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SUPPORTING INFORMATION

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

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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.

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 has 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

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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 blood stream 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.

MATERIALS AND METHODS

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.

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, Nuaillè, France), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies Corp., Carlsbad, CA, USA) at 37°C in 5% CO_2 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

Table 1 *Clinicopathological characteristics of bladder cancer patients*

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

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

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

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

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

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

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

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

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

RESULTS

2-DE immunoblot analysis

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

Identification of proteins recognized by autoantibodies

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

Dot-blot analysis of autoantibodies against identified proteins

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

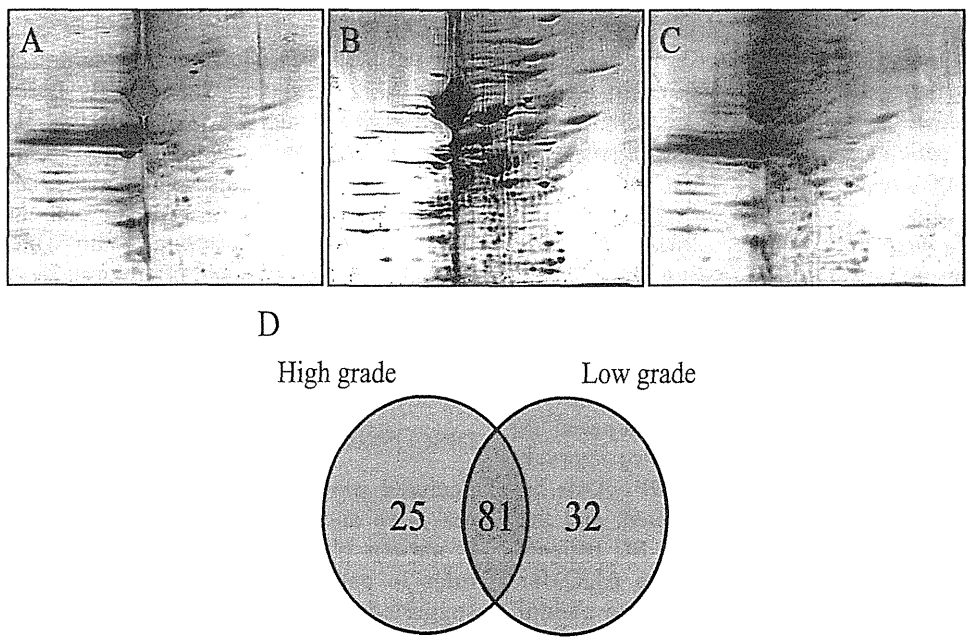

Fig. 1 Detection of autoantibodies by 2-DE immunoblot analysis in sera from BC patients. The proteins extracted from culture supernatants of the BC cell lines were separated by 2-DE and transferred to PVDF membranes. The membranes were incubated with mixed sera from BC patients (A). Protein patterns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

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

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

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

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

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

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

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

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

DISCUSSION

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

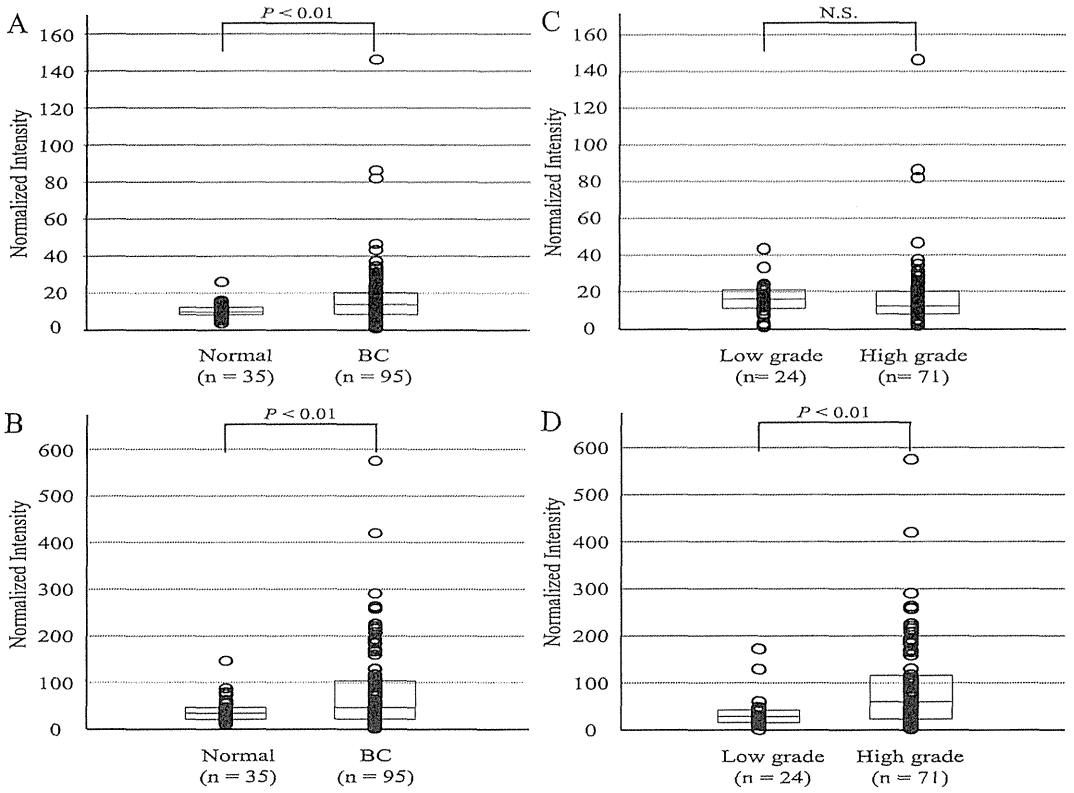

Fig. 2 Dot-blot analysis of serum IgG levels of anti-calreticulin and MMP2 AAbs. Serum IgG levels of anti-calreticulin and MMP2 AAbs in sera from BC patients and normal controls were detected by dot-blot analysis. The normalized intensity was calculated with the Mann-Whitney *U*-test. The serum IgG level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

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

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

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

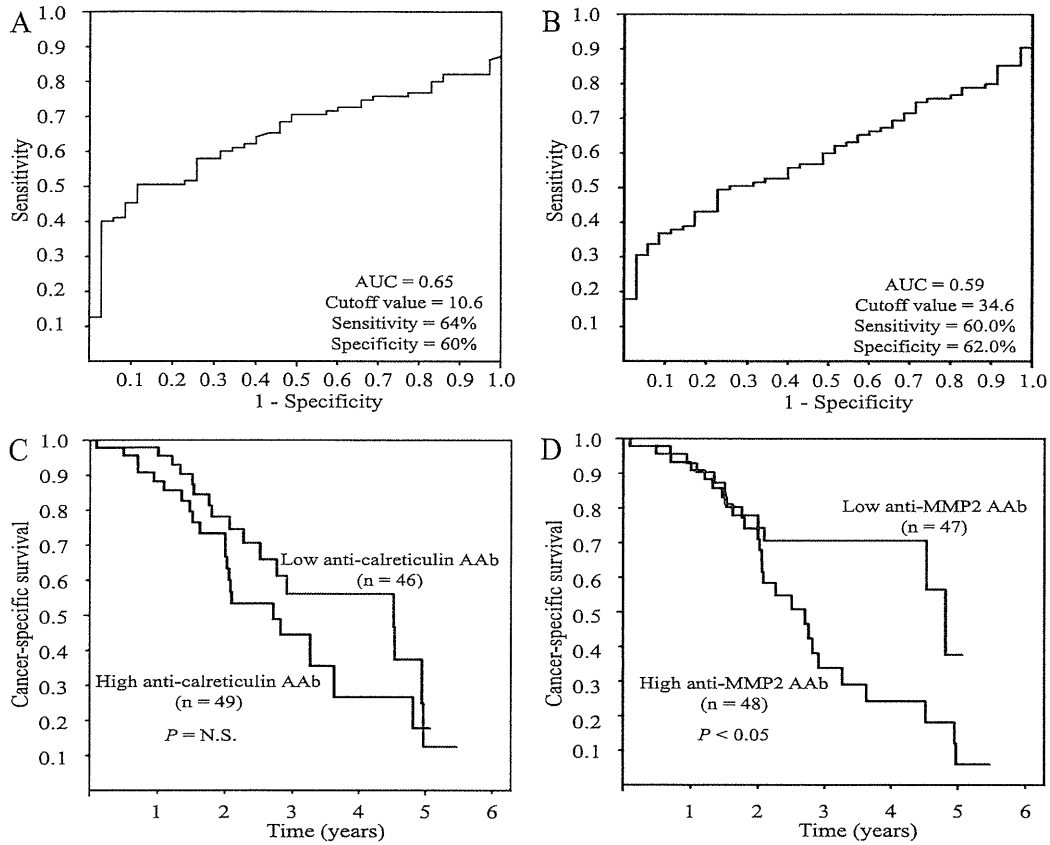

Fig. 3 Receiver-operating characteristic curve (ROC) analysis for BC and the probability of cancer-specific survival according to serum IgG levels of anti-calreticulin and MMP2 AAbs. The corresponding areas under the curves (AUC) of serum IgG levels of anti-calreticulin (A) and anti-MMP2 (B) AAbs in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

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

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

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

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

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

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

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

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Adenovirus-mediated REIC/Dkk-3 gene therapy: Development of an autologous cancer vaccination therapy (Review)

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Abstract. Reduced expression in immortalized cells (REIC)/Dickkopf (Dkk)-3 is a tumor suppressor and therapeutic gene and has been studied with respect to the application of cancer gene therapy. Our previous studies demonstrated that the intratumoral injection of an adenovirus vector carrying the human REIC/Dkk-3 gene (Ad-REIC) suppresses tumor growth in mouse models of prostate, breast and testicular cancer and malignant mesothelioma. The mechanisms underlying these antitumor therapeutic effects have only been clarified recently. It has been demonstrated that Ad-REIC treatment inhibits cancer progression via the upregulation of systemic anticancer immunity. Under experimental conditions, autologous cancer vaccination via cancer-specific apoptosis and anticancer immune activation is a possible therapeutic mechanism. The robust anticancer effects observed in previous preclinical studies support the clinical utility of Ad-REIC. At present, a phase I-IIa study of Ad-REIC gene therapy in prostate cancer patients is ongoing. The current study reviews the observations of previous fundamental studies and summarizes the anticancer mechanisms of intratumoral Ad-REIC treatment in terms of cancer vaccination.

Contents

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Key words: REIC/Dkk-3, cancer vaccine, gene therapy, apoptosis, dendritic cells

1. Introduction

A number of therapeutic cancer vaccines have been previously developed and evaluated in phase II/III clinical trials (1-3). The strategies for immunotherapy include the injection of peptides or proteins in adjuvant treatment and recombinant viruses and plasmids encoding immune factors, as well as the delivery of killed tumor cells and protein- or peptide-activated dendritic cells (DCs) to patients. With respect to the concept of cancer vaccination, controlling tumor-associated antigen (TAA) and systemic immune activation against TAA is essential. A number of previous clinical studies have been conducted focusing on single specific TAA molecules and designing a protocol to target TAA. However, previous trials of cancer vaccines have been unable to demonstrate robust therapeutic effects in spite of the activation of specific cytotoxic T lymphocytes against TAA (4). One possible reason for this is that targeting a single specific TAA is not sufficient to achieve substantial tumor reduction, since not all cancer cells express TAA and the cells without TAA escape the acquired immunity. In addition, there is a possibility that each immunological design of a cancer vaccine, such as specific peptides, is unlikely to cover the range of individual immune systems and may therefore, be ineffective. Hence, it is important to overcome the issues derived from the limited abilities of selected TAA molecules and differences in the immunological characteristics of patients.

A good strategy to address these issues is to kill the cancer cells at the tumor site via the direct injection of anticancer agents. In this way, the various TAAs released from the dead cancer cells are exposed to the individual immune system. The released TAAs are taken up by antigen-presenting cells (APC) and activated APCs upregulate the anticancer

immune response by presenting the TAAs to immune effector cells (1,2). This type of therapy aims to use and vaccinate autologous tumors for anticancer immune activation. This autologous cancer vaccination strategy is predicted to activate individual immunity against the broad range of TAAs present in the cancer cells of the individual. This strategy is also attractive since systemic anticancer immunity may be activated simultaneously with substantial tumor reduction and these two therapeutic effects are predicted to be synergistic.

Gene therapy has been utilized in a number of previous clinical trials of human cancer and exhibits an innovative and attractive therapeutic potential. Adenovirus-mediated gene delivery continues to be the preferred treatment for cancer as the vectors indicate the high transduction efficacy of the therapeutic gene and the safety of the procedure when used for direct local injection (5-7). A number of previous clinical trials have demonstrated the utility and safety of the intratumoral injection of adenoviral vectors in cancer lesions (7). Adenoviral vectors are suitable for use in autologous cancer vaccination strategies as they succeed in robustly killing cancer cells and upregulating specific anticancer immunity pathways (6,8).

The reduced expression in immortalized cells (REIC) gene is identical to Dickkopf (DKK)-3 and REIC/Dkk-3 expression is significantly downregulated in a broad range of human cancer cells (9-17). In our previous study, the therapeutic effects of the REIC/Dkk-3 gene as a tumor suppressor gene were determined by the development of an adenovirus vector carrying the human REIC/Dkk-3 gene (Ad-REIC). The agent was found to significantly induce apoptosis in various cancer cells (13,18-20). Recently, the mechanisms of action of Ad-REIC agents in cancer gene therapy have been clarified *in vitro* and *in vivo* using several mouse tumor models. Under these experimental conditions, autologous cancer vaccination via cancer-specific apoptosis and anticancer immune upregulation is a potential therapeutic mechanism. We herein review the previously reported observations of fundamental studies and summarize the anticancer mechanisms of intratumoral Ad-REIC treatment in terms of cancer vaccination.

2. Characteristics of REIC/Dkk-3

The REIC gene was originally identified at Okayama University (Okayama, Japan) and reported in 2000 (9) as a gene whose expression is decreased via the immortalization of normal human fibroblasts. The authors performed mRNA expression profiling using subtractive hybridization of two types of cell lines, cobalt-irradiated normal fibroblasts, which stop proliferating and immortalized fibroblasts, which continue to proliferate. Subsequently, REIC was identified since expression of the gene was significantly reduced in the immortalized fibroblast cells. The sequence of the REIC gene was found to be consistent with that of the human Dkk-3 gene, a member of the Dkk family that encodes secreted proteins and consists of four primary members in vertebrates (Dkk-1, -2, -3 and -4). The expression of this gene was found to be markedly decreased in a variety of human immortalized cells and was therefore, named REIC. Previously, significant downregulation of the REIC/Dkk-3 expression has been reported in a broad range of human malignant tissues and REIC/Dkk-3 is hypothesized to function as a tumor suppressor gene (9-17).

The REIC/Dkk-3 gene is located on human chromosome 11p15.1 and contains 9 exons spanning >50 kbp (21). The REIC/Dkk-3 gene product is a secretory protein, while the gene itself encodes a deduced 38.3 kDa protein with 350 aa that is detected as two major bands of 60-68 kDa in size, according to variable glycosylation levels. The cDNA possess an N-terminal signal peptide, two cysteine-rich domains and two coiled-coil domains. REIC/Dkk-3 is an N-glycosylated protein, the majority of which intracellularly localizes to the endoplasmic reticulum (ER) (22). REIC/Dkk-3 is expressed in the majority of normal tissues in humans and mice, including the brain, heart, lungs, liver, colon and kidneys and is significantly downregulated in a broad range of human cancer cell lines (9,22). REIC/Dkk-3 has also been found to be downregulated in a variety of cancer tissues compared with surrounding normal tissue, including those of colorectal, lung, gastric, pancreatic, prostate, breast and bladder cancer, hepatocellular and renal cell carcinoma and malignant mesothelioma (10-17). Consistently, the REIC/Dkk-3 expression in cancer specimens is downregulated at the critical transition from low- to high-level malignant disease (10,13,16). Therefore, the lack of REIC/Dkk-3 expression has been found to positively correlate with the malignant grade and progression of cancer in several cancer types. Hypermethylation in the REIC/Dkk-3 promoter region has been previously reported in cancer cells with an absent or reduced expression (14,15,17,23).

3. Physiological functions of REIC/Dkk-3

Previously, the physiological functions of the REIC/Dkk-3 protein have been intensively investigated using knockout or overexpression of intracellular proteins. Previous studies have demonstrated that Dkk-3 modulates fibroblast growth factor and activin/nodal signaling to regulate the mesoderm induction of Xenopus. This suggests that physiological Dkk-3 is required for transforming growth factor β (TGF-β) signaling during early Xenopus development (24). Previously, the Dkk-3 protein has been found to also play an essential role in amphioxus head formation by inhibiting Wnt/β-catenin and nodal signaling (25). The authors identified that the Dkk-3 protein inhibits Wnt/β-catenin signaling in specific mammalian cells and cancer cell lines. However, Wnt/β-catenin signaling is positively regulated by the Dkk-3 protein in the murine retina and in several types of cell lines, including HEK293 cells (25). As for mammalian prostate glands, it has been previously reported that Dkk-3 is involved in prostate acinar morphogenesis and maintains the structural integrity of the prostate gland by limiting TGF-β/Smad signaling (26,27). Consistent with these observations, exogenous REIC/Dkk-3 protein promotes prostate acinar morphogenesis, suggesting that secreted REIC/Dkk-3 protein is also involved in prostate gland differentiation (22). In addition, the increased proliferation of human prostate epithelial cells has been previously confirmed in acini cells formed by epithelial cells stably silenced for Dkk-3 (27). Finally, the Dkk-3 gene has been found to be involved in the mechanisms underlying the differentiation of partially induced pluripotent stem cells to smooth muscle cells, thereby, transcriptionally regulating SM22 via the potentiation of Wnt signaling (28).

These results indicate that intracellular REIC/Dkk-3 protein plays a pivotal role in biology, involved in cell differentiation and proliferation and the development of specific organs via the regulation of the Wnt and TGF-β signaling pathways. Notably, REIC/Dkk-3 protein acts as an inhibitor or inducer of the Wnt and TGF-β signaling pathways based on the cellular conditions of various tissues and organisms, from amphioxus to vertebrates (25). The binding partner of intracellular REIC/Dkk-3 protein has been investigated by several previous studies. It has been reported that Dkk-3 binds to other proteins, such as Kremen1/2 (28,29), β-TrCP (30) and TcTex-1 (31), and that these interactions occur in the cytoplasm. These proteins include substantial molecules that significantly affect and modify intracellular signaling pathways, including the Wnt and TGF-β (16,32). Therefore, the functional varieties of intracellular Dkk-3 protein in the Wnt and TGF-β signaling may be partially explained by the various interaction partners or behavior of the proteins.

4. Cytokine-like aspects of exogenous REIC/Dkk-3 protein in monocyte differentiation

The immunological aspects of exogenous REIC/Dkk-3 protein have been investigated in a previous study (33). Purified recombinant proteins were added to human monocytes obtained from peripheral blood and the cytokine-like actions were examined. To clarify the effects of exogenous REIC/Dkk-3 protein on monocyte differentiation, human $CD14^+$ monocytes were incubated with recombinant proteins at a concentration of 10 μg/ml. Recombinant REIC/Dkk-3 protein was found to induce monocyte differentiation to a DC phenotype. The morphological features of the REIC/Dkk-3-induced cell phenotype and its expression pattern of dendritic markers on the cell surface are similar to those of interleukin (IL)-4- and granulocyte macrophage-colony stimulating factor (GM-CSF)-induced DCs. Consistent with these observations, unpublished data indicates that recombinant REIC/Dkk-3 protein intraperitoneally administered in mice significantly upregulate the ratio of circulating DCs on flow cytometry. Recombinant REIC/Dkk-3 protein also possesses a cytokine-like function in the activation of STAT1 and STAT3 during dendritic phenotype differentiation *in vitro*. In terms of the expression of CD1a and CD14 surface markers, the REIC/Dkk-3-induced dendritic phenotype and IL-4- and GM-CSF-induced DCs are distinctly different. It is likely that these cells are categorized into DC subgroups. In addition, the direct cytotoxic effects of exogenous REIC/Dkk-3 protein were examined in several cancer cell lines. Even at a concentration of 20 μg/ml, no significant cytotoxic effects were associated with REIC/Dkk-3 protein treatment (33).

To date, the molecular mechanisms by which exogenous REIC/Dkk-3 protein differentiates monocytes into the DC phenotype have remained unclear. To clarify the immunomodulatory function of exogenous REIC/Dkk-3 protein, it is essential to determine the cell surface receptor. REIC/Dkk-3 is a secretory protein that is considered to act on cells via a cell surface receptor. However, the definitive cell surface receptors for this protein have not been identified. It has been previously reported that the expression levels of REIC/Dkk-3 modify intracellular Wnt and TGF-β signaling (24,25,27,29,30). There is a possibility that the REIC/Dkk-3 protein, secreted by the cells, interacts with unidentified cell surface receptors and exogenously affect these signaling pathways. Notably, several previous studies have demonstrated that Wnt and TGF-β signaling is involved in the differentiation of specific types of DC phenotypes (34-37). It is conceivable that modification of the signaling of the Wnt and TGF-β pathway by exogenous REIC/Dkk-3 protein triggers the differentiation to the DC phenotype in monocytes.

5. Adenovirus vectors expressing the human REIC/Dkk-3 gene (Ad-REIC) induce cancer cell-specific apoptosis

To examine the possible use of REIC/Dkk-3 as a tool for targeted gene-based therapy, we developed a replication-deficient adenovirus vector encoding the human REIC/Dkk-3 gene (Ad-REIC) (13). The CAG (CMV early enhancer/chicken β-actin) promoter was used to drive REIC/Dkk-3 expression, as this promoter enables strong gene expression (38,39). The overexpression of REIC/Dkk-3 induced by the Ad-REIC agent was found to stimulate apoptosis in a broad range of human cancer cell lines *in vitro* (13,18-20). By contrast, the ability of Ad-REIC to induce apoptosis was reduced in non-malignant cells (13,18,20). These observations indicate that the Ad-REIC agent selectively induces apoptosis in a cancer cell-specific manner. Since REIC/Dkk-3 expression is significantly downregulated in a broad range of human cancer cells, while being typically expressed in non-malignant or normal cells (9-17,22), the endogenous expression level of these proteins appear to correlate with sensitivity to the REIC/Dkk-3 overexpression induced by Ad-REIC.

The molecular mechanisms underlying the apoptosis induced by Ad-REIC have been previously investigated and the pathway is shown in Fig. 1. The phosphorylation (activation) of c-Jun N-terminal kinase (JNK) is a critical step in cancer cell death (13,18,19). REIC/Dkk-3 protein is a secretory protein and the overexpression of this protein induced by Ad-REIC treatment efficiently leads to ER stress-induced apoptosis in cancer cells (19,21,40). ER stress-induced apoptosis is triggered due to a failure in the folding of large amounts of REIC/Dkk-3 protein accumulated in the lumen of the ER. The phosphorylation of JNK occurs downstream of ER stress signaling in cancer cells. As the REIC/Dkk-3 gene expression is absent or lacking in cancer cells (9-17,41), the REIC/Dkk-3 expression and protein folding system in cancer cells does not function well when the protein is overexpressed by Ad-REIC. This implies that, due to the poor capacity for REIC/Dkk-3 gene expression, the cancer cells easily exhibit a failure to fold large amounts of REIC/Dkk-3 protein accumulated in the ER. The differences in the capacity for REIC/Dkk-3 gene overexpression between cancer and normal cells may explain the cancer cell-specific apoptosis induced by Ad-REIC.

Previously, differences in ER stress signaling following Ad-REIC treatment have also been studied in cancer and normal cells (13,19,21,40). As shown in Fig. 1, REIC/Dkk-3-sensitive cancer cells, IRE1α (an ER stress sensor), apoptosis signal-regulating kinase 1 (ASK1) and JNK activation by Ad-REIC, subsequently induce the phosphorylation of c-Jun. As a result of c-Jun activation, translocation

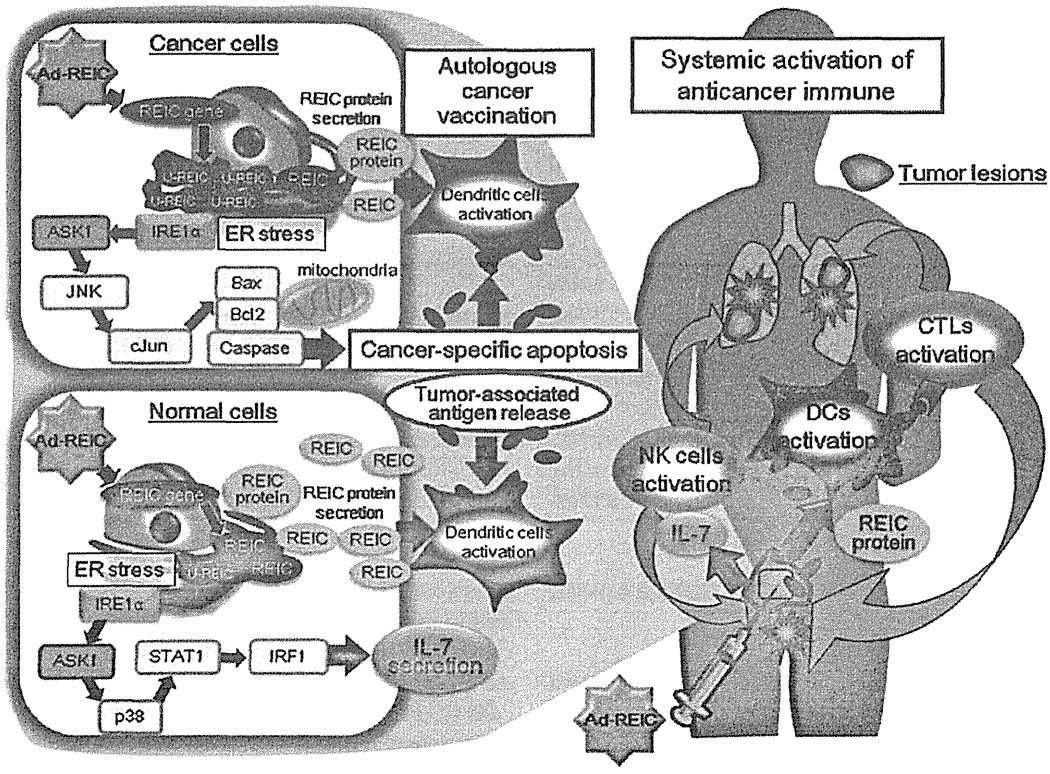

Figure 1. Therapeutic mechanisms of intratumoral Ad-REIC treatment. At the tumor site, Ad-REIC induces cancer cell-specific apoptosis in a phosphorylated JNK-dependent manner via ER stress signaling. Due to the poor capacity for the REIC/Dkk-3 gene expression, the cancer cells exhibit a failure to fold large amounts of REIC/Dkk-3 protein accumulated in the ER. This folding failure and the presence of U-REICs in the ER lead to the stress-induced apoptosis of cancer cells. The activation of JNK by Ad-REIC subsequently induces the phosphorylation of c-Jun, the translocation of Bax to the mitochondria and the downregulation of Bcl2, which subsequently leads to caspase-dependent apoptosis. By contrast, in non-cancer cells, which are typically resistant to Ad-REIC-induced apoptosis, a different ER stress response is observed following treatment. When REIC/Dkk-3 is overexpressed by Ad-REIC in normal cells, for example human fibroblasts, ER stress signaling of the p38, STAT1 and IRF1 pathways is activated. The activation of IRF1 then upregulates IL-7 expression and secretion in the cells. Autologous cancer vaccination with Ad-REIC treatment starts with cancer-specific apoptosis and the subsequent release of TAAs. The REIC/Dkk-3 protein, overexpressed and secreted by Ad-REIC transfection, differentiates monocytes into the DC phenotype at the tumor site. At the same time, abundant TAA fragments are released as a result of cancer cell-selective apoptosis and are supplied to the DCs induced by the secreted REIC/Dkk-3 protein. The activation of the DCs directly enhances cancer cell antigen presentation to CTLs, which upregulates systemic antitumor immunity. In addition, the enhanced IL-7 secretion observed in the normal cells activates NK cells, which also upregulates systemic anticancer immunity. The Ad-REIC-induced synergistic secretion of the REIC/Dkk-3 protein and IL-7 cytokines at the treated tumor site is important for the autologous cancer vaccination induced by the agent. These immunoactive proteins work together to mediate the phase of cancer-specific apoptosis to the anticancer immune effects observed at the injected and distant tumor sites. Ad, adenovirus; REIC, reduced expression in immortalized cells; JNK, c-Jun N-terminal kinase; ER, endoplasmic reticulum; Dkk-3, dickkopf-3; U-REICs, unfolded REIC/Dkk-3 protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; STAT1, signal transducer and activator of transcription 1; IRF1, interferon regulatory factor 1; IL, interleukin; TAAs, tumor-associated antigens; DC, dendritic cell; CTLs, cytotoxic T lymphocytes; NK, *natural killer*.

of Bcl-2-associated X protein to the mitochondria and the downregulation of B-cell lymphoma 2 occur, while the upregulation of caspases leads to the induction of apoptosis. By contrast, in non-cancer cells, which are typically resistant to Ad-REIC-induced apoptosis, a different ER stress response is observed following treatment. When the REIC/Dkk-3 gene is overexpressed by Ad-REIC in normal human fibroblasts, ER stress signaling with IRE1α, ASK1, p38, STAT1 and interferon regulatory factor 1 (IRF1) activation is observed, however, JNK activation is not detected. Furthermore, Ad-REIC treatment induces the upregulation of IL-7 expression and its significant secretion in human fibroblasts, based on IRF1 activation. These differences in the signaling of ER stress responses following REIC/Dkk-3 overexpression between cancer and normal cells may be involved in the various outcomes of apoptosis and the cancer cell-specific apoptosis induced by Ad-REIC agents.

6. Intratumoral Ad-REIC treatment robustly suppresses cancer growth in mouse tumor models

To demonstrate the therapeutic effects of Ad-REIC *in vivo*, mouse tumor models of several cancer types were established by transplanting the cancer cell line and treating the tumor-bearing mice with Ad-REIC (13,18-20,33,42,43). The tumor types of the model included prostate (13,33,42), breast (20), testicular (18) and gastric (43) cancer and malignant mesothelioma (19). Ad-REIC was administered intratumorally (prostate, breast and testicular cancer), intraperitoneally (gastric cancer) or intrapleurally (malignant mesothelioma) to inhibit the growth of cancer cells. In these *in vivo* experiments, significant inhibition of tumor growth was observed in the Ad-REIC-treated group, however, the tumors progressively grew in the control Ad-LacZ-treated groups. In specific experiments, the tumors that developed following Ad-REIC

treatment were resected and examined using TUNEL staining to evaluate the induction of apoptosis by Ad-REIC. Significant numbers of TUNEL-positive cells were observed in broad areas of the Ad-REIC-treated tumors, however, few apoptotic cells were noted in the tumors of the control groups. In an orthotopic prostate tumor model established with a murine prostate cancer RM9 cell line, the progression of orthotopic tumor development and spontaneous metastasis to the retroperitoneal lymph nodes were robustly suppressed by intratumoral Ad-REIC administration (42). In addition, adenoviral vectors encoding the murine REIC/Dkk-3 gene similarly suppressed the progression of RM9 prostate cancer in the mouse model as well as the Ad-REIC encoding the human REIC/Dkk-3 gene. A series of *in vivo* experiments definitively indicated that the REIC/Dkk-3 gene is a promising molecule for cancer gene therapy and that the therapeutic utility of Ad-REIC agents may be applied in a broad range of human cancer types.

7. Adenovirus-mediated REIC/Dkk-3 gene therapy induces autologous cancer vaccination

Adenovirus-mediated REIC/Dkk-3 overexpression has been demonstrated to induce significant apoptosis in treated tumor sites and robust antitumor effects in mouse models (13,18-20,33,42,43). The induction of cancer cell-specific apoptosis by intratumoral Ad-REIC injection is an important therapeutic mechanism. When orthotopic RM9 prostate tumors are injected with Ad-REIC, significant apoptotic induction and tumor growth inhibition are observed in the treated lesions (33,42). Furthermore, treatment of orthotopic prostate tumors with Ad-REIC suppresses the tumor growth of distant lung metastasis in a mouse model (33). Previous *in vitro* cytolytic assays have demonstrated that anticancer immunity against RM9 prostate cancer cells is significantly enhanced in Ad-REIC-treated mice (33). These results indicate that intratumoral Ad-REIC injection in one tumor lesion also suppresses the growth of other distant cancer lesions via the induction of anticancer immunity. Primarily, intratumoral Ad-REIC treatment induces local apoptotic cell death in treated cancer sites and then activates systemic anticancer immunity against cancer cells. In addition, at the Ad-REIC-treated tumor site, secreted REIC/Dkk-3 protein plays a cytokine-like role in inducing monocyte differentiation to a specific DC phenotype and appear to be involved in systemic anticancer immunity (33).

As shown in Fig. 1, there are two mechanisms underlying the anticancer immune activation induced by Ad-REIC gene therapy. The first mechanism is based on autologous cancer vaccination, which is specifically observed in Ad-REIC gene therapy. Cancer vaccination with Ad-REIC starts with the cancer-specific apoptosis and subsequent release of TAAs. Since the REIC/Dkk-3 protein differentiates $CD14^+$ monocytes into the DC phenotype (33), the REIC/Dkk-3 protein overexpressed and secreted by Ad-REIC transfection at the tumor site differentiates and activates DCs. At the same time, abundant TAA fragments are released as a result of cancer cell-selective apoptosis and supplied to the DCs induced by the secreted REIC/Dkk-3 protein. The activation of DCs directly enhances cancer cell antigen presentation to cytotoxic and helper T lymphocytes, which upregulate systemic antitumor immunity (1,2). Therefore, intratumoral Ad-REIC treatment activates the DCs via the actions of secreted REIC/Dkk-3 proteins and TAAs released at the Ad-REIC-injected tumor sites. Ad-REIC-based medicine is predicted to enhance systemic anticancer immunity and achieve antitumor effects in injected and distant lesions as a therapeutic cancer vaccine.

The second mechanism is based on the secretion of IL-7 observed in the normal stromal fibroblasts of the Ad-REIC-injected tumor sites (40). When the REIC/Dkk-3 gene is overexpressed by Ad-REIC in the fibroblasts of the treated tumor, significant levels of IL-7 are expressed and secreted in the cells. As shown in Fig. 1, this phenomenon is triggered by ER stress signaling via the actions of IRE1α and the activation of ASK1 and the p38 kinase system (40). The enhanced IL-7 expression and secretion observed following Ad-REIC treatment activate natural killer cells to play a role in the upregulation of systemic anticancer immunity (40,44). The Ad-REIC-induced synergistic secretion of REIC/Dkk-3 proteins and IL-7 cytokines at the treated tumor site is important for autologous cancer vaccination by the agent. Namely, these immunoactive proteins work together to mediate the phase of cancer-specific apoptosis to the anticancer immune effects observed at the injected and distant tumor sites. For these reasons, Ad-REIC-mediated medicine is predicted to provide autologous cancer vaccination therapy to be applied as an individualized tailor-made vaccine.

8. Future directions of Ad-REIC-mediated cancer vaccination therapy

The field of therapeutic cancer vaccination is currently undergoing a shift in focus, to individualize tailor-made vaccines and the targeting of multiple TAAs. Autologous tumor vaccines must be applicable vaccine formulations, as tumor cells are a clear source of TAAs for vaccination purposes and all relevant candidate TAAs must be contained within them (3). Ad-REIC-mediated cancer vaccination is based on the strategy of developing autologous cancer vaccines to achieve the individualized activation of antitumor immunity. The ultimate goal of Ad-REIC-mediated cancer vaccination is to cure not only the treated primary tumor, but also distant tumor lesions. Strategies of autologous cancer vaccination using intratumoral Ad-REIC treatment must also be available in the clinical setting.

Based on previous preclinical results and concepts, a phase I-IIa study of gene therapy using an Ad-REIC agent was initiated in January 2011 and is currently ongoing at Okayama University Hospital (Okayama, Japan). The aim of this clinical study is to verify the safety, efficacy and anticancer immunological effects of Ad-REIC-based gene therapy in prostate cancer patients. In particular, evidence of the concept of autologous cancer vaccination via Ad-REIC is being tested in clinical trials to clinically develop the agent for large-scale application. The safety and efficacy of Ad-REIC-mediated gene therapy have been verified in patients and the initial impression of the clinical trial has been good. We hypothesize that Ad-REIC-based medicine is likely to provide anticancer immunological effects via the application of autologous cancer vaccination, which is likely to become a promising therapeutic option for treating a wide range of human malignancies as a cancer vaccine.

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新しい治療展開

去勢抵抗性前立腺癌における免疫療法

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KEY WORDS
●前立腺癌
●免疫療法
●ペプチド
●がんワクチン

Immunotherapy for castration-resistant prostate cancer.
Masanori Noguchi (教授)

はじめに

近年，前立腺癌は，罹患率・死亡率ともに上昇している。現在，前立腺癌の標準治療法には，手術療法，放射線療法，ホルモン療法の３種類がある。これらの治療で治療抵抗性となった場合，最終的に去勢抵抗性前立腺癌（ホルモン不応性再燃前立腺癌）となり，多くの施設ではドセタキセルを用いた抗癌剤での治療が行われる。しかし抗癌剤は，根治療法ではなく延命効果を期待した治療法であり，副作用が強く，特に体力の衰えた高齢者は長期の治療継続が困難な症例も少なくない。このようななかで，第４の治療法として注目されるのが，がん免疫療法である。がん免疫療法は2010年頃より新地平がみえてきたといえる。2010年に去勢抵抗性前立腺癌に対して樹状がん関連抗原を発現させた樹状細胞療法（プロベンジ），2011年には悪性黒色腫に対してＴ細胞活性化抑制抗原に対する抗体（イピリムマブ）が米国食品医薬品局（food and drug administration；FDA）の承認を得た。2012年にはがん局所でのＴ細胞活性化抑制抗原に対する抗PD-1および抗PD-L1抗体が肺癌などに有効であろうという結果が得られた。

本稿では，去勢抵抗性前立腺癌に対するがん免疫療法の開発経緯と今後の展望を，特にわれわれが開発に取り組んでいるテーラーメイドがんペプチドワクチン療法について概説する。

Ⅰ．腫瘍細胞ワクチン

自己腫瘍細胞を用いたがんワクチン療法の臨床研究は，種々のがんにおいて以前より行われており，これらの初期臨床試験で効果を認めたもののうちいくつかは，第Ⅲ相臨床試験まで進んだものもある。去勢抵抗性前立腺癌に対して，培養がん細胞株であるLNCaPとPC-3を含む腫瘍細胞ワクチ