

The Thesis Doctor of Medical Science

Detection of tumor-associated antigens in culture supernatants using  
autoantibodies in sera from patients with bladder cancer

(膀胱癌患者血清中の自己抗体を用いた培養上清中の腫瘍関連抗原の検出)

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## 著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

## Abstract

Secreted proteins play essential roles in the process of tumorigenesis, and the analysis of tumor-secreted proteins has been suggested as a promising strategy for identifying cancer biomarkers. In this study, we performed proteomic analysis to identify proteins secreted from bladder cancer cell lines that are recognized by autoantibodies in sera from patients with bladder cancer. In addition, autoantibodies against the identified proteins were validated using a dot-blot array with sera from patients with bladder cancer and normal controls. As the results, we detected twenty-five and thirty-two immunoreactive spots in sera from patients with high- and low-grade bladder cancer, respectively. In addition, validation analysis revealed that serum IgG levels of anti-calreticulin and matrix metalloproteinase-2 (MMP2) autoantibodies were significantly higher in bladder cancer patients than in normal controls (both  $P < 0.05$ ). Furthermore, the serum IgG level of anti-MMP2 autoantibody was significantly higher in patients with high- compared to low-grade bladder cancer ( $P < 0.05$ ). On multivariate analysis, the serum IgG level of anti-MMP2 autoantibody was an independent predictor of cancer-specific survival ( $P < 0.05$ ). Based on these findings, serum IgG levels of anti-calreticulin and MMP2 autoantibodies may be novel biomarker candidates for bladder cancer and its clinical outcome.

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## 1. Introduction

Bladder cancer (BC) is the 7th most common cancer in men and the 17th most common in women in the world. The incidence of BC have been increasing in Japan, and it was about 20 and 5 per 100,000 Japanese males and females in 2002 (22). Approximately 75–85% of BC are diagnosed as non–muscle-invasive bladder cancer (NMIBC) at the first diagnosis, and around 70% of cases present as pTa, 20% as pT1, and 10% as carcinoma in situ lesions (23). NMIBC has a tendency to recur (50-70%) and may progress (10-20%) to a higher grade and/or muscle-invasive BC in time, which might lead to high cancer-specific mortality (46).

The histological tumor grade is one of the clinical factors associated with outcomes of patients with NMIBC. High-grade NMIBC generally shows a more aggressive behavior than the low-grade form, and increases the risk of a poorer prognosis (3, 32). Due to the unfavorable prognosis associated with high-grade NMIBC, differential diagnosis between high- and low-grade NMIBC might be crucial for more appropriate follow-up and aggressive treatment. Cystoscopy and urine cytology are commonly used techniques for the diagnosis and surveillance of BC. Cystoscopy can identify most papillary and solid lesions, but

is markedly invasive to the patients, whereas urine cytology is limited by operator dependence and low sensitivity. For these reasons, some tumor markers have been investigated (e.g. BTAstat, NMP22), but their sensitivity and specificity are limited. Also, they are unable to predict the clinical outcome of BC patients (1, 42, 44). Therefore, one of the most important clinical challenges is the identification of novel biomarkers for aggressive NMIBC destined to recur and progress following initial treatment that can be used to predict the outcome of patients.

Proteins secreted from tumor cells reflect the various states of the tumor in real time and under specific conditions, and their expression patterns are different from normal cell components. Thus, proteins secreted into several body fluids, such as the serum, urine, cerebrospinal fluid, tears, and saliva from tumor cells and conditioned media of cultured tumor cells have been investigated. Approximately 20-25% of cellular proteins are secreted into extracellular spaces, and these proteins play important roles in differentiation, invasion, metastasis, angiogenesis, and the regulation of cell-to-cell and cell-to-extracellular matrix interactions (6, 40, 48). It has been suggested that tumor-secreting proteins are a promising source of diagnostic biomarkers in tumors (33).

Tumor-associated antigens released into the bloodstream could induce a humoral immune response and generate autoantibodies (AAbs) (11). Interestingly, the immune response to such antigens generates marked biological amplification even though tumor-associated antigens are undetectable in sera in the early stage of tumorigenesis (19). Hundreds of tumor-associated antibodies have been identified, and the potential for using AAbs as a novel biomarker useful for cancer diagnosis has been discussed (17). Furthermore, recent studies based on AAb profiling of cancer patients have suggested that AAbs may not be only diagnostic but also prognostic biomarkers (24).

In this study, we performed two-dimensional gel electrophoresis (2-DE) combined with immunoblot analysis to identify tumor-associated secreted antigenic proteins that elicit a humoral response in sera from BC patients. By comparing immunoreactive patterns in sera from high- and low-grade BC patients, novel tumor markers associated with the histological grade were obtained. The identified proteins were further validated by dot-blot analysis with a large number of sera from patients with BC and normal controls. Moreover, the relationships between serum IgG levels of AAbs and clinicopathological factors of BC patients were also evaluated.

## 2. Materials and Methods

### 2-1. Serum samples

Ninety-five serum samples from BC patients who had not received any therapy at Kitasato University Hospital were collected and stored at -80 °C until use. The 2002 TNM and WHO classifications were used for determination of the pathological stage and histological grade of the tumor. Clinical characteristics of BC patients whose sera were collected are shown in Table 1. Thirty-five serum samples from healthy donors were also collected and used as a control. This study was approved by the Ethics Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave consent for the use of their samples.

### 2-2. Cell culture and sample preparation for proteomic analysis

The human bladder cancer cell lines TCCSUP, T24, 5637, and RT4 were purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were grown in RPMI-1640 (SIGMA Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Life Technologies Corp.,



Carlsbad, CA, USA) at 37°C in 5% CO<sub>2</sub> and 95% humidified air and harvested when they reached a confluency of 60-70%. Then, cells were washed 3 times with phosphate-buffered saline without divalent ions (PBS(-)), and additionally incubated with serum-free medium (Hybridoma-SFM; Life Technologies Corp.) for 48 h. The collected culture supernatants were subjected to serial centrifugation to remove cells at 200×g for 20 min at room temperature (R/T) and cell debris at 2,000×g for 30 min at 4 °C, respectively. Forty milliliters of culture supernatants from each cell line was concentrated by ultrafiltration (Amicon Ultra-15 centrifugal filter units with a 30-kDa molecular weight cutoff membrane; Millipore Corp., Billerica, MA, USA), according to the manufacturer's instructions. The concentrated samples were precipitated and components interfering with 2-DE were removed with a 2-D Clean-up Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), according to the manufacturer's instructions. Precipitated samples were solubilized in lysis buffer (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid, 10 mM tris(2-carboxyethyl)-phosphine hydrochloride (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; TAIITEC 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).

### 2-3. 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.

#### 2-4. 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).

## 2-5. 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 overlaid 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  $\text{NH}_4\text{HCO}_3$  until they became colorless. The pieces of gel were dehydrated with 100% acetonitrile and dried under vacuum conditions. They were then rehydrated in 10  $\mu\text{L}$  of trypsin solution containing 10 ng/ $\mu\text{L}$  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  $\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$ . After incubation, the digested tryptic peptide solutions were collected, and the gel was washed once with 7  $\mu\text{L}$  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 was 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](http://www.matrixsciences.com)) and database searches were run using the IPI human database (version 3.82; 92,104 sequences; 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.

## 2-6. 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.

## 2-7. 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).



### 3. Results

#### 3-1. 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).

#### 3-2. 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).

### 3-3. *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 ( $\pm$ SD) of serum IgG levels of anti-calreticulin and matrix metalloproteinase-2 (MMP2) AAbs were  $14.0 \pm 4.4$  and  $47.1 \pm 9.7$  in BC patients, and  $9.9 \pm 2.0$  and  $34.6 \pm 5.1$  in normal controls, respectively. All serum IgG levels of anti-calreticulin and MMP2 AAbs were significantly higher in BC patients than in normal controls ( $P < 0.01$ , Fig. 2A, B). In addition, the mean value ( $\pm$ SD) of serum IgG levels of anti-calreticulin and MMP2 AAbs were  $13.5 \pm 3.1$  and  $40.4 \pm 9.8$  in the patients with low-grade BC, and  $14.0 \pm 4.6$  and  $66.3 \pm 9.0$  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).

### 3-4. 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).

### 3-5. 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 were 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.

#### 4. 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).

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<sup>2+</sup> 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 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.

## 5. 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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## 9. Figures and Tables

Table 1. *Clinicopathological characteristics of bladder cancer patients*

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

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.

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	<i>P</i>	HR	95%CI	<i>P</i>
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.



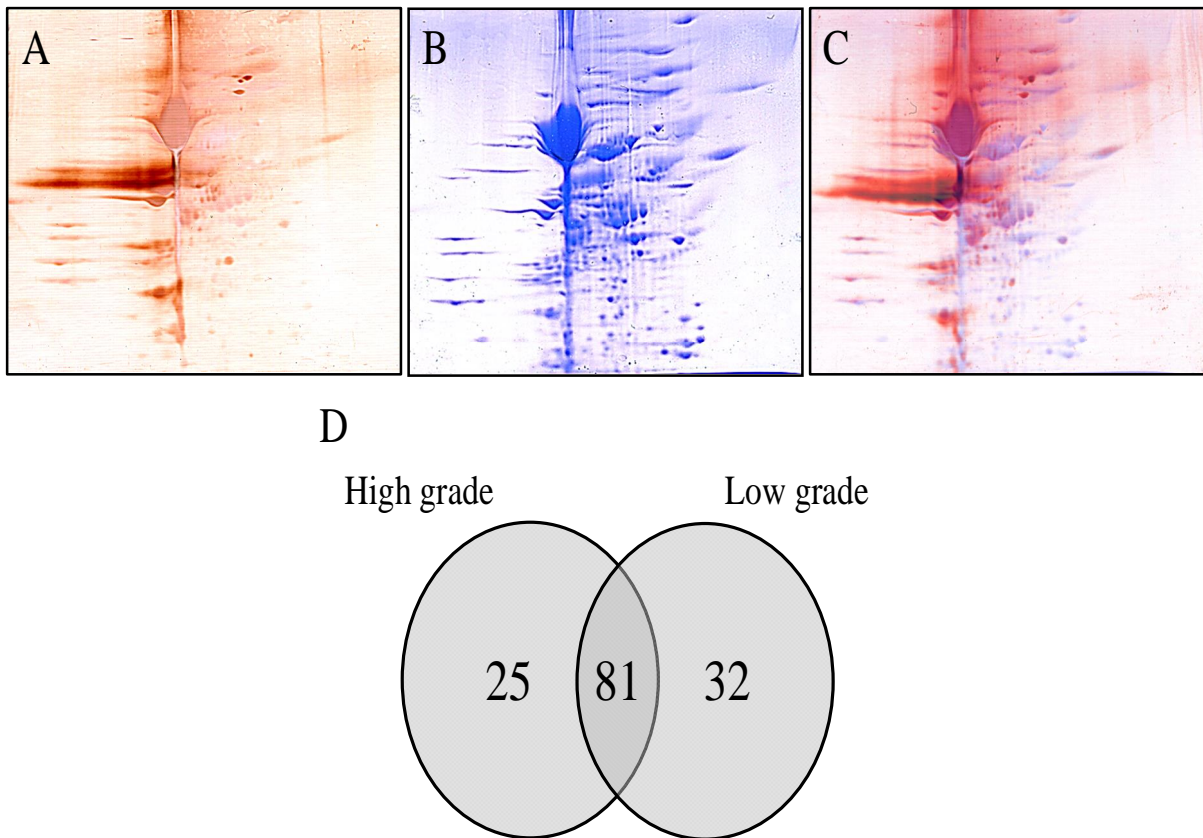


Figure 1. Detection of autoantibodies by 2-DE immunoblot analysis in sera from BC patients.

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.

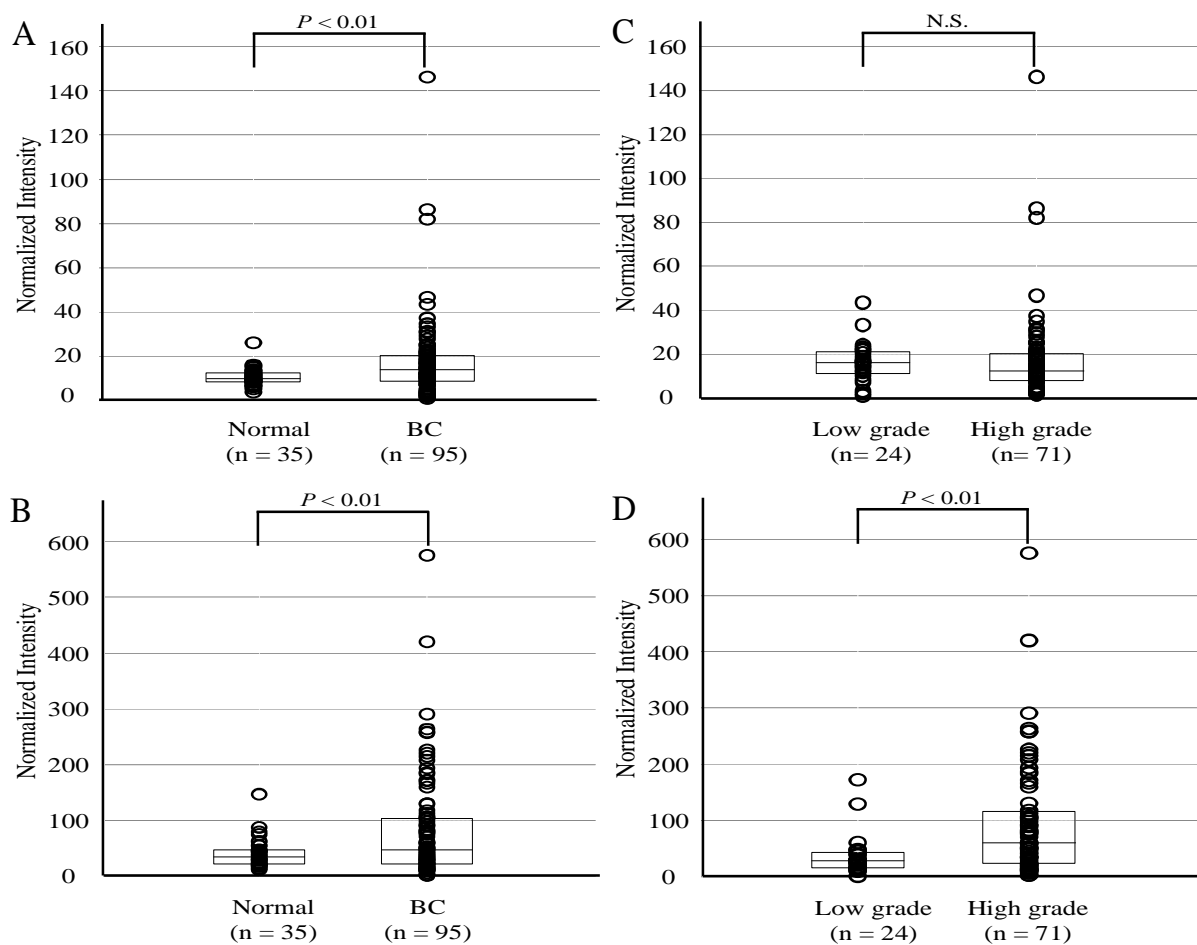


Figure 2. Dot-blot analysis of serum IgG levels of anti-calreticulin and MMP2 AAbs.

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

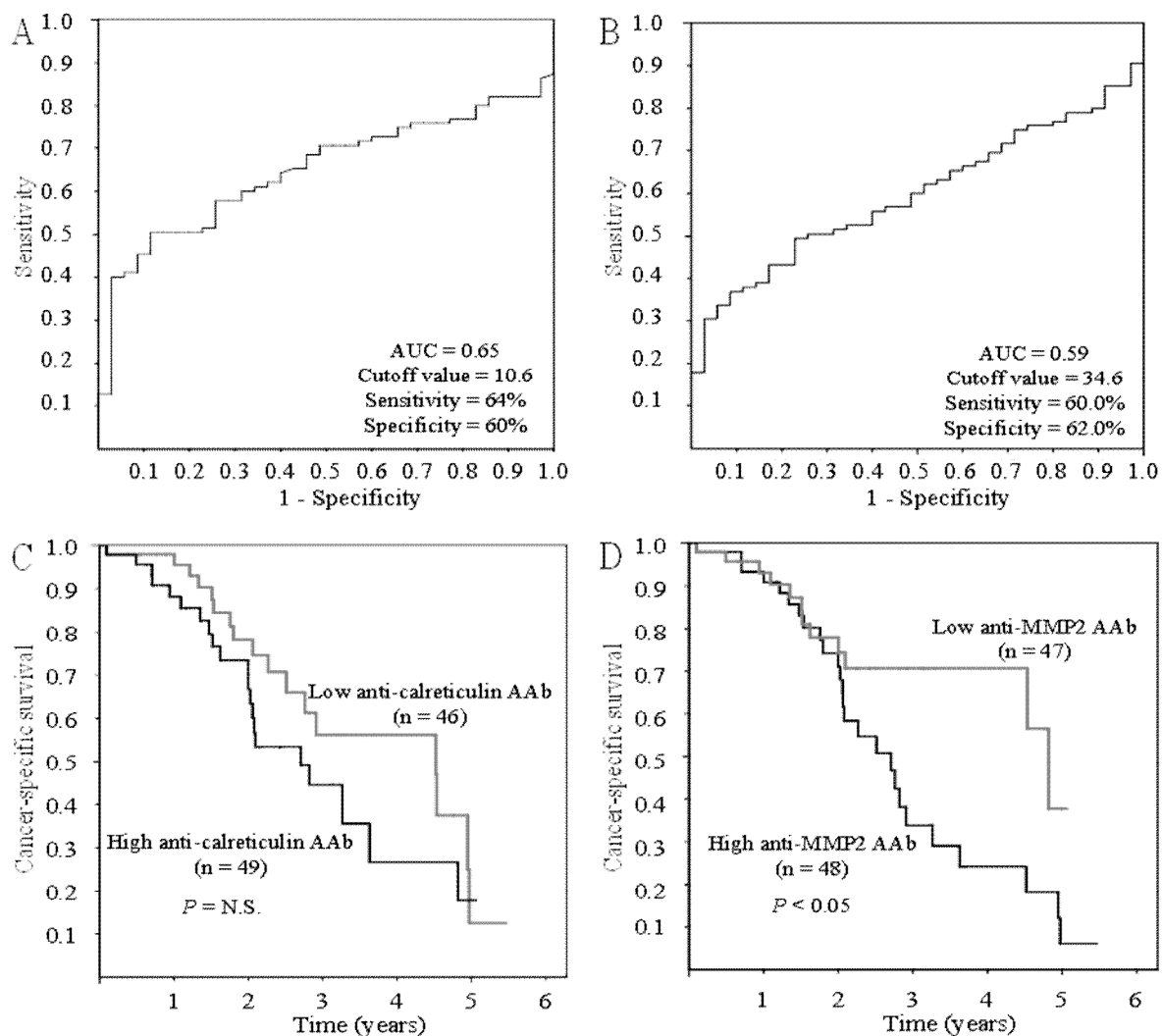


Figure 3. Receiver-operating characteristic curve (ROC) analysis for BC and the probability of cancer-specific survival according to serum IgG levels of anti-calreticulin and MMP2 AAbs.

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