored at −80°C from the following case and control groups: 81 patients with newly diagnosed breast cancer before any therapy including surgery, chemotherapy, and hormone treatment at the Department of Surgery, Seoul National University Hospital from June 2005 to July 2007; 73 healthy control subjects, who visited our Healthcare System Gangnam Center and had a comprehensive medical check-up from September 2006 to February 2007 and the results of all check-ups, including breast mammography, showed nothing that could be considered evidence of malignancy. The mean age of all 73 healthy female volunteers at the time of blood sampling was 45.4 y. Blood from all 73 female volunteers, who fully understood the aim of this study and also provided informed consent, was drawn and prepared in the same way as blood from the 81 patients were managed. Urine samples were obtained from five patients with newly diagnosed breast cancer as infiltrating duct carcinoma before removal of the primary tumor (typically 200 mL of clean catch urine first thing in the morning). None of the patients had other malignancies. Clinicopathologic descriptions were obtained from the pathologic reports and medical records, and tumor staging was determined according to 6th American Joint Committee on Cancer tumor-node-metastasis (TNM) classification. The study was approved by the Institutional Review Board of the Seoul National University Hospital, and all patients gave their informed consent in order to participate in the study.

Preparation of Sera, Urine, and α 2-HS Glycoprotein. Sera were obtained at the time of diagnosis from 81 patients with breast cancer, after informed consent was given. Sera from 73 healthy individuals were used as controls. For individual screening, α 2-HS glycoprotein (AHSG; Calbiochem) was used as an antigen. Urine samples collected in separated polypropylene tubes (NUNC) were centrifuged at 1,000 × *g*, at 4 °C for

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20 min to remove particulates. The supernatants were loaded onto a Centricon 5 kDa membrane (Millipore) in order to concentrate the proteins and to remove small molecules. Briefly, the Centricon filters were centrifuged at 2,000× *g* at 4°C until the final volume of each sample reached 200 μL. Concentrated urine samples then were incubated with three volumes of cold acetone. The mixture was stored at −20°C for 3 h and a pellet was obtained by centrifugation at 1,500 × *g* at 4°C for 15 min. The pellet was dried and stored at −70°C until used. For two-dimensional electrophoresis, five urine samples were pooled with an equal amount of 100 μg.

Two-Dimensional Electrophoresis. Immobiline DryStrips, linear pH 4-7, 13 cm long (Amersham Biosciences), were in-gel rehydrated for 12 h with pooled urinary protein in a rehydration buffer. The first dimensional separation (IEF) was done in the Ettan IPGphor II IEF System (Amersham Biosciences). After completion of the IEF, proteins on the strip were equilibrated for 15 min. The immobilized pH gradient strip was then transferred onto a 12% acrylamide slab gel and the second dimensional separation was done in a SE260 Mini-Vertical Electrophoresis Unit (Amersham Biosciences). The separated proteins were transferred onto a nitocellulose membrane (Amersham Biosciences) or visualized by silver staining. The two-dimensional electrophoresis images were captured using ImageScanner (Amersham Biosciences) and analyzed by Progenesis software (GE Healthcare).

Immunoblot Analysis. For two-dimensional immunoblot, fractionated proteins were transferred electrophoretically onto a nitrocellulose membrane (Amersham Biosciences). The membranes subsequently were incubated for 2 h at 4°C with pooled sera (1:250 dilution) and washed three times with TBS. The membrane was incubated with horseradish peroxidase–conjugated sheep antihuman immunoglobulin (Amersham Biosciences) at 1:5,000 dilution for 1 h at 4°C. The reaction was visualized with a chemiluminescence reagent (Amersham Biosciences) followed by autoradiography on Hyperfilm MP (Amersham Biosciences). For individual validation, 1 μg of purified AHSG was loaded onto a 12% acrylamide slab gel (8 × 9.5 cm). Separated proteins were transferred onto a nitrocellulose membrane. After blocking, the membranes were incubated for 2 h at 4°C with pooled sera (1:250 dilution) or individual sera (1:100 dilution).

In-Gel Enzymatic Digestion and Mass Spectrometry. The two-dimensional gel was stained with silver nitrite and spots corresponding to Western blot–positive spots were excised. The spots were destained by sodium-thiosulfate and potassium ferricyanide. The transparent gels were washed alternately with 25 mmol/L ammonium bicarbonate (pH 8.5) and acetonitrile and finally dehydrated with acetonitrile. After drying completely, the spots were covered with 25 μL of sequencing-grade modified trypsin (Promega) in NH_4HCO_3 buffer (25 mmol/L, pH 8.5) and left at 37°C overnight. After enzymatic digestion, the resulting peptides were extracted using 100 μL of 50% acetonitrile, 25 mmol/L ammonium bicarbonate followed by 100 μL of 50% acetonitrile, and 0.05% (v/v) trifluoroacetic acid. Extractions were conducted in an ultrasonic bath for 15 min each time. The peptides were dehydrated completely in a Speedvac at 43°C. Prepared peptides were rehydrated with 2 μL of 0.2% trifluoroacetyl or trifluoroacetic acid and the solution (1 μL) was loaded onto the matrix-assisted laser desorption/ionization (MALDI) target plate by mixing 1 μL of each solution with the same volume of a matrix solution that was prepared fresh every day by dissolving 0.3 g/mL a-cyano-4-hydroxycinnamic acid (Wako Purified Reagent) in acetone/ethanol (1:1, v/v) solvent. Measurements were done using MALDI-TOF/TOF (4700 Proteomics Analyzer; Applied Biosystems). The peptide mass fingerprint was used for protein identification from the tryptic fragment size using the Mascot Search engine (version 2.2, released 28.02.2007; Matrix Science Ltd.) based on the entire NCBInr (version 22.09.2007, 670,092 mammalian entries) and UniProtKB/Swiss-Prot 54.2 (17,252 human entries) databases.

Statistical Methods. Data were stored in Excel (Microsoft Corporation) and statistical analyses were done using SPSS software version 12.0 (SPSS) and SAS (SAS Inc.). Qualitative data were compared using Pearson's correlation test; quantitative data were expressed as means (SD) and were compared using Student's *t*-tests.

Table 1. List of identified tumor antigens by MALDI-TOF

Spot	Protein name	Accession no.	Isoelectric point		MW (Da)		Mascot Score	Sequence coverage (%)	Immuno-reactivity
			Theor.	Observ.	Theor.	Observ.			
S1	Kininogen-1 precursor	P01042	6.34	5.32	72,984	65,432	106	24	Normal sera
S2	Kininogen-1 precursor	P01042	6.34	5.28	72,984	67,654	132	24	Normal sera
S3	Kininogen-1 precursor	P01042	6.34	5.22	72,984	69,223	113	34	Normal sera
S4	α-2-HS-glycoprotein precursor	P02765	5.43	5.10	40,098	49,998	61	13	Cancer sera
S5	α-2-HS-glycoprotein precursor	P02765	5.43	5.13	40,098	48,100	75	22	Cancer sera
S6	α-2-HS-glycoprotein precursor	P02765	5.43	5.16	40,098	47,875	70	20	Cancer sera
S7	Monocyte differentiation antigen CD14	P08571	5.84	6.22	40,678	68,396	61	20	Normal sera
S8	Zinc-α-2-glycoprotein precursor	P25311	5.57	5.61	34,079	38,560	68	29	Cancer sera
S9	Cathepsin D precursor	P07339	6.10	6.07	45,037	45,201	75	40	Cancer sera
S10	Cathepsin D precursor	P07339	6.10	6.21	45,037	44,898	100	42	Cancer sera
S11	Clusterin precursor	P10909	5.89	5.49	53,031	34,315	74	30	Cancer sera
S12	Ig α-2 chain C region	P01877	5.71	6.69	37,283	57,435	63	24	Normal sera
S13	Ig α-2 chain C region	P01877	5.71	6.87	37,283	56,998	64	16	Normal sera

NOTE: The calculation of experimental isoelectric point and MW was based on migration of the protein spot on two-dimensional electrophoresis gels.
Abbreviations: Theor., theoretical; Observ., observed.

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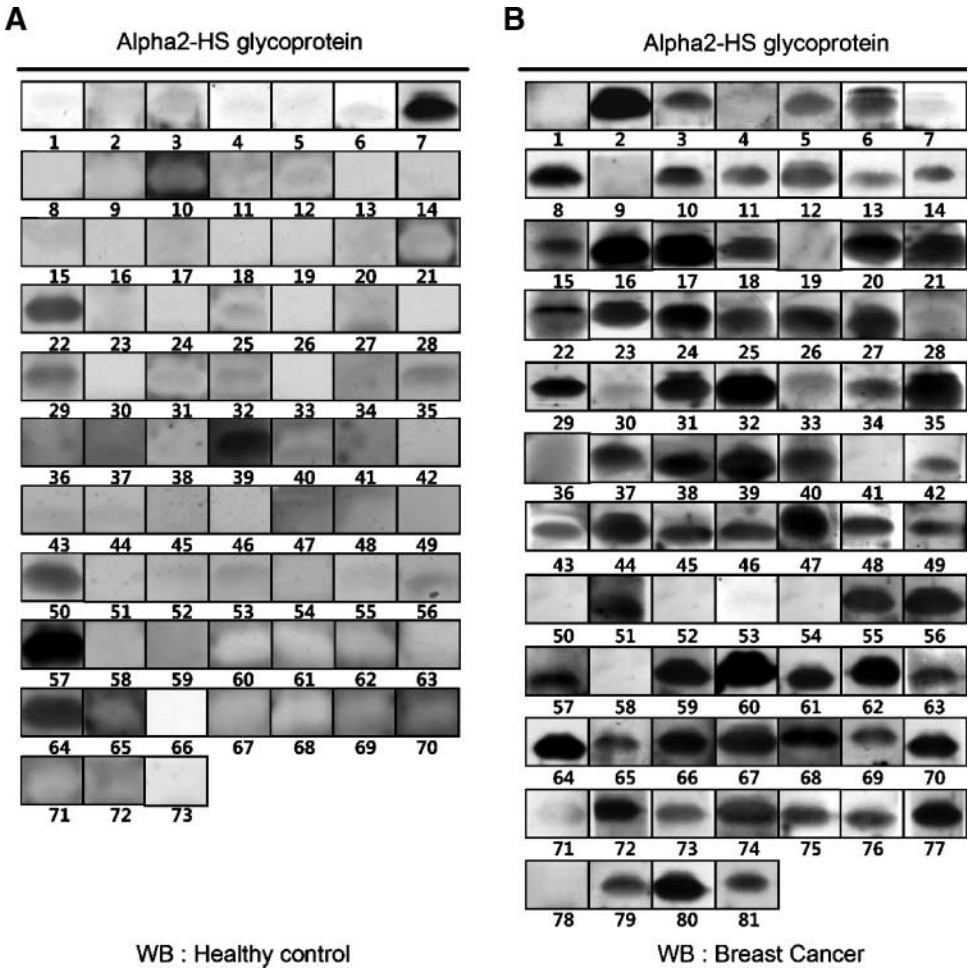

Figure 2. Individual screening of AHSG autoantibody. **A.** AHSG (1 μg) was separated by SDS-PAGE and transferred to nitrocellulose membrane. Each lane was cut into a piece and incubated with individual 73 healthy sera. Immunoreactivity was visualized by antihuman IgG-horseradish peroxidase and enhanced chemiluminescence reagent. **B.** AHSG was probed by 81 sera from breast cancer patients, and its immunoreactivity was detected by enhanced chemiluminescence reagent.

Receiver operating characteristic curve was constructed (MedCalc for Windows, version 9.3.3.0) with the expression intensity values obtained from immunoblotting results calculated by a densitometer to assess their diagnostic accuracy in distinguishing patients with breast cancer from control subjects. By using the receiver operating characteristic method, we calculated sensitivity, specificity, error rate, and area under the curve to determine the diagnostic accuracy of our findings. To investigate the significance of AHSG autoantibodies as a detection marker for breast cancer, the AHSG-positive group was compared with the AHSG-negative group according to tumor-node-metastasis stage, T stage, N stage, and patient age. All tests were two-tailed and $P \leq 0.05$ was considered to indicate a significant difference.

Results

Screening of Autoantibodies to Urinary Proteins by Two-Dimensional Immunblotting. For the screening of autoimmunogenic tumor antigen, urinary proteins were separated by two-dimensional electrophoresis and visualized by silver staining (Fig. 1A). After collection, urine was concentrated and precipitated. The urinary proteins were solubilized in 9.5 mol/L urea solution and subjected to two-dimensional electrophoresis. The separated urinary proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with the pooled serum from 10 patients with breast cancer or 10 healthy volunteers as a primary antibody. Finally, reactivity was visualized by horseradish peroxidase–conjugated antihuman immunoglobulin as secondary antibody. The serum from patients with breast cancer reacted at multiple spots (Fig. 1B). Seven spots (S4, S5, S6, S8, S9, S10, and S11) were newly appeared in breast cancer patient sera, but S1, S2, S3, S7, S12, and S13 were decreased in breast cancer patient sera (Fig. 1C and D). Especially three of the reactive spots (S4, S5, S6) separated at isoelectric point 5 and molecular weight was estimated to be approximately 51 kDa by migration distance on two-dimensional electrophoresis (Fig. 1C). These spots showed strong reactivity with sera from

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breast cancer patients, but the same spots were not reactive with control sera.

Identification of Immunoreactive Spots by Mass Spectrometry. Autoimmunogenic tumor antigens were identified by MALDI-TOF/TOF mass-spectrometry. After in-gel trypsin digestion, extracted peptides were analyzed by mass spectrometry. A total of 13 immunoreactive spots with healthy subjects or cancer sera were identified and are summarized in Table 1. Among them, S4 to S6 spots in Fig. 1C were identified as the same protein, AHSG. Bold letters indicate the matched peptide by mass spectrometry analysis (Supplementary Fig. S1A). The mass spectrometry spectrum of AHSG is shown in Supplementary Fig. S1B. Furthermore, 10 peptides chosen from the peptide mass fingerprinting were subjected to sequence analysis by MALDI-TOF/TOF. Representative tandem mass spectrometry analysis of QLKEHAVEGDCGFQLLK is shown in Supplementary Fig. S1C.

Detection of Serum Autoantibody to AHSG. To confirm whether breast cancer patient sera contained autoantibodies to AHSG, purified AHSG was probed with pooled sera from 10 healthy controls or 10 breast cancer patients. Different concentrations (1,000, 500, 250, and 125 ng) of purified AHSG were separated by SDS-PAGE and transferred to nitrocellulose membrane. The duplicate membrane was probed by each pooled serum from healthy and breast cancer subjects. We found that the combined patient sera used above was able to detect as little as 125 ng of AHSG whereas the control sera could not detect even 1 μg of the protein (Supplementary Fig. S2A and B) by Western blot analysis. The AHSG loading control on the nitrocellulose membrane was stained by Coomassie Brilliant R250 and is shown in Supplementary Fig. S2C. These data indicate that breast cancer patient sera contain autoantibodies against purified AHSG. We then screened individual patient serum for AHSG by Western blot analysis. Individual analysis against 1 μg of AHSG showed that 7 (9.6%) positive signals were detected from 73 control sera (Fig. 2A), whereas sera from 64 (79%) of the 81 breast cancer patients exerted immunoreactivity (Fig. 2B).

Statistical Analysis of Autoantibody Expression Levels. The mean ages of breast cancer patients and normal controls were 47.2 years (SD, 8.7) and 45.4 years (SD, 9.9), respectively. There were no significant differences between breast cancer patients and normal controls with respect to age. The primary tumor size was ≤2.0 cm in 43.2% of the patients, 56.8% of the patients had node-negative disease, and 53.1% of the patients had estrogen receptor–positive tumors. None of the patients had distant metastasis and 84% of the patients were stage I or II. Based on our findings, the usefulness of serum AHSG as a biomarker for the diagnosis of breast cancer was evaluated. Serum reactivity against AHSG was significantly higher in breast cancer patients than in normal controls (79% and 9.6%, respectively; $P < 0.0001$; Table 2). There were no significant differences between AHSG-negative cancer patients and AHSG-positive cancer patients with respect to age, tumor size, nodal status, TNM stage, and estrogen receptor/progesterone receptor and Her-2 status (Table 3). Moreover there were no differences in sensitivity and specificity among subgroups classified according to tumor size, nodal status, TNM stage, and estrogen receptor expression (data not shown).

Frequency of AHSG Autoantibodies in the Sera of Benign Breast Tumor, Thyroid Cancer, Cervical Cancer, and Control Groups. We next did immunoblotting experiments to determine the frequency of AHSG autoantibodies in the sera of benign breast tumor, thyroid cancer, cervical cancer, and control groups. We screened 40 obtained sera from benign breast tumor (n = 10), thyroid cancer (n = 10), cervical cancer (n = 10), negative control group (randomly selected from the 73-control group), and positive control group (randomly selected from the 81-case group). The frequencies of AHSG autoantibodies were 10% (1 of 10), 0% (0 of 10), and 10% (1 of 10) in benign tumor, thyroid cancer, and cervical cancer, respectively, compared with 100% (5 of 5) in breast cancer and 0% in normal controls.

Discussion

Many tumor-specific biomarkers such as autoantibodies and autoantigens have been suggested and evaluated for the early diagnosis of breast cancer (9, 10, 16-23-27). However, these have not been useful clinically because their sensitivity and specificity are not sufficient to detect early disease. This also has been the case with the use of several biomarkers in combination in an attempt to improve specificity and sensitivity (28).

Numerous serum tumor markers have been described for breast cancer, including members of the MUC-1 family of mucin glycoproteins (e.g., CA 15-3, CA 27.29), carcinoembryonic antigen, oncoproteins (e.g., HER-2/c-erbB-2), and cytokeratins (e.g., tissue polypeptide antigen, tissue polypeptide–specific antigen; refs. 29-33). Among these, the two best-established serum biomarkers for breast cancer are carbohydrate antigen 15-3 (CA 15-3) and carcinoembryonic antigen (4, 34). Nevertheless, there are no markers currently recommended for routine use in diagnosing breast cancer because their sensitivity and specificity are not high enough, particularly in early-stage breast cancer (4, 32). In the present study, we utilized the concept of autoantibody against cancer-associated antigens and identified the AHSG autoantibody as a novel biomarker for breast cancer.

Although it is not clear why some patients develop immunogenicity to a particular antigen, most tumor-associated antigens are not products of mutated genes but rather are overexpressed proteins or antigens related to cell differentiation (35). The present study showed that several urinary proteins are immunoreactive with serum from breast cancer patients. These autoimmunogenic

Table 2. Diagnostic results of AHSG

AHSG	Group		
	Normal	Cancer	Total
Negative	66	17	83
Positive	7	64	71
Total	73	81	154

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Table 3. AHSG reactivity in cancer patients according to clinicopathologic variables

Factors (n)	AHSG negative (%)	AHSG positive (%)	*P**
Age in y			0.182
≤40 (19)	5 (29.4)	14 (21.9)	
41-50 (35)	9 (52.9)	26 (40.6)	
>51 (27)	3 (17.6)	24 (37.5)	
T stage	0.895		
T_1 (35)	7 (41.2)	28 (43.8)	
T_2 (44)	10 (58.8)	34 (53.1)	
T_3 (1)	0	1 (1.6)	
T_4 (1)	0	1 (1.6)	
N stage	0.465		
N_0 (46)	8 (47.1)	38 (59.4)	
N_1 (23)	6 (35.3)	17 (26.6)	
N_2 (8)	2 (11.8)	6 (9.4)	
N_3 (4)	1 (5.9)	3 (4.7)	
TNM stage (AJCC 6th)	0.554		
I (28)	4 (23.5)	24 (37.5)	
II (40)	10 (58.8)	30 (46.9)	
III (13)	3 (17.6)	10 (15.6)	
IV (0)	0	0	
Estrogen receptor status			0.989
Negative (38)	8 (47.1)	30 (46.9)	
Positive (43)	9 (52.9)	34 (53.1)	
Progesterone receptor status			0.656
Negative (42)	8 (47.1)	34 (53.1)	
Positive (39)	9 (52.9)	30 (46.9)	
Her2 status			0.736
0 (48)	11 (64.7)	37 (57.8)	
1+ (11)	1 (5.9)	10 (15.6)	
2+ (16)	4 (23.5)	12 (18.8)	
3+ (6)	1 (5.9)	5 (7.8)	

*Significance was tested using Pearson χ tests.

tumor antigens were identified using a proteomics approach including two-dimensional immunoblot and mass-spectrometry. Among these, cathepsin D seems to be involved in the pathogenesis, growth, and progression of breast cancer. However, cathepsin autoantibody was already reported in endometrial cancer patients (36). Both Pro- and mature forms were detected by patient's sera. Because both forms were also detected by breast cancer patient's sera, we excluded them from our further screening. In a prior study, an autoantibody (S11) against clusterin was detected in patients with immunologic infertility (37). S1-3, a kininogen of autoimmunity, was studied in recurrent pregnancy losses as well. Several studies have reported the presence of autoantibodies against kininogen in patients with recurrent early pregnancy losses (38). The expression of Zn-α 2-glycoprotein, which functions in antigen processing and presentation of peptide antigen via major histocompatibility complex class I, occurs in human breast cancer cell lines not expressing human HER2 protein (39). In addition, IFN-γ protein increases expression of human Zn-α 2-glycoprotein mRNA (40). The occurrence of Zn-α 2-glycoprotein as an immunogen in breast cancer may have utility in diagnosis. Therefore, we selected AHSG for further validation.

AHSG displays the role as a major growth promoter in serum that can influence tumor establishment and growth. Recently, it has been suggested that AHSG may have biological activity as a transforming growth factor-β1 antagonist (41). Transforming growth factor-β1 is associated with tumor progression and resistance to chemotherapy in established cancers as well as host immune suppression (42). AHSG blocks transforming growth factor-β1 binding to cell surface receptors and suppresses transforming growth factor-β1 signaling (43). From this point of view, the formation of autoantibody may neutralize the function of AHSG, which normally would suppress breast cancer progression. We currently are investigating the mechanisms involved in the action of AHSG autoantibody in breast cancer cells and its functional relevance in breast cancer development.

AHSG, also known as fetuin-A, is a liver secretory glycoprotein found at high levels in serum, urine, saliva, cerebrospinal fluid, and bone (44). A common observation is that the protein must be phosphorylated to be physiologically active in mammals, because the non-phosphorylated version has minimal or no effects (45). In our data, three spots (S4, S5, S6) were identified as a same protein on two-dimensional electrophoresis (i.e., AHSG). This protein could undergo posttranslational modification such as phosphorylation. In another report, this typical pattern of AHSG was also observed on two-dimensional electrophoresis (46). We also confirmed three spots (S4, S5, S6) by Western blot analysis with anti-AHSG antibody (data not shown).

Our preliminary result needs more verification and further validation. Especially, the reproducibility has to be proved by an independent large case-control set. To verify the result, we plan to build an ELISA setting for the verification of 1,000 or >1,000 case-control series. Likewise, further verification by the samples of ductal carcinoma *in situ* patients is needed to confirm the specificity in addition to the verification which was done

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with benign breast disease and other malignancy samples.

In conclusion, we screened 81 sera from breast cancer patients and found that the frequency of positive expression of AHSG autoantibody in breast cancer patients was high enough to consider AHSG autoantibody as a potential biomarker for breast cancer. Additionally, AHSG autoantibody showed low reactivity with other solid cancers such as cervical cancer, thyroid cancer, and benign breast tumor (Supplementary Fig. S3). The significantly high frequency and considerable specificity of autoantibody against AHSG in breast cancer patients implies its potential in clinical settings and also provides a starting point for further studies. Our findings indicate that AHSG in breast cancer induces a humoral immune response and autoantibodies against it can be used as potential biomarkers for clinical serologic screening of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplementary Figure 1. Identification of alpha 2-HS glycoprotein as an autoimmunogenic tumor antigen. A. Total sequence of AHSG and matched tryptic peptides were shown in Bold. **B**. MS-spectrum of AHSG by MALDI-TOF MS (●: matched peptide ●●: peptide chosen for MS/MS #: the modification at a Cys residue) C. The peptide 2030.0734 chosen for the AHSG was sequenced by TOF/TOF.

Supplementary Figure 2. Detection of purified α2-HS glycoprotein by its autoantibodies from serum of breast cancer patients. A. Purified α2-HS glycoprotein (1000, 500, 250 and 125 ng) was separated by SDS-PAGE and transferred to N.C membrane. This membrane was probed with combined sera from 10 healthy controls and subjected to Western blot anaysis. **B**. The duplicate membrane was incubated with pooled sera form 10 breast cancer patients and analyzed by Western blotting. **C**. Each membrane was stained by Coomassie brilliant R250

Supplementary Figure 3. Frequency of AHSG autoantibodies in the Sera of benign breast tumor, thyroid cancer, cervical cancer and control groups. A.α2-HS glycoprotein (1 μg) was separated by SDS-PAGE and transferred to N.C membrane. Each lane was cut into a piece and incubated with benign breast tumor, thyroid cancer, cervical cancer, normal control and breast cancer, respectively. Immunoreactivity was visualized by anti-human IgG-HRP and ECL reagent. **B**. loading control stained with ponceau S.

Supplementary Figure 4. Individual screening of Cathepsin D and Zinc-alpha-2-glycoprotein autoantibody. A. Cathepsin D (1 μg) was separated by SDS-PAGE

and transferred to N.C membrane. Each lane was cut into a piece and incubated with individual 21 healthy sera. Immunoreactivity was visualized by anti-human IgG-HRP and ECL reagent. **B**. Cathepsin D was probed by sera from 21 breast cancer patients. **C**. Zinc-alpha-2-glycoprotein was probed by sera from 14 healthy subjects. **D**. Zinc-alpha-2-glycoprotein was probed by sera from 14 patients with breast cancer

A

1 MKSLVLLLCLAQLWGCHS**APHGPGL**IYRQP

31 NCDDPETEEAALVAIDYINQNLPWGYK**HTL**

61 **NQIDEVK**VWPQQPSGELFEIEIDTLETTCH

91 VLDPTPVARCSVR**QLKEHAVEGDCDFQLLK**

121 **LDGKFSVVYAK**CDSSPDSAEDVRKVCQDCP

151 LLAPLNDTRVVHAAKAALAAFNAQNNGSNF

181 QLEEISR**AQLVPLPPSTYVEFTVSGTDCVA**

211 **KEATEAAK**CNLLAEKQYGFCKATLSEKLGG

241 AEVAVTCTVFQTQPVTSQPQPEGANEAVPT

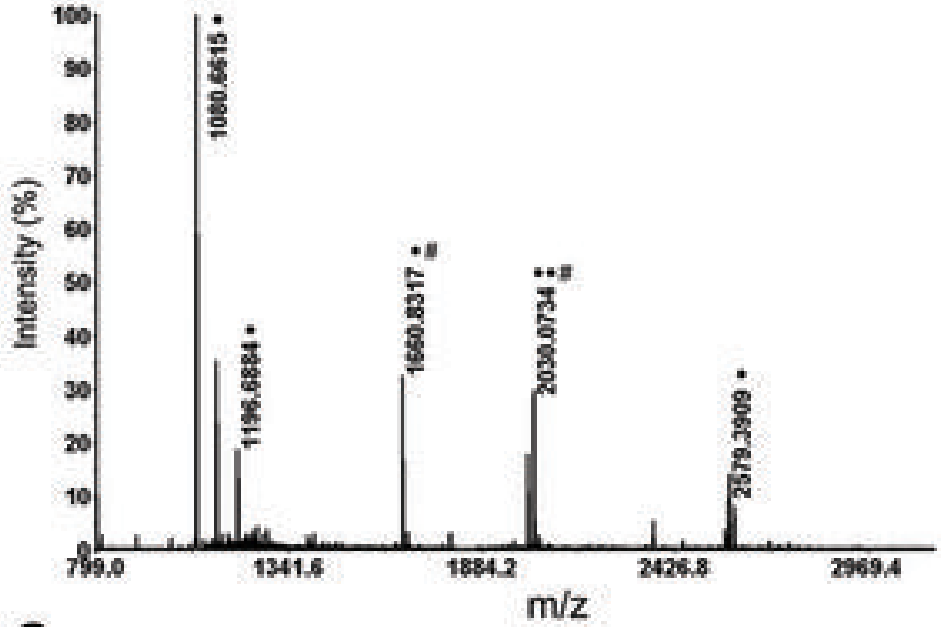
271 PVVDPDAPPSPPLGAPGLPPAGSPPDSHVL

301 LAAPPGHQLHRAHYDLRHTFMGVVSLGSPS

331 GEVSHPRKTRTVVQPSVGAAAGPVVPPCPG

361 RIRHFKV

B



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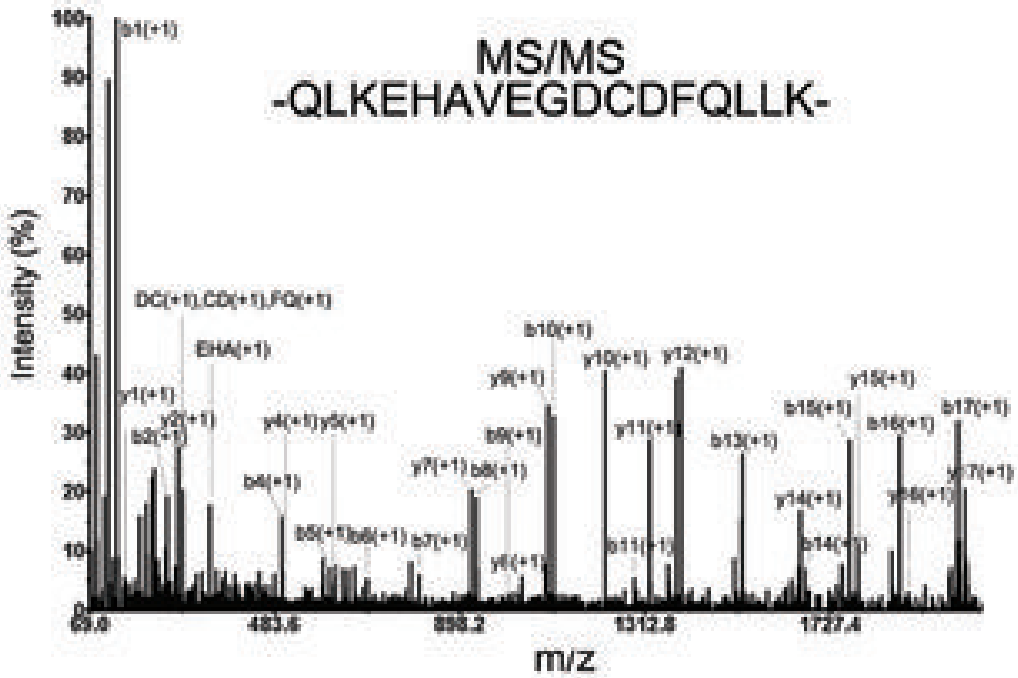
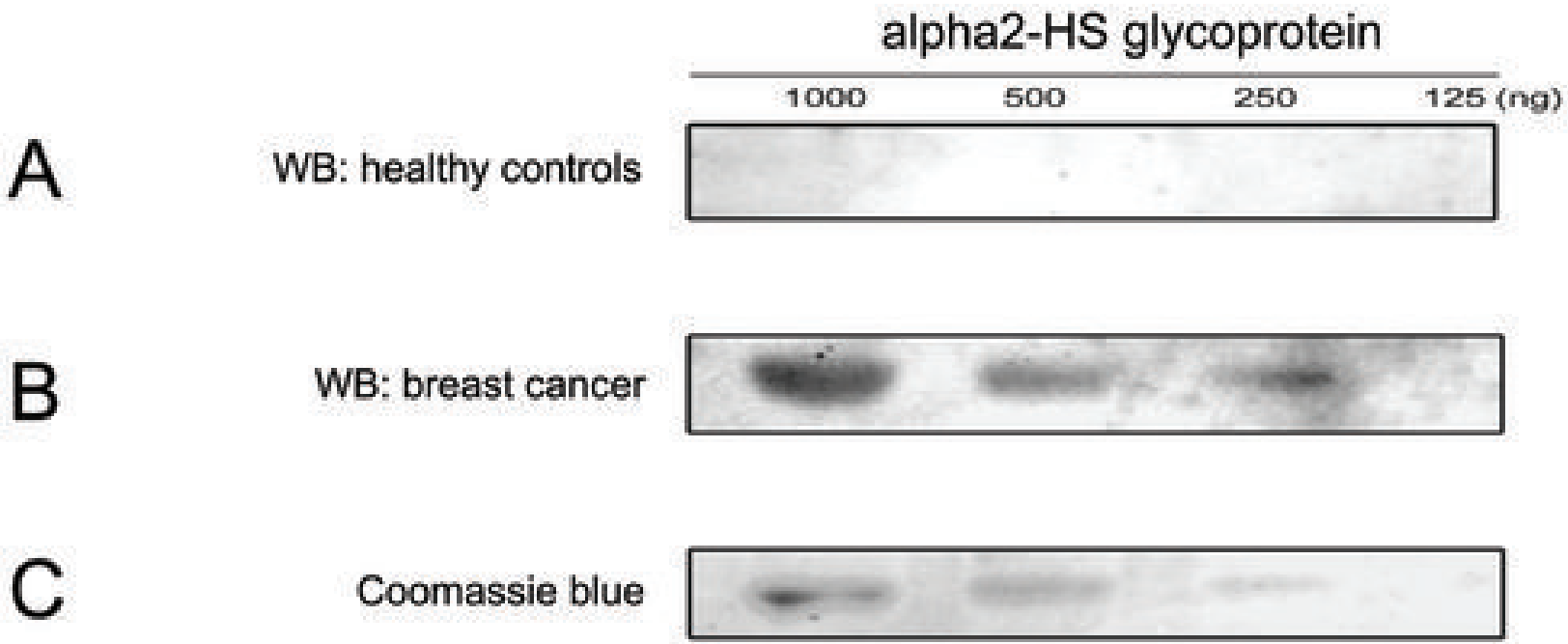
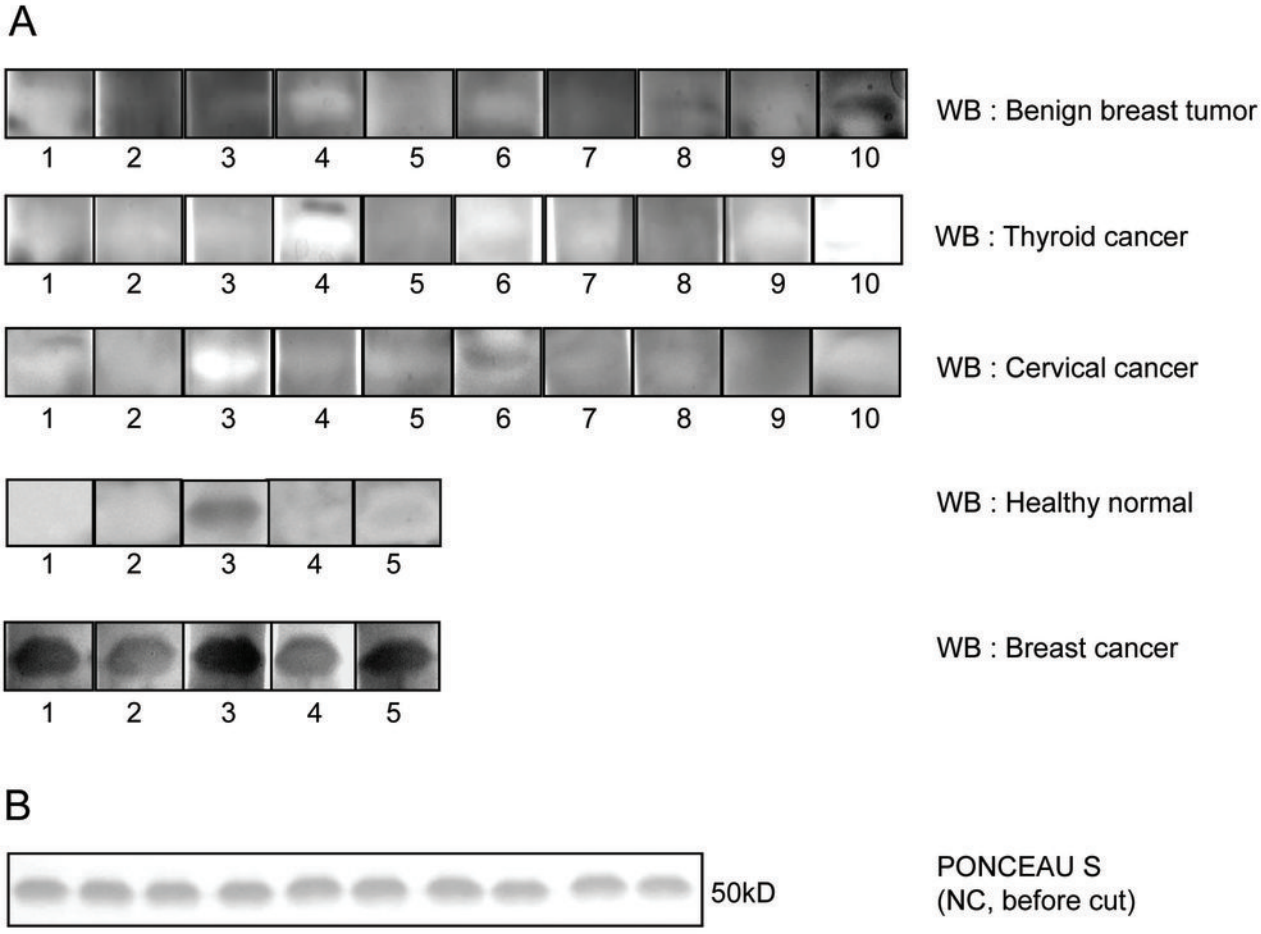


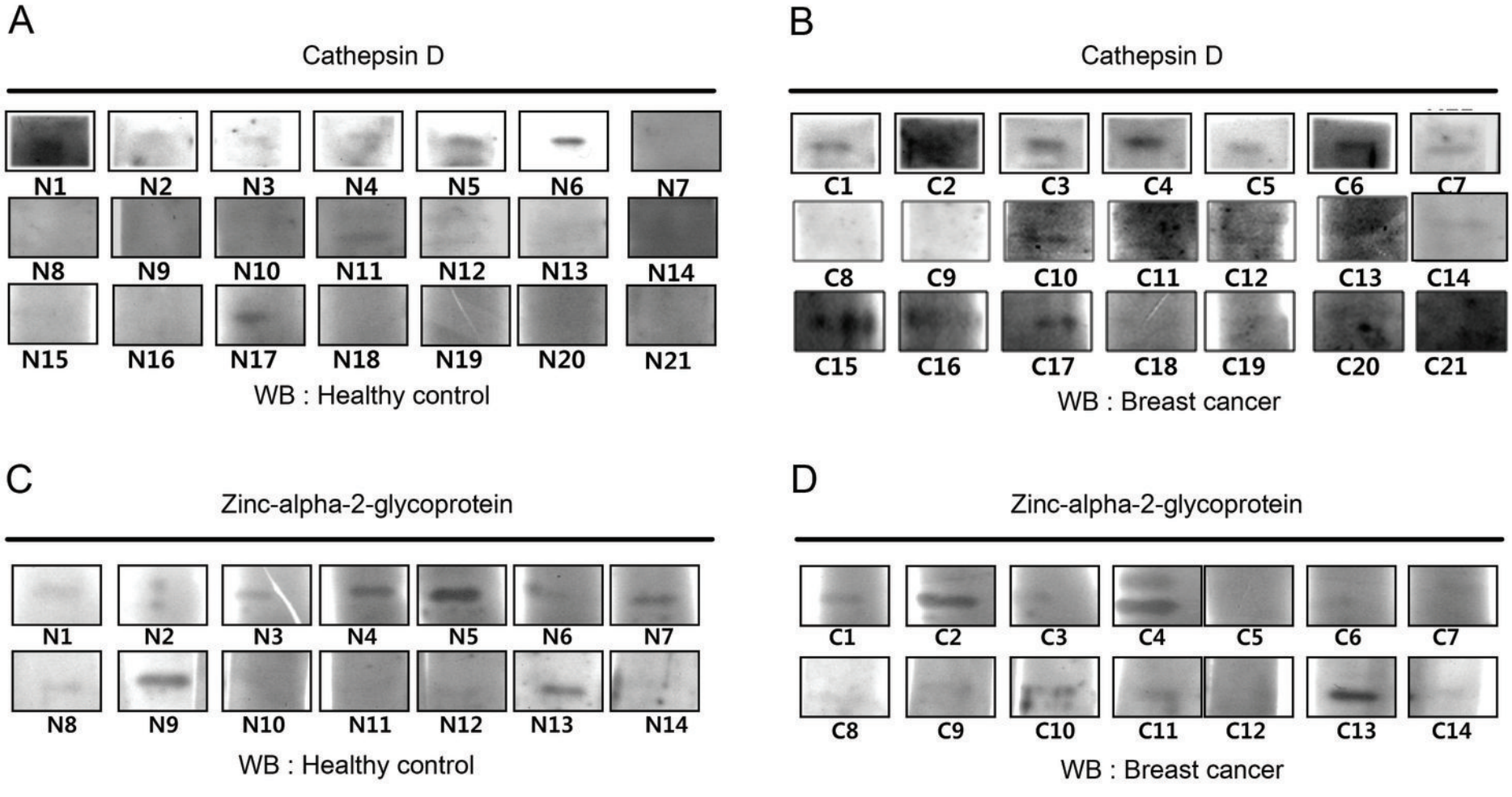
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

Supplementary Table 1.

Clinical information of urine samples

Samples	Age	Histology	T	N	M	Stage	ER/PR	Her2	Sample type
U1	63	Infiltrating duct carcinoma	2	0	0	IIA	-/-	0	urine
U2	48	Infiltrating duct carcinoma	1	0	0	I	+/+	3+	urine
U3	32	Infiltrating duct carcinoma	2	1	0	IIB	+/+	0	urine
U4	45	Infiltrating duct carcinoma	1	0	0	I	+/+	0	urine
U5	48	Infiltrating duct carcinoma	1	1	0	IIA	+/+	3+	urine

Supplementary Table 2.

Clinical information of normal patients who express AHSG antibody at high level

sample	status	Sex	ANA(FANA)	RF
N7	Normal	female	(-)	(+)
N15	Normal	female	(-/+)	(-)
N22	Normal	female	(-/+)	(-)
N32	Normal	female	(-)	(-)
N43	Normal	female	(+)	(-)
N50	Normal	female	(-)	(+)
N57	Normal	female	(-)	(-)

BI(No.)	Patient No.	Sex	collection date	reactivity
1	3xxxxxx	F	8/19/2005	0
2	3xxxxxx	F	9/23/2005	1
3	3xxxxxx	F	9/28/2005	1
4	3xxxxxx	F	11/2/2005	0
5	3xxxxxx	F	1/11/2006	1
6	3xxxxxx	F	8/12/2005	1
7	3xxxxxx	F	10/5/2005	0
8	3xxxxxx	F	9/14/2005	1
9	2xxxxxx	F	9/21/2005	0
10	3xxxxxx	F	9/30/2005	1
11	3xxxxxx	F	10/19/2005	1
12	2xxxxxx	F	10/26/2005	1
13	3xxxxxx	F	11/2/2005	1
14	3xxxxxx	F	11/4/2005	1
15	2xxxxxx	F	6/29/2005	1
16	3xxxxxx	F	7/20/2005	1
17	3xxxxxx	F	7/22/2005	1
18	3xxxxxx	F	8/17/2005	1
19	3xxxxxx	F	8/24/2005	0
20	3xxxxxx	F	9/14/2005	1
21	3xxxxxx	F	10/26/2005	1
22	3xxxxxx	F	11/2/2005	1
23	3xxxxxx	F	12/14/2005	1
24	3xxxxxx	F	12/21/2005	1
25	3xxxxxx	F	1/4/2006	1
26	3xxxxxx	F	1/4/2006	1
27	3xxxxxx	F	1/4/2006	1
28	3xxxxxx	F	1/11/2006	0
29	3xxxxxx	F	1/13/2006	1
30	3xxxxxx	F	1/13/2006	0
31	3xxxxxx	F	1/13/2006	1

32	3xxxxxx	F	2/8/2006	1
33	3xxxxxx	F	4/26/2006	0
34	3xxxxxx	F	5/3/2006	1
35	2xxxxxx	F	6/9/2006	1
36	3xxxxxx	F	6/21/2006	0
37	3xxxxxx	F	6/22/2006	1
38	2xxxxxx	F	6/28/2006	1
39	3xxxxxx	F	7/5/2006	1
40	3xxxxxx	F	7/26/2006	1
41	3xxxxxx	F	6/22/2007	0
42	3xxxxxx	F	6/22/2007	1
43	3xxxxxx	F	6/22/2007	1
44	3xxxxxx	F	6/27/2007	1
45	2xxxxxx	F	6/27/2007	1
46	3xxxxxx	F	6/27/2007	1
47	3xxxxxx	F	6/27/2007	1
48	2xxxxxx	F	6/27/2007	1
49	3xxxxxx	F	6/27/2007	1
50	3xxxxxx	F	6/27/2007	0
51	3xxxxxx	F	6/27/2007	1
52	3xxxxxx	F	6/28/2007	1
53	3xxxxxx	F	6/29/2007	0
54	3xxxxxx	F	6/29/2007	0
55	3xxxxxx	F	6/29/2007	0
56	2xxxxxx	F	7/4/2007	1
57	2xxxxxx	F	7/4/2007	1
58	2xxxxxx	F	7/4/2007	0
59	3xxxxxx	F	7/4/2007	1
60	3xxxxxx	F	7/4/2007	1
61	3xxxxxx	F	7/4/2007	1
62	3xxxxxx	F	7/11/2007	1
63	3xxxxxx	F	7/11/2007	1
64	3xxxxxx	F	7/11/2007	1

65	2xxxxxx	F	7/11/2007	1
66	3xxxxxx	F	7/11/2007	1
67	3xxxxxx	F	7/11/2007	1
68	3xxxxxx	F	7/11/2007	1
69	3xxxxxx	F	7/13/2007	1
70	3xxxxxx	F	7/13/2007	1
71	3xxxxxx	F	7/13/2007	0
72	3xxxxxx	F	7/18/2007	0
73	3xxxxxx	F	7/18/2007	1
74	3xxxxxx	F	7/18/2007	1
75	3xxxxxx	F	7/18/2007	1
76	3xxxxxx	F	7/18/2007	1
77	3xxxxxx	F	7/18/2007	1
78	3xxxxxx	F	7/18/2007	1
79	3xxxxxx	F	7/19/2007	1
80	3xxxxxx	F	7/19/2007	1
81	3xxxxxx	F	7/20/2007	1