Of the 497 patients, only four (1%) patients were lost to follow-up. Survival was updated in February 2002, with a minimum follow-up period of 5 years for univariate and multivariate analyses. Univariate analyses were performed by log-rank testing using the following seven categories: (i) age >60 versus ≤60 years old; (ii) male versus female; (iii) PS 0 versus 1 or 2; (iv) macroscopically scirrhous-type cancer (Japanese classification type 4) versus non-scirrhous type; (v) histologically intestinal type versus diffuse type; (vi) with versus without history of gastrectomy; and (vii) one versus two versus three or more metastatic sites. Multivariate analysis of prognostic factors using a Cox proportional hazard model was carried out with these categorized variables to calculate relative risks and their 95% confidence intervals (CIs).

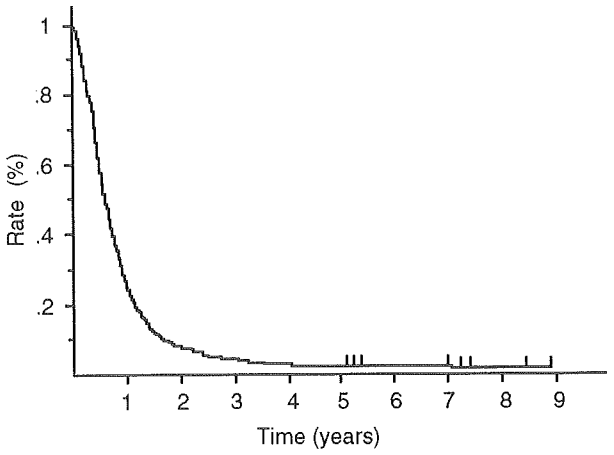

Figure 1. Overall survival of patients.

RESULTS

Patient Characteristics

Characteristics of the 497 patients are summarized in Table 2. Most of the patients had a good PS at registration, while 86 (17%) had a PS of grade 2. Histologically, 228 (46%) patients had an intestinal type of adenocarcinoma, 266 (54%) had a diffuse type and three had an unknown type. One hundred and thirty-seven patients (28%) had macroscopically scirrhous-type primary gastric tumors. Eighty-four (17%) patients had undergone gastrectomy before registration. The sites of metastases documented in the 497 case report forms were: abdominal lymph nodes in 232 (47%); liver in 236 (47%); peritoneum in 86 (17%), and others in 70 (14%) patients. The number of metastatic sites consisted of one in 315 (63%), two in 148 (30%) and three or more in 34 (7%) patients, respectively.

Response and Survival

Of the 497 patients, six (1%) achieved a complete response (CR) and 121 (24%) achieved partial responses, giving an overall response rate of 26%. The response rates in each regimen are listed in Table 1, ranging from 8% in the FTM group to 55% in the EAP group. Figure 1 shows survival curves of all 497 patients, indicating a median survival time (MST) of 7.2 months. The MSTs in each regimen are listed in Table 1, ranging from 6.0 to 9.3 months. Of the 497 patients, 39 (8%) and 11 (2%) have survived longer than 2 and 5 years, respectively. The numbers of 2- and 5-year survivors in each regimen are listed in Table 1.

Characteristics of Long-term Survivors

Twenty-six (67%) of the 39 2-year survivors responded to the initial chemotherapy. These 39 patients included 11 with para-aortic node metastasis alone as an 'unresectable factor'. All of the 39 patients had been classified into PS grades 0 or 1 at registration. Twelve patients had prior gastrectomy before starting chemotherapy. There were no significant differences in histological types between the 2-year survivors and the others.

Table 3. Characteristics of 5-year survivors

Age	G	PS	Macro	H	MS	Surg.	First R	Response 1st/2nd	Surv	Pre
75	M	0	N	D	Liver	–	5-FUci	CR/–	60	D
65	M	0	N	I	A-LN	B	5-FUci	PR/PR	61	A
46	M	0	N	D	A-LN	B	5-FUci	PR/–	63	A
55	M	1	N	I	Liver	–	UFT	PR/CR	65	A
47	M	0	N	I	A-LN	B	FP	CR/–	85	A
52	M	1	N	I	A-LN	–	5′FP	CR/–	86	D
57	M	1	N	D	A-LN	A	EAP	PR/–	87	D
53	M	0	N	D	A-LN	A	EAP	CR/–	88	A
49	F	0	N	D	A-LN	B	FP	NC/CR	90	A
58	M	0	N	I	A-LN, C-LN	A	EAP	CR/–	103	A
62	M	1	N	I	A-LN	A	5′FP	PR/–	108	A

G=gender; M=male; F=female; PS=performance status; Macro=macroscopic type; N=non-scirrhous; H=histology; I=intestinal; D=diffuse; MS=metastatic site; A-LN = abdominal lymph node; C-LN = cervical lymph node; Surg. = surgical resection (A = after chemotherapy; B = before chemotherapy); R = regimen (for definitions see text); CR = complete response; PR = partial response; Surv = survival (months); Pre = present status (A = alive; D = dead).

Characteristics of the 11 5-year survivors are summarized in Table 3. These patients consisted of eight with para-aortic node metastases alone as an 'unresectable factor', one with para-aortic and cervical node metastases, and two patients with only liver metastases. Ten of the 11 patients achieved overall responses to the initial chemotherapy: five patients achieved CR at the initial chemotherapy and one patient achieved CR by the second-line chemotherapy. One patient, who had not achieved an objective response to the initial chemotherapy (FP) achieved CR in the third line chemotherapy, consisting of 5-FU + doxorubicin + mitomycin C. Of the 11, eight patients received surgical resections, four patients before initiating the chemotherapy and four after achieving tumor regression

Table 4. Univariate analysis by each variable

Variable	*n*	MST	2-year survival (%)	5-year survival (%)	*P*-value
Age (years)					
<60	219	7.8	10.5	3.7	
≥60	278	6.8	5.8	1.1	0.04
Gender					
Male	364	7.2	8.2	2.7	
Female	133	7.2	6.8	0.8	0.9
Performance status					
0	175	9.9	11.0	4.0	
1	236	6.8	8.5	1.7	
2	86	5.1	0	0	<0.01
Histological type					
Intestinal	228	7.8	9.2	2.6	
Diffuse	266	6.5	6.8	1.9	0.3
Macroscopic type					
Scirrhous	137	6.0	4.4	0	
Non-scirrhous	360	7.6	9.2	3.1	0.04
History of gastrectomy					
Yes	84	8.3	14.3	4.8	
No	413	6.8	6.5	1.7	0.02
No. of metastatic sites					
1	315	8.3	9.5	3.2	
2	148	5.9	5.4	0.7	
≥3	34	5.4	2.9	0	<0.01

Table 5. Relative risk of prognostic factors

Variable	*n*	RR	95% CI	*P*-value
Age (years)				
<60	219	–		
≥60	278	1.16	0.97–1.40	0.2
Gender				
Male	364	–		
Female	133	0.93	0.75–1.14	0.5
Performance status				
0	174	–		
1	235	1.16	1.08–1.25	<0.01
2	85			
Histological type				
Intestinal	228	–		
Diffuse	266	1.13	0.97–1.30	0.11
Macroscopic type				
Scirrhous	137	–		
Non-scirrhous	360	1.27	1.02–1.25	0.04
History of gastrectomy				
Yes	84	–		
No	413	1.01	0.92–1.10	0.9
No. of metastatic sites				
1	315	–		
2	148	1.32	1.14–1.53	0.01
≥3	34			

Performance status and no. of metastatic sites are ordered categories.

in the initial chemotherapy, including two with a pathological CR in the surgically resected specimen. The remaining three patients did not receive surgical resection during the follow-up period. Ten of the 11 5-year survivors presented with no evidence of disease at 5 years, while two patients died after 5 years because the primary disease recurred.

Univariate and Multivariate Analyses

Results of the univariate and multivariate analyses are summarized in Tables 4 and 5. Univariate analysis revealed significantly better survival in patients in five categories: age <60 years, PS = 0, macroscopically non-scirrhous-type tumors, a prior history of gastrectomy and a small number of metastatic sites. Figure 2 shows the survival curves of the patients with only one metastatic site: 77 with abdominal lymph nodes, 44 with peritoneal tumors and 117 with liver metastases alone. Their MSTs were 9.6, 8.2 and 7.7 months, with 2-year survival rates of 14.3, 15.9 and 6.8%, and with 5-year survival rates of 10.4, 0 and 1.7%, respectively. One hundred and seventeen patients with only liver metastases had the worst MST among the three groups and showed significantly poorer survivals than the remaining patients ($P = 0.04$). Seventy-seven patients with only abdominal lymph node metastases had a remarkably higher 5-year survival rate than other groups, while their MSTs and 2-year survival rates were similar to those of 44 patients with only peritoneal metastases.

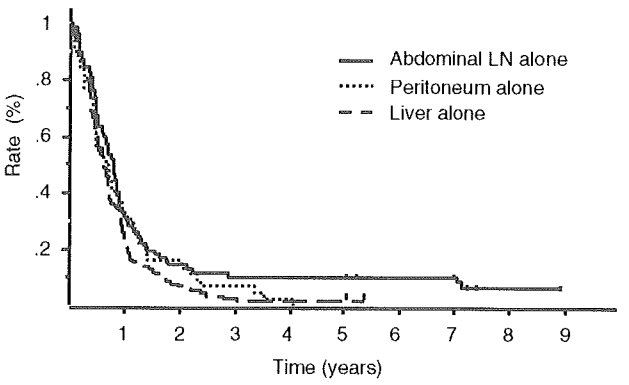

Figure 2. Survival of patients with a single metastatic site: 77 patients had a metastasis to an abdominal node, 117 had a liver metastasis and 44 had a peritoneal metastasis. LN, lymph node.

Multivariate analysis revealed that the presence of only one metastatic site, a macroscopically non-scirrhous-type tumor

and a good PS score were each significantly associated with better prognosis (Table 5).

DISCUSSION

We have already reported the preliminary long-term results of 226 patients with unresectable gastric cancer and treated with systemic chemotherapy, which revealed 2- and 5-year survivals of 10 and 4%, respectively (10). In the present analysis, an additional 271 patients registered in the subsequent phase III trial (9205) were included to confirm the previous results and to carry out multivariate analysis for prognosis. With regard to the long-term results, 2- and 5-year survivals in the additional 271 patients were 6 and 1%, respectively. These survivals were lower than those obtained previously (10), where long-term survivals in cisplatin (CDDP)-containing regimens (8804, 8903 and 9001) were better than non-CDDP-containing regimens (8501). One possible reason for the lower long-term survivals in trial 9205 might be that only one of the three arms included a CDDP-containing regimen (FP). However, this superiority of a CDDP-containing regimen was not observed in the additional 271 patients enrolled into the phase III study (9205): 2- and 5-year survivals in the FP group were 7 and 0%, whereas those in the 5-FUci group were 7 and 3%, respectively. Based on these results, the superiority of CDDP-containing regimens in the phase II series (8804, 8903 and 9001) in terms of long-term survival might have been caused by selection bias: for example, the incidence of patients with a single metastatic site was 77% in phase II and 52% in phase III.

Was the long-term survival of a few patients truly achieved by chemotherapy, or was it simply related to the natural history of these patients? Because there have been no prospective reports using adequate sample sizes on the long-term survival of patients not treated with chemotherapy, it is hard to establish the effectiveness of chemotherapy for long-term survival. However, there have been two randomized trials comparing best supportive care with combination chemotherapy (1,2). Although these studies had only a few patients, no patient treated solely with supportive care survived longer than 1 year. Additionally, most of the long-term survivors in the present analysis achieved good responses to chemotherapy, particularly the 5-year survivors: 10 of the 11 patients were alive with no evidence of disease at 5 years. These results thus support the value of chemotherapy for achieving long-term survival.

Because the case report forms in the earlier study frequently lacked laboratory reports of serum data including tumor markers, these data were excluded from this multivariate analysis. Univariate analysis revealed that there were significant differences in survival in terms of PS grade, numbers of metastatic sites, having a history of gastrectomy, age and macroscopic tumor type. However, multivariate analysis showed there were only three variables significantly and independently associated with a good prognosis: having a better PS grade, having fewer metastatic sites and the presence of macroscopically non-scirrhous-type tumors. Better PS grade and fewer metastatic sites are also known to be better prognostic factors in patients with advanced colorectal cancer treated with chemotherapy (13). In addition, patients with macroscopically scirrhous-type tumors showed significantly poorer survival than those with non-scirrhous types, and this seems to be specific for patients with gastric cancers. Scirrhous tumors are also known to lead to poorer survival than other macroscopic types in patients treated by surgical resection (14). Thus, these forms of tumors appear to be especially malignant and exhibit a higher resistance to chemotherapeutic agents.

Another objective of this study was to clarify the characteristics of the long-term survivors. The 11 5-year survivors had some specific characteristics. All patients had good PS grades of 0 or 1 and macroscopically non-scirrhous-type tumors. Ten had only one metastatic site, achieved a CR through the initial chemotherapy and had no evidence of disease at 5 years. Another significant characteristic was that eight of the patients had only a para-aortic node metastasis as an unresectable factor. In the whole study series, 77 such patients had significantly better 5-year survival (10.4%) than the other patients with single metastatic sites, such as in the liver or peritoneum. Thus patients with para-aortic node metastases alone have a greater chance of achieving long-term survival than other patients; this suggests that potentially curative strategies such as adjuvant surgery may be effective for them. A phase II study of this strategy for this subpopulation (neoadjuvant chemotherapy followed by surgery) by the JCOG is now underway.

The role of surgery in patients with potentially incurable disease remains controversial. Although patients with prior surgery showed better survival than others in the univariate analysis, this was not found in the multivariate analysis. This might have been caused by 'leading bias'—early detection of recurrence—because of periodic follow-up surveys after surgery. It is also difficult to evaluate the role of adjuvant surgery after achieving downstaging by chemotherapy because of the small number of such cases. However, of the 11 5-year survivors, eight received surgical resections for primary sites, including four patients with adjuvant surgery. Thus adjuvant surgery might have value, particularly for patients with para-aortic node metastasis alone, if they achieve downstaging by chemotherapy. Of course, these advantages should be evaluated further in the ongoing neoadjuvant study.

In conclusion, there were a few long-term survivors in patients with unresectable gastric cancer treated with chemotherapy. This suggests that some patients with only abdominal lymph node metastases may achieve long-term survival with successful chemotherapy. Better PS scores, small numbers of metastatic sites and macroscopically non-scirrhous-type tumors were independent favorable factors for survival in the multivariate analysis.

Acknowledgments

Presented in part at the 37th Annual Meeting of the American Society of Clinical Oncology, San Francisco, May 2001.

This work was supported by Grants-in-aid (5S-1, 8S-1, 11S-3, 11S-4) from the Ministry of Health, Labor, and Welfare, Japan.

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Frequent Immune Responses to a Cancer/Testis Antigen, CAGE, in Patients with Microsatellite Instability – Positive Endometrial Cancer

Takashi Iwata,[1,2] Tomonobu Fujita,[1] Nobumaru Hirao,[1,2] Yuriko Matsuzaki,[1] Tsutomu Okada,[1] Hiroshi Mochimaru,[1] Nobuyuki Susumu,[2] Eri Matsumoto,[4] Kokichi Sugano,[4] Naohide Yamashita,[3] Shiro Nozawa,[2] and Yutaka Kawakami[1]

Abstract **Purpose:** Identification of cancer/testis antigens useful for diagnosis or immunotherapy of cancers was attempted by cDNA expression cloning with patients' sera (SEREX).

Experimental Design: cDNA expression libraries made from testis or endometrial cancer cell lines were screened using sera from patients with endometrial cancer or melanoma patients immunized with dendritic cells pulsed with autologous tumor lysates. Tissue-specific expression by RT-PCR and immunogenicity by Western blotting of the bacterial recombinant antigen with sera from cancer patients were evaluated.

Results: A cancer/testis antigen, CAGE, was isolated by two independently performed SEREX. CAGE was expressed in various cancer cell lines including endometrial cancer, colon cancer, and melanoma in 7 of 10 endometrial cancer tissues and in 1 of 3 atypical endometrial hyperplasia, but not in normal tissues including the endometrium and testis. The protein expression on cancer cells was confirmed by Western blot analysis with the recombinant CAGE protein, anti-CAGE IgG antibody was detected in sera from 5 of 45 endometrial cancer, 2 of 24 melanoma, and 2 of 33 colon cancer patients, but not in sera from healthy individuals. By ELISA analysis, anti-CAGE antibody was detected in 12 of 45 endometrial cancer, 2 of 20 melanoma, and 4 of 33 colon cancer patients. Intriguingly, anti-CAGE antibody was highly positive in 7 of the 13 (53.8%) microsatellite instability (MSI)-H patients with endometrial cancer, but negative in 20 non – MSI-H patients ($P = 0.001$).

Conclusion: CAGE may be useful for immunotherapy and diagnosis of various cancers particularly MSI-positive endometrial cancer.

Identification of human tumor antigens is important not only for the analysis of antitumor immune responses and development of immunotherapy, but also for development of diagnostic methods (1). Various methods for the identification of tumor antigens have recently been applied, including cDNA expression cloning with tumor-reactive T cells and patients' serum IgG antibodies, as well as a reverse immunology strategy, which evaluates induction of T cells against candidate molecules identified by various techniques such as systematic gene expression analysis and cDNA subtraction.

Among the representative tumor antigens recognized by T cells, cancer/testis antigens, which are expressed in various cancers and in some normal tissues including testis and placenta, are good candidates as tumor-specific common antigens for use in the immunotherapy. Cancer/testis antigens have previously been isolated by various methods. MAGE1 was first isolated by cDNA expression cloning with melanoma-reactive T cells (2), and NY-ESO-1 was isolated by cDNA expression cloning (SEREX) with serum from a patient with esophageal cancer (3). CT15, 16, and 17 were isolated DNA homology search using public gene databases (4), and MAGEC-1 was isolated by cDNA subtraction (RDA) between testis cDNA library and normal tissues (5).

A cancer/testis antigen, CAGE, was originally isolated by SEREX with serum from a patient with gastric cancer (6). Although its expression in some tumors was reported by RT-PCR analysis, expression of the CAGE protein in tumor cells and the presence of serum IgG antibodies in various cancer patients has not yet been evaluated. Thus, further analysis of

Authors' Affiliations: [1]Division of Cellular Signaling, Institute for Advanced Medical Research, [2]Department of Obstetrics and Gynecology, School of Medicine, Keio University, [3]Department of Advanced Medical Science, Institute of Medical Science, University of Tokyo, Tokyo, and [4]Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, Tochigi, Japan

Received 8/23/04; revised 1/15/05; accepted 1/26/05.

Grant support: Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (12217132, 12557109, 12671630, 14104013, 16591686), Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Grant-in-Aid from the Ministry of Health and Welfare, Japan, for Second-term Comprehensive 10-year Strategy for Cancer Control, the Promotion and Mutual Aid Cooperation for Private Schools for Japan, Mitsui Life Social Welfare Foundation, Keio University Special Grant-in-Aid for Innovative Collaborative Research Projects, Keio Gijuku Academic Development Funds, and Keio University Grant-in-Aid for Encouragement of Young Medical Scientists.

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Requests for reprints: Yutaka Kawakami, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjukuku, Tokyo 160-8582, Japan. Phone: 81-3-5363-3778; Fax: 81-3-5362-9259; E-mail: yutakawa@sc.itc.keio.ac.jp.

the CAGE protein and its immunogenicity in various cancers remains to be investigated.

In this study, we isolated CAGE by screening a testis cDNA library with sera from melanoma patients who were frequently immunized with dendritic cells pulsed with autologous tumor lysates (7), and by screening an endometrial cancer cDNA library with sera from patients with endometrial cancer. Through evaluation of the tissue-specific expression and immunogenicity by screening serum IgG antibodies specific for the recombinant CAGE protein, we revealed that CAGE was expressed frequently in various cancers including endometrial cancer and melanoma, and serum IgG antibody was frequently detected in sera from patients with microsatellite instability (MSI)-positive endometrial cancers, indicating the possible use of CAGE for immunotherapy of endometrial cancer and melanoma as well as for diagnosis of MSI-positive cancers.

Materials and Methods

Patients. In this study, sera of seven endometrial cancer patients, one esophageal cancer patient, and three melanoma patients were used for SEREX method. Of the seven patients with endometrial cancer, one was classified as stage II, three were classified as stage III, and three were classified as stage IV. One esophageal cancer patient was stage III, and all of three melanoma patients were stage IV. These three melanoma patients were frequently (8-10 times) immunized with dendritic cells pulsed with autologous tumor lysates, but no effective regression of disease was seen, and all died within 6 months (7). In addition to these sera, all sera of other cancer patients and healthy individuals, cancer tissues, and normal endometrial tissues used in this study were obtained with informed consent and written agreement.

Cell lines and tissues. The human endometrial cancer cell line SNG-II (8), ovarian clear cell adenocarcinoma cell lines, RMG-I (9) and RMG-II (10), were established by our group. The human endometrial cancer cell line Hec-Ib (11) was kindly provided by Dr. Kuramoto (Kitasato University, Kanagawa, Japan), and the Ishikawa line (12) was kindly provided by Dr. Nishida (Kasumigaura National Hospital, Ibaraki, Japan). Ishikawa, Hec-Ib SNG-II, RMG-I, and RMG-II cell lines were cultured in F12 (Sigma Aldrich Co., St. Louis, MO) supplemented with 10% FCS and 100 μg/mL kanamycin. The melanoma cell lines SKmel23, 888mel, A375mel, Groves mel, 501 mel, 586 mel, 526 mel, and 501Amel; the lung cancer cell lines LU99, EBC1, and RERF-LC-MA; the renal cell cancer cell lines Saito, RCC6, RCC7, and RCC8; the bladder cancer cell line KU7; the prostate cancer cell line PC3; the breast cancer cell line MDA231; leukemia cell lines HL60, K562, and Molt 4 were cultured in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The esophageal cancer cell lines, TE8 and TE10, were cultured in DMEM (Sigma) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The pancreatic cancer cell line PK59 was cultured in a complete medium consisting of RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 6 μg/L epidermal growth factor, 150 units/L insulin, 0.5 mg/L hydrocortisone, 10 mg/L transferrin, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Melanocyte was cultured in serum-free MM-4 medium (Morinaga, Yokohama, Japan). Primary cultured fibroblasts were cultured in DMEM (Sigma) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in our laboratory. Normal tissues used in RT-PCR were obtained from Clontech (Palo Alto, CA). Normal endometrium, endometrial cancer tissues, and atypical endometrial hyperplasia tissues were obtained from surgical operation with informed consent and stored at −80°C until use.

Construction of cDNA libraries. We used two kinds of cDNA library in this study, testis cDNA library and endometrial cancer cDNA library. Total RNA of testis was obtained from Clontech and that of endometrial cancer was isolated from the endometrial cancer cell lines Ishikawa, Hec-Ib, and SNG-II by guanidine isothiocyanate and CsCl gradient ultracentrifugation. These endometrial cancer cell lines, SNG-II, Hec-Ib, and Ishikawa, are frequently used for research work, Ishikawa is established from well-differentiated endometrioid adenocarcinoma and expresses both estrogen receptor and progesterone receptor (11, 12), SNG-II is established from well-differentiated endometrioid adenocarcinoma and expresses CA125 antigen, Hec-Ib is established from moderately differentiated endometrioid adenocarcinoma and expresses progesterone receptor (11). We mixed these three kinds of total RNAs of endometrial cancer cell lines for constructing the library. We purified poly(A)+RNA with latex beads, synthesized cDNA by RT-PCR, and inserted cDNA into the bacteriophage expression vector -Zap express (Stratagene, La Jolla, CA) as described (13). Testis cDNA library and endometrial cancer cDNA library consisted of 2.5×10^6 and 1.2×10^6 primary recombinants.

Immunoscreening of the cDNA library with sera. The SEREX method was carried out as described previously (14). The testis cDNA library was screened with mixed sera of one esophageal cancer patient and three melanoma patients who received immunization with dendritic cells pulsed with autologous tumor lysates. The endometrial cancer cDNA library was screened using sera of seven endometrial cancer patients. The positive clones were picked up and PCR was conducted by Ex Taq kit (Takara, Kyoto, Japan), and then the PCR products were sequenced on ABI Prism 3100 sequencer (Perkin-Elmer, Branchburg, NJ).

Expression of CAGE gene in tumor cell lines or tissues. Total RNA was isolated from cell lines by guanidine isothiocyanate and CsCl gradient ultracentrifugation, and total RNA from normal tissues was purchased from Clontech. Total RNA from endometrial cancer tissues and normal endometrial tissues was obtained by TRIzol method (Invitrogen, Carlsbad, CA) for a higher yield and treated with DNase I (Takara) to avoid DNA contamination. Reverse transcription was done using Super Script II reverse transcriptase (Invitrogen) and gene-specific PCR was done with Ex-Taq DNA polymerase (Takara). The primers for CAGE were 5′-CTTCCAACCGTATGTAGGCGAG (forward), 5′-CTCCTTGCGTCTTTGTCCAGGT (reverse), and were used in RT-PCR consisting of initial denaturation at 94°C for 2 minutes and 35 amplification cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 1.5 minutes at 72°C, followed by 5 minutes at 72°C. The primers for glyceraldehyde-3-phosphate dehydrogenase were 5′-TGAACGGGAAGCTCACTGG (forward), 5′-TCCACCACCCTGTTGCTGTA (reverse), and were used in RT-PCR consisting of initial denaturation at 94°C for 2 minutes and 25 amplification cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, followed by 5 minutes at 72°C.

Preparation of recombinant his-tagged CAGE and production of anti-CAGE polyclonal antibodies. His-tagged CAGE were generated. Due to the fact that the open reading frames of genes of DEAD box family are highly homologous, if the full length of the CAGE protein is used for antibody generation, it will produce nonspecific antibodies. Therefore, we selected a part that is specific for CAGE as the target sequence for pET16a partial construct, from codons 1,261 to 1,873 in the open reading frame. The PCR products contained sites of the restriction enzymes *Bam*HI (5′) and *Sal*I (3′). The primers for partial protein were 5′-taaaaggatccTATTTGAAAGATCCTATGAT (forward), 3′-taaaaagtcgacTCAACTTAAAAAATAAAACT (reverse). The PCR product was digested with *Bam*HI and *Sal*I, cloned into the pET16a (Novagen, Darmstadt, Germany) which was modified to contain multiple cloning sites, and then expressed in *E. coli*, AD494(DE3)pLys S (Novagen). The recombinant CAGE proteins were purified using the affinity resin HiTrap Chelating (Amersham Biosciences Corp., Pitscataway, NJ). There were 235 amino acids in the recombinant His-tagged CAGE protein and the predictive molecular weight was 31.1 kDa. The rabbit anti-CAGE polyclonal antibody of this recombinant CAGE protein was made by the Protein Purification Company (Tochigi, Japan).

pcDNA3.1 construction and transfection. pcDNA vector (Invitrogen) was used for construction of CAGE. PCR was conducted by using the following primers to generate the full length of the open reading frame of the CAGE gene with the sites of *Bam*HI on the 5′ end and *Not*I on the 3′ end; 5′-taaaaaggatccATGTCCCACTGGGCCCCAGAG (forward), 3′-taaaaagcggccgcTCAACTTAAAAAATAAAACT (reverse). Then the PCR product was digested by *Bam*HI and *Not*I cloned into the pcDNA3.1 vector. The pcDNA3.1-CAGE was transfected by LipofectAMINE method (Invitrogen) into fibroblast cells. After 48 hours incubation at 37°C, the transfected cells were collected and lysed by SDS sample buffer.

Protein expression of CAGE in tumor cell lines and normal cell line. The expression of the CAGE protein was evaluated by Western blotting, generating rabbit anti-CAGE antibodies. The human endometrial cell lines Ishikawa and Hec-Ib, and the normal melanocyte cell line were diluted into 1.0×10^4 cells/μL in SDS sample buffer. Loading 5.0×10^4 cells per lane, electrophoresed on SDS-PAGE (10% gel) and then transferred to nitrocellulose membrane (Hybond Extra C; Amersham Biosciences Corp.). After blocking, the membrane was incubated overnight at 4°C with 1:1,000 diluted anti-CAGE antibodies in 5% skim milk solution, the membrane was washed in TBST, incubated for 2 hours with 1:4,000 diluted goat anti-rabbit Fc antibody conjugated with alkaline phosphatase (Cappel, Aurora, OH), and then washed in TBST. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used for the enzymatic detection.

Immunocytochemistry. The PCR product of full-length CAGE was digested with *Sal*I and *Bam*HI and subcloned into pFLAG-CMV-2 vector (Sigma-Aldrich Co.). COS7 cells were cultured in eight-well glass slides (Becton Dickinson, Bedford, MA) and transfected by LipofectAMINE (Invitrogen). After 24 hours, the expression of the fusion protein was determined immunocytochemically with anti-Flag M5 murine antibody (Sigma-Aldrich Co.) and Alexa 568 conjugated anti-mouse IgG antibodies. The stained cells were visualized with Carl Zeiss LSM5 Pascal confocal microscope (63×).

Immunoscreening of CAGE by Western blotting. One microgram of recombinant CAGE protein was loaded per gel, and electrophoresed on 10% SDS-PAGE gel. After being transferred to the membrane, it was cut into 10 strips and the strips were incubated overnight at 4°C with 1:100 diluted serum samples or 1:4,000 diluted monoclonal anti-His antibody (Amersham Biosciences Corp.). The strips were washed in TBST, incubated for 2 hours with 1:4,000 diluted goat anti-human Fc antibody conjugated with alkaline phosphatase (Cappel) or goat anti-mouse Fc antibody conjugated with alkaline phosphatase (Cappel).

ELISA for detection of anti-CAGE antibodies. The recombinant CAGE protein was diluted in PBS buffer to a final concentration of 3 μg /mL. The CAGE solution of 3 μg /mL were dispensed into 96-well plates (100 μL/well) and incubated overnight at 4°C. Serum samples (100 μL, 1:100 dilution) were added per well and incubated for 2 hours at room temperature. After incubation with 100 μL of 1:5,000 dilution of goat anti-human IgG Fc labeled with horseradish peroxidase (Cappel), the plates were washed with PBST, and developed by tetramethylbenzidine solution for 20 minutes. After stopping the reaction by adding H_2SO_4, the absorbance was measured at 450 nm. All serum samples were run in duplicate and randomly dispensed on the plates. Sera from cancer patients and sera from healthy controls were tested simultaneously. Statistical analysis was conducted by using χ^2 test.

Microsatellite instability detection. MSI detection was done as described previously (15). Genomic DNA was extracted from tumor tissues and peripheral lymphocytes. Fragments of microsatellite repeat loci *D2S123*, *D5S346*, *D17S250*, *BAT26*, *BAT25*, *MSH3*, *MSH6*, *TGFβRII*, *BAX*, *MBD4A10*, and *MBD4A6* were amplified PCR, using the above DNA as the template with that from peripheral lymphocytes serving as the normal control. If ≥30% of the above markers showed MSI, the tumor was defined as MSI-H, based on the standard proposed at the Workshop of the National Cancer Institute Workshop (16). If the MSI rate was <30%, the tumor was defined as MSI-L, and if MSI rate was zero, the tumor was defined as MSS.

Detection of serum CA602 antigen. Serum CA602 (17) levels were measured by enzyme immunoassay at Mitsubishi BML Co. (Tokyo, Japan). Serum CA602 cutoff was set at 63 units/mL based on the values measured in normal individuals (10).

Results

Isolation of cancer/testis antigens by SEREX. To isolate cancer/testis antigens, we have screened a total of 1.2×10^6 clones of a testis cDNA library with the mixture of sera from three melanoma patients who were frequently immunized with dendritic cells pulsed with autologous tumor lysates, and an esophageal cancer serum which was known to contain the MAGE cancer/testis antigens (as a positive control for isolation of cancer/testis antigens). A total of 87 positive clones, representing 26 distinct genes including cancer/testis antigens such as MAGE1a, MAGE2b, MAGE4a, MAGE4b, MAGE6, MAGE9a, and NY-ESO-1, were isolated (Supplemental Table 1). We have also screened a cDNA library made from a mixture of mRNA of three endometrial cancer cell lines with sera from seven patients with endometrial cancer, because endometrial cancer antigens have not yet been isolated by SEREX. A total of 5.0×10^6 cDNA clones were screened, and 193 positive clones representing 59 distinct genes were isolated (Supplemental Table 2). By evaluating tissue-specific expression using gene databases including serial analysis of gene expression databases and expressed sequence tag databases, and RT-PCR analyses, one of the isolated clones which showed cancer/testis antigen–like expression, and identified from both screenings of testis and endometrial cancer libraries, was found to be a cancer/testis antigen CAGE, which was originally identified by Cho et al. (6) using SEREX with sera from gastric cancer patients. They reported that by RT-PCR analysis, CAGE was frequently expressed in gastric cancer, cervical cancer, lung cancer, and liver cancer, but the serum recognition was only tested with one gastric cancer patient's serum, which was used for screening the cDNA library. The expression of CAGE in other cancers including endometrial cancers and melanoma used in this study, and its immunogenicity in patients with various cancers, remains to be investigated. Therefore, we attempted further analysis of the CAGE expression and its immunogenicity in various cancers.

Expression of the CAGE mRNA and protein in various cancers including melanoma and endometrial cancers. By RT-PCR analysis, CAGE was expressed only in testis among normal tissues and also in various cancer cell lines including lung and renal cell cancer as previously reported (Fig. 1*A* and *B*). Although it was previously reported that any of the melanoma and breast cancer cell lines tested did not express CAGE, we observed that four of seven melanoma, and one breast cancer cell line expressed CAGE. In addition, two of three endometrial cancer cell lines, one of three chronic myelogenous leukemia cell lines, and one pancreatic cancer line were found to express CAGE (Fig. 1*B*). We further revealed that 7 of 10 endometrial cancer tissues (four in grade 1, four in grade 2, and two in grade 3) and one of three atypical endometrial hyperplasia tissues expressed CAGE, whereas none of the eight normal endometria (four in the proliferation phase and four in the secretory phase) expressed CAGE (Fig. 2). The expression of CAGE did not have any correlation with the differentiation grade in endometrial cancer.

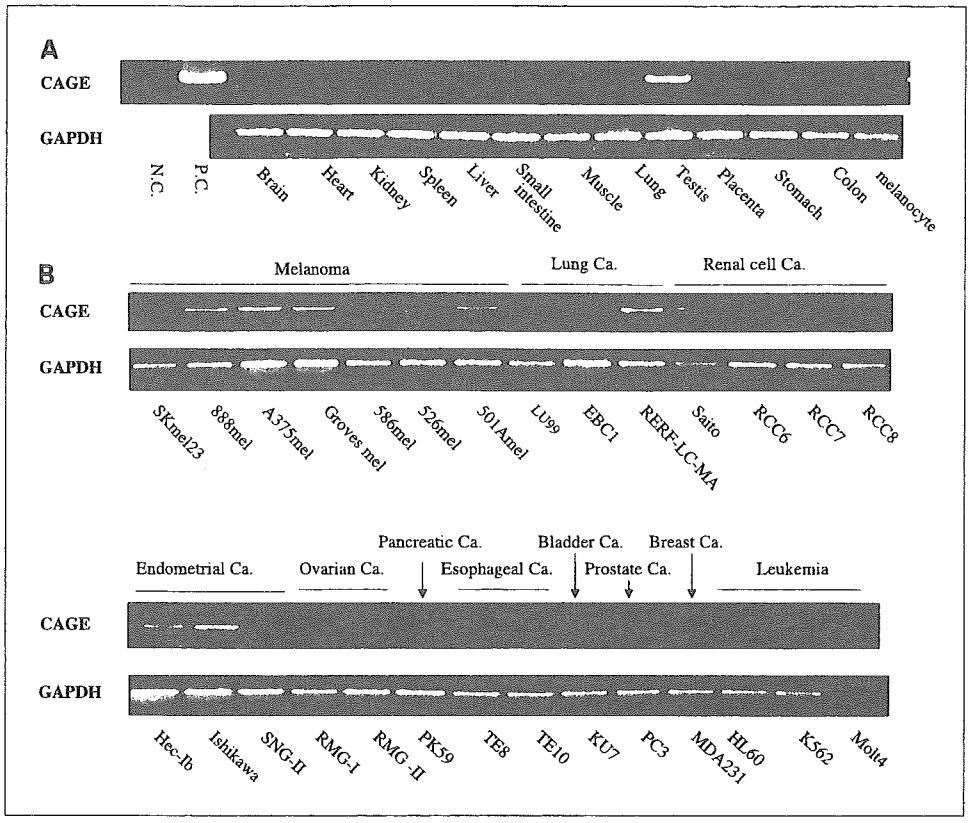

Fig. 1. Expression of CAGE in various cancers and normal testis evaluated by RT-PCR analysis. *A*, CAGE was expressed only in testis among normal tissues. *B*, CAGE was expressed in four of seven melanoma, one of three lung cancer, two of four renal cell cancer, two of three endometrial cancers, one pancreatic cancer, one bladder cancer, one breast cancer, and one of three chronic myelogenous leukemia cell lines.

Because expression of the CAGE protein in tumor cells has not been previously evaluated, we examined the CAGE protein in various cancer cell lines, including endometrial cancer and melanoma by Western blot analysis with the anti-CAGE rabbit antibody which was produced by immunization with the bacterial recombinant CAGE protein as described in Materials and Methods. This polyclonal antibody detected the predicted 82.5 kDa band in lysates from fibroblast cells transfected with pcDNA-CAGE, but not in lysates from untransfected fibroblast cells, indicating specific recognition of CAGE (Fig. 3). The same specific bands were shown with this antibody in lysates from two endometrial cancer cell lines, Hec-Ib and Ishikawa, which were CAGE-positive when evaluated by RT-PCR analysis, but were not shown in lysates from PCR-negative cultured melanocytes, demonstrating that the CAGE protein was present in various cancer cell lines. Although we further attempted immunohistochemical study with this antibody to confirm the CAGE protein expression in fresh tumor tissues, reliable results could not be obtained by relatively strong background staining with the rabbit serum. We then evaluated subcellular localization of CAGE. Although CAGE with green fluorescent protein-tag have previously been reported in the nucleus of the transfected cervical cancer cell line C33A (6), the smaller Flag-tagged CAGE was found to be mainly present in the cytoplasm

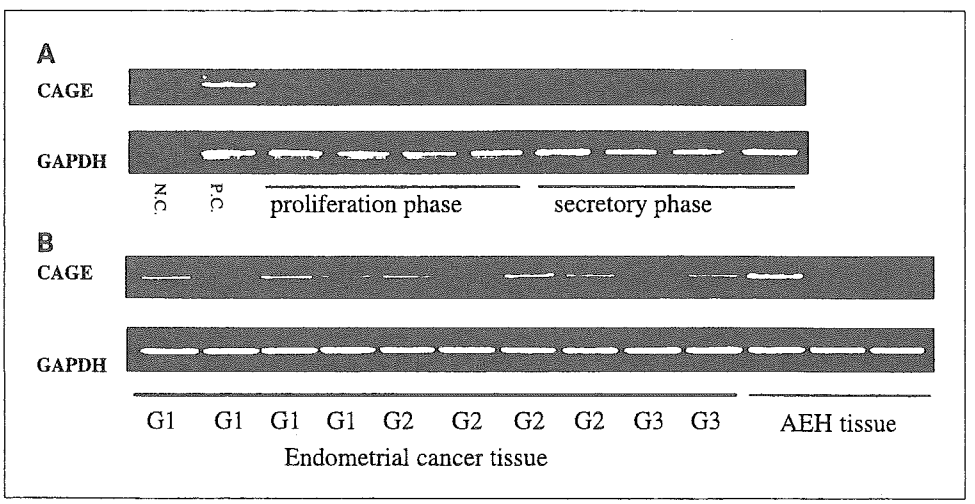

Fig. 2. Expression of CAGE in endometrial cancer tissues, but not in normal endometrial tissues. *A*, CAGE was expressed in none of the eight normal endometrium tissues (four in the proliferation phase and four in the secretory phase). *B*, 7 of 10 endometrial cancer tissues [four in grade 1 (G_1), four in grade 2 (G_2), and two in grade 3 (G_3)] and one of three atypical endometrial hyperplasia tissues expressed CAGE. AEH, atypical endometrial hyperplasia; NC, negative controls; PC, positive controls.

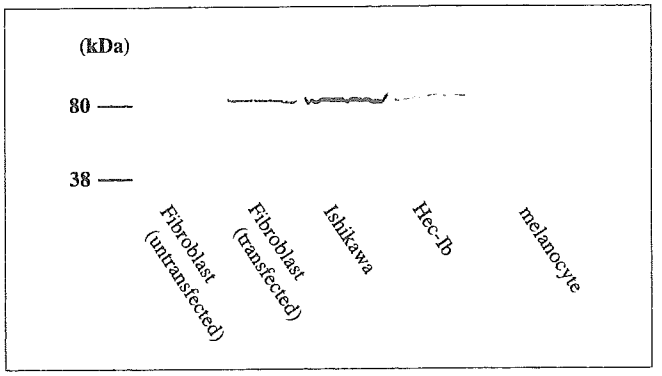

Fig. 3. Expression of the CAGE protein in various cancer cell lines. The specific CAGE band was detected in lysates from two endometrial cancer cell lines, Hec-1b and Ishikawa, which were CAGE-positive in RT-PCR analysis, but was not shown in lysate from PCR-negative cultured melanocytes. Fibroblast cells transfected with pcDNA-CAGE were positive controls and untransfected fibroblast cells were negative controls.

of COS cells transfected with pFLAG-CAGE in our experiment, when evaluated by immunostaining with anti-FLAG M5 monoclonal antibodies (Fig. 4).

Detection of anti-CAGE IgG antibody in sera from patients with various cancers including endometrial cancer and melanoma. Because the recognition of CAGE by serum from cancer patients was previously tested only with a single patient with gastric cancer, we have evaluated the recognition of CAGE by serum IgG antibody in patients with various cancers by Western blot analysis with the His-tagged bacterial recombinant CAGE protein fragment. Representative Western blot results are shown in Fig. 5 and Table 1. Anti-CAGE IgG antibody was detected in sera from 5 of 45 patients with endometrial cancers, 2 of 24 patients with melanomas, and 2 of 33 patients with colon cancers, but was not detected in sera from 20 patients with renal cell cancers, 18 patients with prostate cancers, 12 patients with pancreatic cancer, or in 40 healthy individuals. The positive sera did not stain the negative control proteins, recombinant His-tagged VEGFC, assuring the CAGE-specific recognition of these positive sera (data not shown). CAGE-specific recognition was also confirmed by phage plaque assay with the same sera (data not shown).

We next attempted quantitative analysis of anti-CAGE IgG antibodies in sera from patients with various cancers using ELISA. By setting up the cutoff for positive anti-CAGE antibody at the average absorbance of the healthy individuals plus 2 SD (0.058), positive sera were found in 12 of 45 (26.7%) patients with endometrial cancer, 4 of 33 (12.5%) patients with colon cancer, 2 of 20 (10.0%) patients with melanoma, and 1 of 40 (2.5%) age-matched healthy individuals, but not in 10 patients with ovarian cancer (Fig. 6). Among these positive patients, five endometrial cancers, two colon cancer, and two melanoma patients showed high CAGE antibody titers >0.12 OD, for whom positive bands were clearly detected in the Western blot analysis (Fig. 5).

Frequent detection of anti-CAGE serum IgG antibody in patients with microsatellite instability-positive endometrial cancer. Correlation of the positive serum CAGE antibody with various clinicopathologic features was then evaluated. Although age, Federation Internationale des Gynaecologistes et Obstetristes stage, grade, and positive CA602 tumor marker, did not correlate with the positive antibody, surprisingly, the MSI status was found to correlate with the positive antibody in patients with endometrial cancer (P = 0.001; Table 2). Among 33 endometrial cancer patients whose MSI status were evaluated, 7 of 13 (53.8%) patients with MSI-H had positive serum CAGE antibody, whereas none of 20 non-MSI-H patients, including one MSI-L and 19 MSS patients, had anti-CAGE antibody (Fig. 6). Interestingly, two patients with colon cancer with positive CAGE antibody also turned out to be MSI-positive cancers that developed in patients with hereditary non-polyposis colon cancer.

Because the CAGE protein has a six-thymine repeat in the coding region, we investigated the possibility that altered CAGE protein through slippage mutations by abnormal DNA mismatch repair in MSI-positive tumor cells might lead to induction of IgG, by sequencing the CAGE gene in tumors obtained from five CAGE antibody-positive patients with MSI-H endometrial cancer. However, no mutation was observed in the entire CAGE sequences. We further evaluated correlation between CAGE expression and MSI status in 13 endometrial cancer tissues using real-time PCR analysis, and found that four of five MSI-H and six of eight MSS tumors expressed CAGE, indicating no correlation between CAGE expression and MSI status (Fisher exact test, P = 1.0) in these 13 patients (Supplemental Fig. 1). These results suggest that anti-CAGE antibodies do not simply correlate to CAGE expression between different MSI-H and MSS. Therefore, the mechanism for induction of IgG response to CAGE in MSI-positive patients has not been clear and needs further investigation.

Discussion

In this study, we attempted to isolate cancer/testis antigens by screening a testis cDNA library with sera from melanoma patients who were frequently immunized with dendritic cells

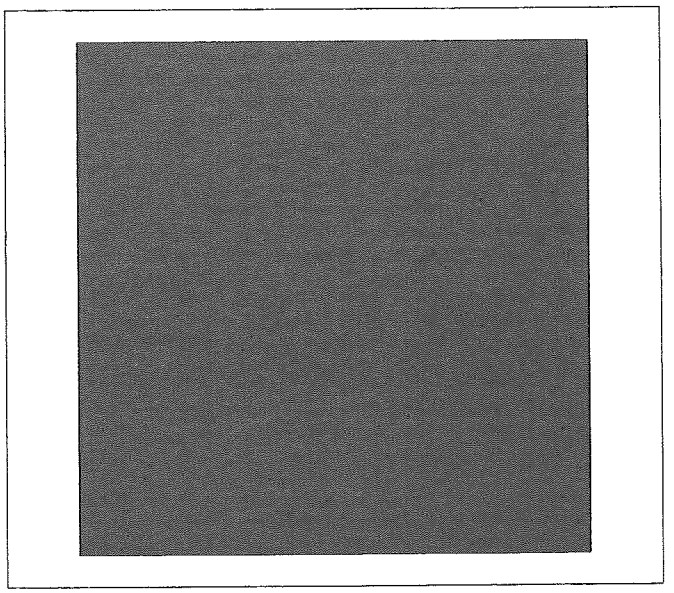

Fig. 4. Presence of FLAG-CAGE in the cytoplasm of COS cells transfected with pFLAG-CAGE. After transfection of COS7 cells with FLAG-tag CAGE, FLAG-CAGE was immunostained with anti-FLAG-mouse monoclonal antibodies (M5) and Alexa 568 conjugated anti-mouse goat IgG, and visualized with confocal microscope (63×).

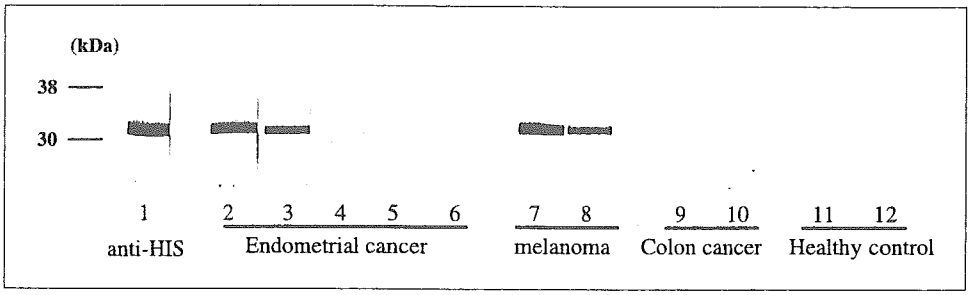

Fig. 5. Presence of anti-CAGE IgG antibodies in sera from various cancer patients detected by Western blot analysis with bacterial recombinant CAGE protein. By Western blot analysis, the recombinant His-tagged CAGE protein fragment containing NH_2-terminal 211 amino acids of CAGE (molecular weight = 31.1 kDa) was recognized by IgG antibodies in sera from some of the patients with various cancers. Lane 1, staining of CAGE with anti-His antibody; lanes 2-12, staining with 1:100 diluted sera; lanes 2-6, sera from endometrial cancer patients; lanes 7 and 8, sera from melanoma patients; lanes 9 and 10, sera from colon cancer patients; lanes 11 and 12, sera from healthy controls. Only positive cancer samples are shown in this representative experiment. No band was shown in the lanes with sera from two healthy individuals. One microgram of recombinant CAGE protein was loaded per lane.

pulsed with autologous tumor lysates, because mRNA for cancer/testis antigens are often expressed at higher levels in testis and cancer cells, and sera from patients immunized with autologous tumor constituents may contain higher titer of antibody specific for immunogenic tumor antigens. We also attempted to isolate endometrial cancer antigens by screening a cDNA library made from endometrial cancer cell lines with sera from patients with endometrial cancer, because SEREX has not been previously applied for endometrial cancer. Endometrial cancer is the most common invasive neoplasia of the female genital tract and the fourth most frequently diagnosed cancer in the U.S. Worldwide, approximately 150,000 cases are diagnosed each year, making endometrial cancer the fifth most common cancer in women (18). Because radiation and chemotherapy are not so effective, development of an alternative therapeutic strategy, such as immunotherapy, is required for patients with advanced endometrial cancer.

From these independently done SEREX studies, a cancer/testis antigen CAGE was isolated. CAGE was originally isolated by Cho et al. (6) using SEREX with sera from a gastric cancer patient. CAGE mapped to X chromosome p22.13 was previously shown to be expressed in normal testis and various cancers. Although the function of CAGE has not yet been defined, CAGE has helicase domains and DEAD box, and seems to be one of the DEAD box families with a conserved Asp-Glu-Ala-Asp (DEAD) motif, which have RNA-dependent ATPase activity and RNA helicase activity. The DEAD box family proteins are reported to play important roles in a wide range of cellular regulations including RNA metabolism, embryogenesis, spermatogenesis, and cellular growth (19-21). Some DEAD box family proteins, including rck/p54 (22), DDX1 (23), and HAGE are overexpressed in various cancer cell lines, and the expression of DDX1 is correlated with poor prognosis in patients with neuroblastoma (24). Mutations in helicases involved in DNA repair mechanisms were found in cancer-prone syndromes such as xeroderma pigmentosum, Bloom's syndrome, Werner's disease, X-linked mental retardation associated with α-thalassemia, and Cockayne's syndrome. With regard to the immunogenicity of DEAD box protein, a mutated murine DEAD box protein, named p68, was found to encode an antigens recognized by CTL on a UV-induced sarcoma (25). A mutated peptide of MUM-3 homologous to RNA helicase with a DExH motif was isolated with human leukocyte antigen-A28 restricted autologous melanoma-specific CTL (26).

CAGE was previously reported to be expressed in various cancer cell lines through hypomethylation of the promoter region (27). However, its protein expression and immunologic recognition has not been thoroughly evaluated. Therefore, we further analyzed the protein expression and immunogenicity of CAGE isolated by our two independent SEREX experiments using sera from patients with endometrial cancer and melanoma. In addition to the previously reported cancers, we found that CAGE was also expressed in other types of cancers, including endometrial cancer, melanoma, breast cancer, bladder cancer, pancreatic cancer, renal cell cancer, and leukemia, and in particular it was expressed frequently (7 of 10 patients) in endometrial cancer tissues. Cancer/testis antigen frequently expressed in various cancers, MAGE-A4 or NY-ESO-1, was previously reported to be expressed only in 12% or 19% of endometrial cancers, respectively (28). CAGE was also expressed in one of three atypical endometrial hyperplasia tissues, but not in normal endometria in either proliferation or secretory phase, although cell cycle-dependent expression of CAGE was suggested (6). Hypomethylation of the CAGE promoter was reported not only in cancer cells, but also in precancerous states including chronic gastritis and liver cirrhosis, suggesting that CAGE expression may occur in the relatively early stages of cancer development.

We next examined the immunogenicity of CAGE in patients with various cancers and found that anti-CAGE IgG antibody were present in the sera of patients with various cancers, including endometrial cancers, melanoma, and colon cancer.

Table 1. Number of anti-CAGE antibodies detected in various cancer patients' sera by Western blotting analysis

Healthy controls	Endometrial cancer	Ovarian cancer	Melanoma	Colon cancer	Renal cell cancer	Prostate cancer	Pancreatic cancer
0 of 40	5 of 45	0 of 10	2 of 24	2 of 33	0 of 20	0 of 18	0 of 12

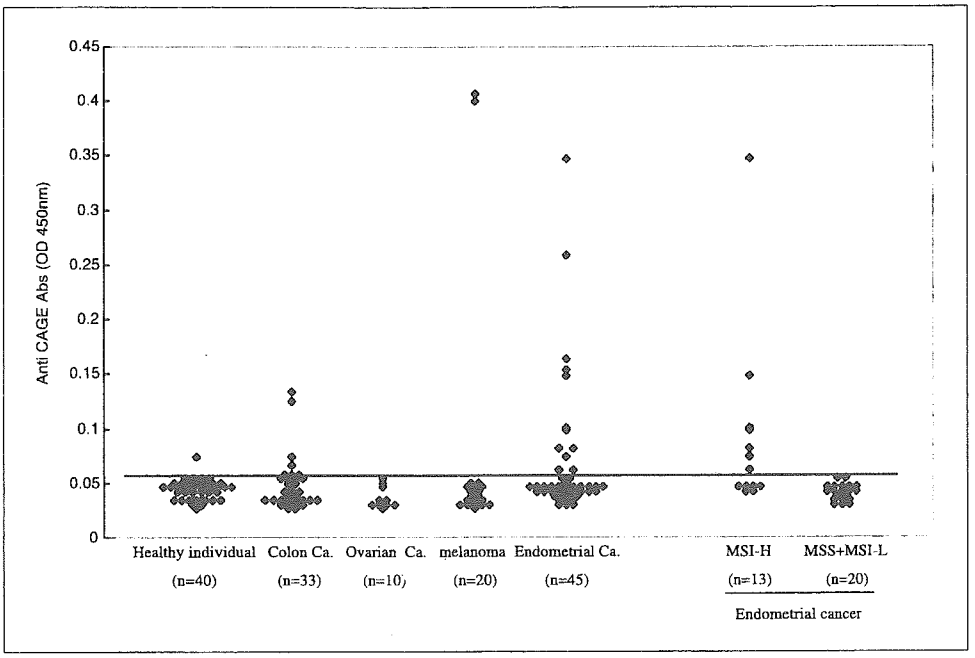

Fig. 6. Frequent detection of anti-CAGE antibodies in sera from patients with MSI-H endometrial cancer evaluated by ELISA. ELISA was done with the recombinant CAGE protein. The horizontal line indicates the cutoff value for positivity (OD = 0.058: the average absorbance of the healthy individuals plus 2 SD). Positive sera were found in 12 of 45 (26.7%) patients with endometrial cancer, 4 of 33 (12.5%) patients with colon cancer, 2 of 20 (10.0%) patients with melanoma, and 1 of 40 (2.5%) age-matched healthy individuals, but not in 10 patients with ovarian cancer. Among 33 patients with endometrial cancer whose MSI status were evaluated, 7 of 13 (53.8%) patients with MSI-H had positive serum CAGE antibody, whereas none of 20 non – MSI-H patients including 1 MSI-L and 19 MSS patients had anti-CAGE antibody.

Because we have been working on immune responses in patients with MSI-positive cancers, and a subpopulation of endometrial cancers and colon cancers is known to be MSI-positive through either mutation of DNA mismatch repair enzyme genes such as MLH1 or silencing of promoters for the repair enzyme genes by methylation, we have evaluated the correlation between CAGE antibody positivity and MSI status. In particular, endometrial cancer was reported to be frequently MSI-positive due to hereditary non-polyposis colon cancer or silencing of the MLH1 promoter by methylation (29). Sporadic endometrial cancers (9-30%) were reported to be MSI-positive. Surprisingly, anti-CAGE antibody was detected in sera from 7 of 13 (53.8%) patients with MSI-H, but not in sera from 20 non-MSI-H patients including one MSI-L and 19 MSS patients. Interestingly, two patients with colon cancer with positive CAGE antibody also had MSI-positive cancers developing with hereditary non-polyposis colon cancer. Two melanoma patients with positive CAGE antibody may suggest possible MSI in melanoma, although this was not evaluated because of the unavailability of tumor samples. Because one of the two melanoma patients with the positive CAGE antibodies was immunized with dendritic cells pulsed with autologous tumor lysate, we evaluated the titer of the anti-CAGE antibodies before and after the dendritic cell immunization in the patient, and found that this patient had a high titer of antibodies even before the immunization, and no significant change was observed after the vaccination.

Table 2. The correlation of anti-CAGE antibodies and clinicopathologic features

		Anti-CAGE antibodies (OD 450 nm)		
	n	≤0.058	>0.058	*P**
Age				
<60	28	20	8	
>60	17	13	4	0.98
Federation Internationale des Gynaecologistes et Obstetristes stage				
I + II	32	24	8	
III + IV	13	9	4	0.98
Grades				
1 + 2	35	28	7	
3	10	5	5	0.14
MSI				
MSI-H	13	6	7	
MSS + MSI-L	20	20	0	0.001
Not examined	12			
CA602 value				
≤63	31	24	7	
>63	14	9	5	0.58

*P value was calculated by χ^2 test.

Defective DNA mismatch repair frequently causes frameshift changed, unique COOH-terminal peptides, particularly by slippage mutation in the repetitive sequence in the protein coding region. We have previously reported that the CDX2 COOH-terminal peptide generated by the frameshift mutation induced IgG responses specific to both altered COOH-terminal peptides and NH_2-terminal wild-type peptides in a patient with hereditary non-polyposis colon cancer (14). Anti-p53 antibody, which recognizes wild-type p53, was known to be induced through conformational changes of mutated p53. Because CAGE has six repeated thymine sequence in the protein coding region, we sequenced this region of genomic DNA obtained from tumor samples of five MSI-H endometrial cancer patients, but could not find any alteration in this region. Thus, the mechanism of induction of IgG response to CAGE in MSI-positive patients is still unclear. Mutations in other regions of CAGE or other molecules generated by MSI may be involved in modification of the antigen processing and induction of T cells and B cells specific for CAGE.

Because cancer/testis antigens are often expressed in human leukocyte antigen – negative cells in immunologic privilege sites such as spermatogonia and spermatocytes in tests, they are not recognized by specific T cells, indicating that some of the cancer/testis antigens may be tumor-specific common antigens and one of the promising targets for cancer immunotherapy. Immunization trials have been in progress for MAGE and NY-ESO-1 (30). The recognition by IgG antibodies suggests that the same antigen activated CD4+ helper T cells in patients, meaning that the antigens are immunogenic in cancer patients. In addition, many SEREX-defined antigens, including MAGE and NY-ESO-1, have been shown to also induce CD8+ CTL. Positive correlation was observed between positive serum IgG antibody and induction of CD8+ CTL against a cancer/testis antigen NY-ESO-1 (31). Patients with MSI-positive colon cancer have relatively good prognosis despite poor histologic results. Because predominant infiltration of T cells, particularly CD8+ T cells, is observed in MSI-positive colon cancer tissues, immune responses to frameshift antigens may contribute to the maintenance of tumor-free status after treatment. We have previously shown the immune response to both frameshift-mutated and wild-type peptides of CDX2 in MSI-positive colon cancer patients (14), and T cell response to the frameshift-mutated TGFβ-RII frequently detected in MSI-positive colon cancer was also reported (32). Although prognosis of MSI-positive endometrial cancer is still controversial, there are reports showing better prognosis of patients with MSI-positive endometrial cancer (33). If immune response is involved in the good prognosis, CAGE may be one of the target antigens besides the frameshift antigens. Therefore, CAGE may be a good candidate antigen for immunotherapy, at least as CD4+ T cell antigens, particularly for MSI-positive endometrial cancer patients with positive CAGE serum antibody.

Serum anti-CAGE antibody may be used as a tumor marker. We often observed the disappearance of serum antibody in the SEREX-defined antigens after curative treatment in patients with various cancers (13, 14). Use of serum antibodies against p53 (34), cyclin B1 (35), hTERT (36, 37), and survivin (37), were recently reported. A positive rate of 15% for anti-p53 antibody in patients with colon cancers and that of 21.6% or 7.8% for anti-survivin antibody in patients with lung or colon cancers were reported. A positive rate of anti-CAGE antibody in 7 of 13 (53.8%) patients with MSI-positive endometrial cancer and in 1 of 3 patients with atypical endometrial hyperplasia indicated possible use of anti-CAGE serum antibody for prognostic or early diagnosis for patients with MSI-positive endometrial cancers. Further analysis with a larger numbers of patients is necessary for confirmation and usefulness of this possibility. CA602, a part of CA125 antigen, is one of the most commonly used tumor markers for endometrial cancers. No correlation was observed between anti-CAGE antibody and CA602 in this study. Although CA602 produced by tumor cells correlates with tumor volume, the induction of antibody was defined by the immune response of patients through antigen processing and immune response of T cells and B cells. Therefore, these tumor markers can be independently used for diagnosis of endometrial cancer.

In summary, we have shown that CAGE is expressed in various cancers including endometrial cancers and melanoma, and frequent detection of specific serum IgG antibody in patients with MSI-H endometrial cancers, indicating the highly immunogenic nature of CAGE in MSI-positive endometrial cancers. These results suggest that CAGE may be useful not only for immunotherapy of various cancers, but also for diagnosis of some cancers, particularly MSI-positive endometrial cancers.

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Loss of blood group A antigen expression in bladder cancer caused by allelic loss and/or methylation of the *ABO* gene

Yoshitomo Chihara[1,2], Kokichi Sugano[1], Ayumi Kobayashi[1], Yae Kanai[3], Hidenobu Yamamoto[4], Masaaki Nakazono[4], Hiroyuki Fujimoto[5], Tadao Kakizoe[5], Kiyohide Fujimoto[2], Setsuo Hirohashi[4] and Yoshihiko Hirao[2]

[1]*Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, Tochigi, Japan;* [2]*Department of Urology, Nara Medical University, Nara, Japan;* [3]*Pathology Division, National Cancer Center Research Institute, Tokyo, Japan;* [4]*Department of Urology, Tochigi Cancer Center Hospital, Tochigi, Japan and* [5]*Department of Urology, National Cancer Center Hospital, Tokyo, Japan*

Loss of ABO blood group antigen expression has been reported in transitional cell carcinoma (TCC) of the bladder. Synthesis of the ABO blood group antigen was genetically determined by allelic variants of the *ABO* gene assigned on 9q34.1. We analyzed loss of heterozygosity (LOH) and promoter hypermethylation of the *ABO* gene in TCC and compared them with alterations of A antigen expression in TCC, dysplasia and normal urothelium. A total of 81 samples of TCC of the bladder obtained from transurethral resection (TUR) ($n=44$) and radical cystectomy ($n=37$) were examined. Expression of the A antigen was evaluated by immunohistochemical staining (IHC) using anti-A antigen monoclonal antibody. LOH of the *ABO* gene locus was examined by blunt-end single-strand DNA conformational polymorphism (SSCP) analysis using flouresence-based auto sequencer. Promoter hypermethylation of the *ABO* gene were examined by bisulfite PCR-SSCP (BiPS) analysis and/or methylation-specific PCR (MSP). Loss of A allele and/or hypermethylation were significantly associated with abnormal expression of the A antigen in cases undergoing TUR ($P=0.02$) and radical cystectomy ($P=0.0005$). For the analysis of the concomitant dysplasia in 23 cases with TCC of the bladder, the expression of the A antigen was maintained, regardless of the A allelic loss or methylation status in the tumor. In conclusion, A allelic loss and hypermethylation in the promoter region of the *ABO* gene showed significant correlation with reduction of A antigen expression in TCC, while the expression of the A antigen is maintained in concomitant dysplasia or normal urothelium, suggesting that loss of the *ABO* gene and/or its promoter hypermethylation is a specific marker for TCC.
Laboratory Investigation (2005) **85,** 895–907. doi:10.1038/labinvest.3700268; Published online 9 May 2005

Keywords: bladder cancer; *ABO* gene; LOH; promoter hypermethylation; dysplasia

Superficial bladder cancers often show multifocal occurrences or metachronous recurrence after transurethral resection (TUR), and eventually develop into invasive bladder cancer. Allelic loss on chromosome 9 is the most frequent genetic event in transitional cell carcinomas of the bladder,[1–4] that is observed in 70% of invasive bladder cancers and even in 50% of superficial bladder cancers at Stage G1.[4] Whether or not loss on chromosome 9 arises in urothelial lesions such as dysplasia is crucial to the understanding of early genetic events in bladder carcinogenesis. Some authors have reported on the allelic loss of chromosome 9 that occurs in the small urothelial lesions and normal bladder urothelium in their attempts to trace genetic alterations using microsatellite markers.[5,6] However, it is still difficult to analyze allelic status in small epithelial regions obtained from formalin-fixed, paraffin-embedded tissues, and a few data have been reported regarding early genetic alterations in bladder dysplasia.[3,7] ABO (H) blood group antigens are constitutively expressed on epithelial cells such as those found in the gastrointestinal tract and urothelium. A reduction in blood-group A antigen (GalNAcα1-3[Fucα1-2]Galβ1-3GlcNAc-R) expression was reported in transitional cell carcinoma (TCC) of the bladder

Correspondence: Dr K Sugano, MD, PhD, Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, 4-9-13 Yohnan, Utsunomiya-shi, Tochigi Pref. 320-0834, Japan.
E-mail: ksugano@tcc.pref.tochigi.jp
Received 16 April 2004; revised 1 January 2005; accepted 14 January 2005; published online 9 May 2005

and showed significant correlation with an invasive phenotype.[8–11] Orntoft and Wolf[12] examined the correlation between blood-group antigen expression and the activity of glycosyltransferases in TCC of the bladder and reported that the activity of A glycosyl transferase was severely reduced in tumors showing loss of A antigen expression. This phenomenon drew our attention, due to the fact that the determinant of the ABO blood-group antigen is synthesized by the action of the *ABO* gene encoding ABO glycosyltransferase assigned to chromosome 9q34.1, where loss of heterozygosity (LOH) was frequently reported in bladder cancer.[1–4] The *ABO* gene is composed of seven exons and six introns and encodes ABO glycosyltransferase, of which substrate specificity is determined by genetic polymorphisms in exons 6 and 7 (Figure 1).[13,14] Blood-group A antigen is synthesized by α-*N*-acetylgalactosaminyltransferase (A-GalNAc transferase), which catalyzes the transfer of *N*-acetylgalactosamine to the subterminal β-galactosyl residue of the blood-group H carbohydrate chain. Blood-group B-antigen is synthesized by B-galactosyl transferase, which catalyzes the transfer of galactose to the subterminal β-galactosyl residue of the blood-group H carbohydrate chain. The *ABO* gene in blood-group O donors lacks glycosyltransferase activity, for it has a deletion on a guanine residue at the nucleotide position 261 in exon 6, causing protein truncation at codon 117.[13–16] Immunohistochemistry using anti-A monoclonal antibody in bladder cancer may be useful to evaluate the allelic status of the *ABO* gene locus at 9q34.1 in those who are heterozygous for ABO genotypes. Expression of blood-group A antigen is stable enough even in formalin-fixed paraffin-embedded specimens, and this could be applicable in the analysis of small lesions that are too small to be examined by genetic analysis. Two papers were so far reported as to the correlation between reduced expression of A antigen and A allelic loss in TCCs of the bladder.[17,18] Meldgaard *et al*[17] analyzed 22 bladder tumors for LOH of the 9q allele by PCR-restriction fragment length polymorphism (RFLP) analysis of the *ABO* locus at 9q34. Seven tumors from heterozygous informative individuals were sorted by flowcytometry. LOHs were detected in the most aneuploid subpopulation of cells in two cases, but both cases were losing O-alleles. No LOHs were detected in analysis of the low aneuploid subpopulation. As all tumors showed loss of blood group ABH antigen expression, they concluded that LOH of the *ABO* locus on chromosome 9q34 is not the cause of loss of blood group ABH expression in human bladder cancer.[17] Orlow *et al*[18] analyzed 19 patients with bladder cancer serologically typed as blood group A. Expression of A antigen was maintained in 14 samples in normal urothelium, while it was reduced in nine tumors. PCR-RFLP analysis showed loss of the A allele in one tumor sample showing reduced expression of the A antigen. They indicated that the lack of the A antigen expression in certain bladder tumors is due to the allelic loss of the *ABO* gene and that in some of these tumors, the loss involved the surrounding chromosomal region at 9q34.1–4.[18] These two reports did not support the correlation between A-allelic loss and the reduced expression of the A antigen in the majority of bladder cancers. Recent advance in cancer epigenetics shed light on the reduced expression of A antigen in malignant cells. Kominato *et al*[19,20] reported that hypermethylation of the promoter region of the *ABO* gene induced *ABO* gene silencing in their study using a human stomach carcinoma cell line. Iwamoto *et al*[21] established subclones with positive or negative expression of the A antigen from parental colonic cancer cell lines and reported a distinct difference in the methylation pattern of the CpG island of the promoter region of the ABO glycosyltransferase, that is densely methylated in a subclone lacking the expression of the A antigen. Gao *et al*[22] examined 30 oral squamous carcinomas for expression of the A and B antigens and A/B glycosyltransferase, together with LOH at the *ABO* locus and hypermethylation of the *ABO* gene promoters. Loss of A or B antigen expression was found in 21 of 25 tumors (84%), while the expression of the glycosyltransferase was absent in all of tumors showing negative expression of A or B antigens. Loss of the A or B allele was found in 3/20 tumors (15%) heterozygous for the *ABO* locus and hypermethylation of the promoter region in 10 of 30 tumors (33.3%).[22] Furthermore, Habuchi *et al*[23] reported that the region 9q32–9q33, which is in the vicinity of the *ABO* gene locus at 9q34.1, is a frequent target of LOH and methylation in bladder cancer. These findings prompted us to hypothesize that deletion of blood-group A antigen expression in TCC of the bladder might be regulated by a combination of genetic and epigenetic mechanisms, that is, an LOH of the *ABO* gene locus and hypermethylation of the *ABO* gene promoter region. The purpose of this study was to elucidate the relevant mechanisms underlying the loss of blood group A antigen expression in TCC of the bladder and whether it could be used as a phenotypic marker to estimate any underlying genetic and epigenetic abnormalities in normal urothelium and concomitant bladder dysplasia in patients with bladder cancer.

Materials and methods

Samples and DNA Extraction

A total of 81 cases of TCC of the bladder were studied, of which 44 underwent TUR and 37 underwent radical cystectomy (Table 1). The histo-blood group for all cases was A (72 cases) or AB (nine cases) examined by routine hemagglutination tests at hospital. Tumors were graded and staged according to the WHO classification or the 1997 UICC TNM classification system. Based on patients'

Table 1 Patient background

	TUR-BT	*Radical cystectomy*	P-*value*
No. of cases examined	44	37	
Gender			NS
Male	37 (84.1%)	33 (89.2%)	
Female	7 (15.9%)	4 (10.8%)	
Age (median)	66 (45–79)	66 (39–89)	NS
Pathological stage			$P<0.01$
pTa	14 (31.8%)	0 (0%)	
pT1	25 (56.8%)	11 (29.8%)	
pT2	4 (9.1%)	8 (21.6%)	
pT3	0 (0%)	10 (27.0%)	
pT4	1 (2.3%)	8 (21.6%)	
Hitological grade			$P<0.01$
G1	7 (14.9%)	0 (0%)	
G2	21 (51.1%)	2 (5.4%)	
G3	16 (34.0%)	35 (94.6%)	
Blood group			
A	38	34	
AB	6	3	

history, the proportion of cases with advanced stage or high-grade tumors was significantly higher in those who underwent radical cystectomy than those who underwent TUR ($P<0.01$). In 44 patients who underwent TUR, DNA was extracted from fresh specimens and normal DNA was extracted from peripheral blood lymphocytes (PBL) by a standard procedure using proteinase K digestion followed by phenol–chloroform extraction. In 37 cases that underwent radical cystectomy, a total of 1130 paraffin-embedded specimens obtained from mapping study of the bladder were histologically confirmed by hematoxylin and eosin staining as being composed of tumor, dysplasia and normal tissues. DNA was extracted from manually dissected tumors and corresponding normal tissues using DEXPAT (TAKARASHUZO Co., Ltd, Shiga, Japan) according to the manufacturer's recommendation.

Expression of Blood-Group A Antigen by Immunohistochemical Staining

In all, 4-μm-thick sections from formalin-fixed, paraffin-embedded specimens of resected tissues that underwent TUR or radical cystectomy were used for immunohistochemical staining (IHC). A mapping study of the bladder specimens revealed concomitant dysplastic lesions in 23 cases that underwent radical cystectomy, and they were then subjected to IHC performed as described previously.[24] Mouse monoclonal antibody (mAb) directed against A antigen (clone 81FR2.2; DAKO, Carpinteria, CA, USA) was used as the primary antibody and the avidin–biotin-conjugated immunoperoxidase technique was performed with a DAKO LSAB2 Kit (DAKO, Carpinteria, CA, USA). Reportedly, the specificity of the mAb 81FR2.2 was characterized by transfection experiment of the A-glycosyl transferase gene to the HeLa cell (genotype OO).[25] Erythrocytes, normal epithelium and vascular endothelium were used as internal positive controls, while muscle and connective tissues served as negative controls. To determine the specificity of A antigen, IHC was performed for normal urothelium of blood group B and O donors. Immunohistochemistry for A antigen was classified as follows: 'negative' if the section had no positively (0%) stained tumor cells, 'positive' if staining was seen across the section (>70% positively stained tumor cells), and 'heterogenous' if <70% of tumor cells stained positively. As to the correlation with A allelic loss or methylation status, cases showing positive or heterogenous expression were compared with those showing negative expression.

Allelic Status on 9q Loci Defined by Blunt-End Single-Strand DNA Conformation Polymorphism Analysis

LOH of the *ABO* gene locus was examined by blunt-end Single-strand DNA conformation polymorphism (SSCP) analysis,[26] using genetic polymorphisms at nucleotide positions 261 and 297 in exon 6 of the *ABO* gene. Genotypes and their allelic frequencies in Japanese population were previously reported[15] and shown in Figure 1. Four groups of alleles, A (A101, A102, A103), B (B101, B102, B103, A104), O1 (O101, O102, O202, O203) and O2 (O103, O201) were identified by the analysis of two genetic polymorphisms (nucleotides 261, 297) in exon 6 of the *ABO* gene. The 5′-terminus of the reverse primer

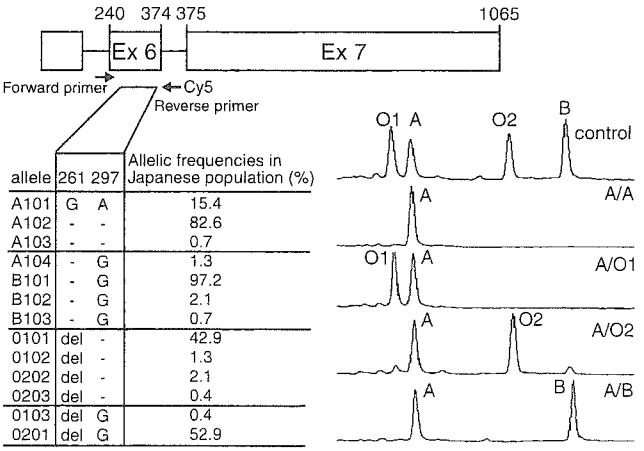


Figure 1 Schema of single nucleotide polymorphisms (SNPs) in exons 6 of the *ABO* gene and electropherogram of the blunt-end SSCP analysis showing examples of normal DNA from blood group A or A/B donors. SNPs in nucleotide positions 261 and 297 were used for analysis in this study. DNA variants and their allelic frequencies reported in the Japanese are indicated.[15] The blood group *O* gene has a single base deletion at position 261 resulting in a frame-shift mutation and causing protein termination at codon 117.

was labeled with Cy5 flourescent dye. The nucleotide sequences of the forward and reverse primers were 5′-TCTCCATGTGCAGTAGGAAGGATG-3′ and 5′Cy5-ATGGCAAACACAGTTAACCCAATG-3′, respectively. PCR conditions were as follows: 0.5–1.0 μg of genomic DNA as a template, 0.2 μmol/l of each primer, 0.125 mmol/l deoxynucleoside triphosohate (dNTP), 0.25 units of AmpliTaq Gold DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT, USA) in a total reaction volume of 25 μl. After the first denaturation step at 95°C for 12 min, 40 cycles were performed for amplification consisting of 30 s at 95°C, annealing for 30 s at 57°C, and extension for 30 s at 72°C followed by a final extension at 72°C for 7 min. PCR products were then treated with Klenow fragment (TAKARA SHUZO Co., Ltd, Shiga, Japan) to generate DNA fragments with blunt ends. To 1 μl of each PCR product, 0.5 units of Klenow fragment was added, and the mixture was incubated at 37°C for 30 min. One microliter of this reaction mixture was diluted with 10 μl of loading solution (90% deionized formamide, 20 mM EDTA, 0.05% bromophenol blue) and heat denatured at 95°C for 5 min. An ALF red automated DNA sequencer™ (Pharmacia, Tokyo, Japan) was used for blunt-end SSCP analysis. One microliter of the diluted mixture was applied onto a 15% polyacrylamide gel (30:1, acrylamide:bisacrylamide ratio) containing Tris/glycine buffer (25 mM Tris, 192 mM glycine). Electrophoresis was performed at 30 W for 16 h using a continuous buffer system consisting of 25 mM Tris and 192 mM glycine. During electrophoresis, the gel was maintained at a constant temperature of 18°C by a circulating water bath. The data were analyzed using the ALF Win Fragment analyzer 1.02™ software package (Pharmacia, Tokyo, Japan). LOH was determined by measuring the signal ratio between the opposing alleles and defined as tumor cellularity according to the equation that we previously reported.[4,26,27] Supposing that the A1 allele is lost in a heterozygote carrying A1 and A2 alleles, T is the peak height of the signal from the tumor samples and N is the peak height of the signal from normal control. The tumor cellularity in the sample is thus given as follows:

$$\text{Tumor cellularity (\%)} = [(N_{A1}/N_{A2}) - (T_{A1}/T_{A2})] \times 100/(N_{A1}/N_{A2})$$

Genomic DNA from normal PBL was analyzed to set the cutoff values for tumor cellularity. As previously reported, the mean + 3s.d. values of the normal heterozygous DNA were used as a cutoff value for tumor cellularity, and tumor samples showing tumor cellularities above the cutoff level were considered to have LOHs.[4] A104 allele was indistinguishable from B allele in this analysis, while the observed frequency of the A104 allele in the Japanese is reported to be as low as 1.3%. In fact, in all samples tested, the genotypes coincided with the patient's ABO isotypes. In addition, two single base nucleotide polymorphism markers (*ALDOB*, 9q21.3 and *VAV2*, 9q34.1) were used to assess the allelic status on 9q according to the method that we previously reported;[4] the former is centromeric and the latter is telomeric to the *ABO* gene locus, respectively (Figure 4). Nucleotide sequences of the forward and reverse primers for *ALDOB* and *VAV2* were as follows: 5′Cy5-GGGCTTGACTTTC CAACACG-3′ and 5′-TCTAGCCTCAATCCTCATAC-3′ (*ALDOB*), 5′-GTGTCTGCACTGGCCACACT-3′ and 5′Cy5-TCCAAAGGACCTTCTCCAAA-3′ (*VAV2*).

Bisulfite PCR-SSCP Analysis and Methylation-Specific PCR

In cases that underwent TUR, methylation status in the promoter region of the *ABO* gene was analyzed by bisulfite PCR-SSCP (BiPS) and methylation specific PCR (MSP).[24,28,29] Seven primer sets were designed to amplify seven overlapping regions spanning the CpG island located from −765 to +21 relative to the translation start site (Figure 2). Primer sets *re 1* through *re 6* were designed for BiPS analysis and *RE7*.M and *RE7*.UM were for MSP. Bisulfite treatment was performed using the CpGenome DNA Modification Kit (Intergen Co., New York, NY, USA). In all, 1 μg of tumor-derived DNA was treated with Na-bisulfite according to the manufacturer's recommendations. PCRs were performed in 25 μl reaction volumes containing 10 × buffer, 1.0 μl bisulfite-modified DNA corresponding to 50 ng of genomic DNA as a template, 0.2 μmol/l of each primer, 0.125 mmol/l dNTP and 0.25 units of AmpliTaq Gold DNA polymerase. PCR conditions were 95°C for 9 min for heat denaturation, 40 cycles of 94°C for 1 min, 1 min at the different annealing temperatures for each primer set (Table 2), 72°C for 2 min for amplification, followed by a final extension at 72°C for 10 min. The BiPS procedure was performed as previously described.[28,29] Nondenaturing polyacrylamide gels of 8% for *re 2* and *re 6*, 10% for *re 1*, *re 4* and *re 5*, and 15% for *re 3* were used for the analysis. CpGenome™ Universal Methylated DNA (CHEMICON International, Temecula, CA, USA) was used as a positive control, and PBL obtained from healthy control donors were used as a negative control. When extra bands were observed, they were cut from the gels, reamplified and subjected to direct sequencing using ABI 3100 PRISM sequencer with a Big-Dye terminator sequencing kit (Perkin-Elmer). In analysis of cases that underwent radical cystectomy, BiPS analysis was not employed due to the technical difficulty for reliable amplification of relatively long sized DNA fragments from formalin-fixed paraffin-embedded sections. In cases that underwent radical cystectomy, methylation status was assessed by MSP of region 7, the most proximal to the translation start site. The size of the PCR product was as short as 96 bp and amplifiable from archival samples with

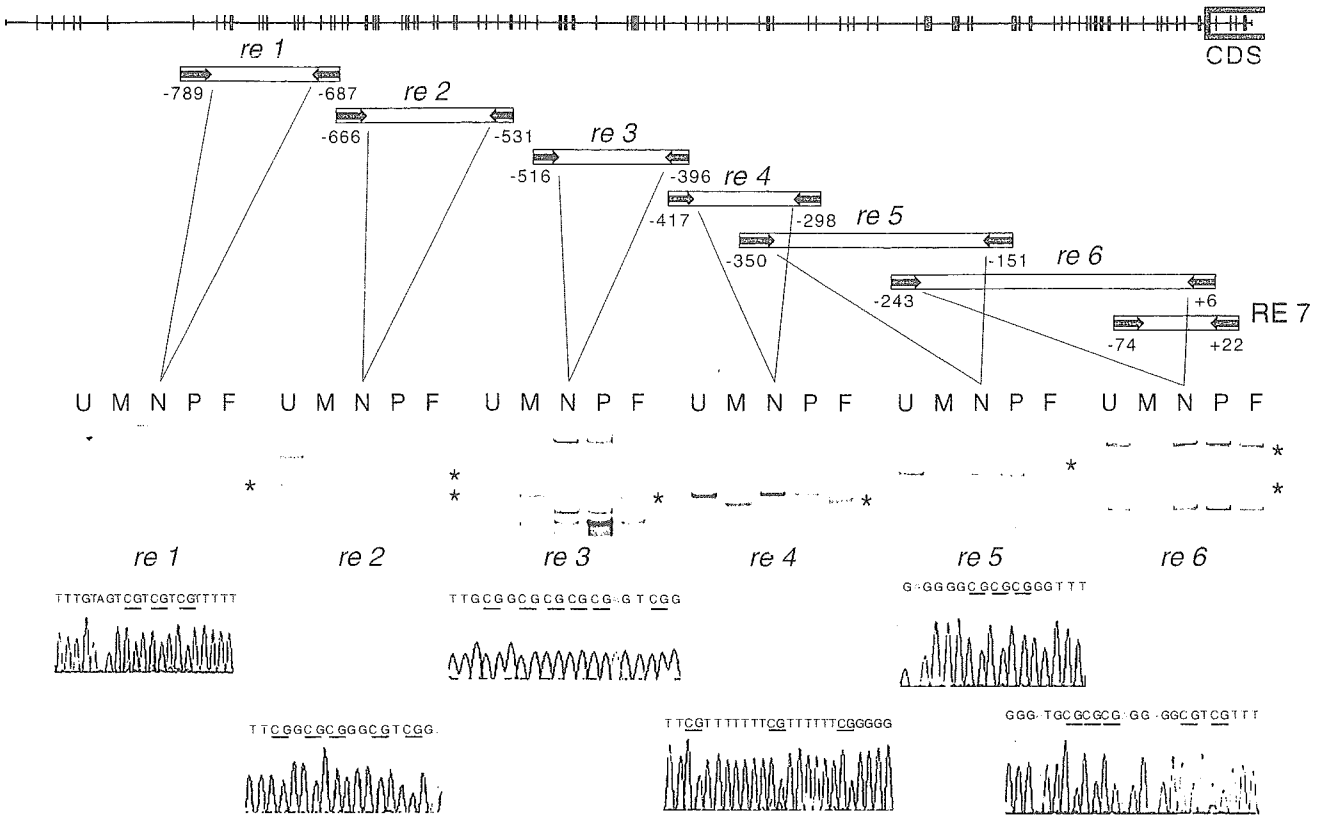


Figure 2 Map of the 5′ CpG island of the *ABO* gene and result of BiPS analysis. (Top) CpG sites in the promoter region of the *ABO* gene are indicated by vertical lines. (Middle) The amplified DNA fragments from regions 1 to 7 are indicated. PCR primer set of each region was indicated by arrows. (Bottom) BiPS analysis of the *ABO* gene. Extra bands are indicated by asterisks. After SSCP analysis, the extra bands were excised from gels, reamplified by PCR, and sequenced. Results of the direct sequencing of the case with full methylation were shown in the lower panel. U: unmethylated control, M: methylated control, N: no methylation, P: partial methylation, F: full methylation, *extra band showing mobility shift.

Table 2 Primer sequences for BiPS analysis and MSP

Primer name	*Forward primer sequence*	*Reverse primer sequence*	*Products length (bp)*	*No. of CpG sites*	*Annealing temperature (°C)*
re 1	5′-TTGGGATTTTTCGGGAGGTAATTT-3′	5′-CCCCGCTACGACCCCCGCCCTTAC-3′	103	11	54
re 2	5′-GGGCGGAGCGGGGTTTTGTTTACG-3′	5′-CGCGACCCCACGAAACTCTACGTC-3′	136	20	48
re 3	5′-AGCGATTTTGTTTAGGGGGA-3′	5′-ACTACGACCCCAAAACCCAC-3′	121	15	59
re 4	5′-TCGTGGGTTTTGGGGTCGTAGTTT-3′	5′-CCCCGTCCCCGAAAACCCCTTAAC-3′	120	11	54
re 5	5′-GGGGGTCGTTTTCGTTCGGGAGAT-3′	5′-CGAATCCCCAAAAACCCCTACTAA-3′	200	19	48
re 6	5′-TAAGGTATTAGGGTTACGAGG-3′	5′-GACCATAACTCCGCGTCTAAT-3′	248	33	49
RE 7.M	5′-GAGGGGGCGTTTCGGGTTTATTTC-3′	5′-ACGTCCGCAACACCTCGACCATAA-3′	96	16	70
RE 7.UM	5′-GGAGGGGGTGTTTTGGGTTTA-3′	5′-ATCCACAACACCTCAACCATAACT-3′	96	13	60

M, methylated; UM, unmethylated.

relative ease; however, five out of 37 cases that underwent radical cystectomy failed in PCR amplification. Methylation status of region 7 was used as the surrogate indicator for extensive methylation of the CpG sites or full methylation.

Statistical Analysis

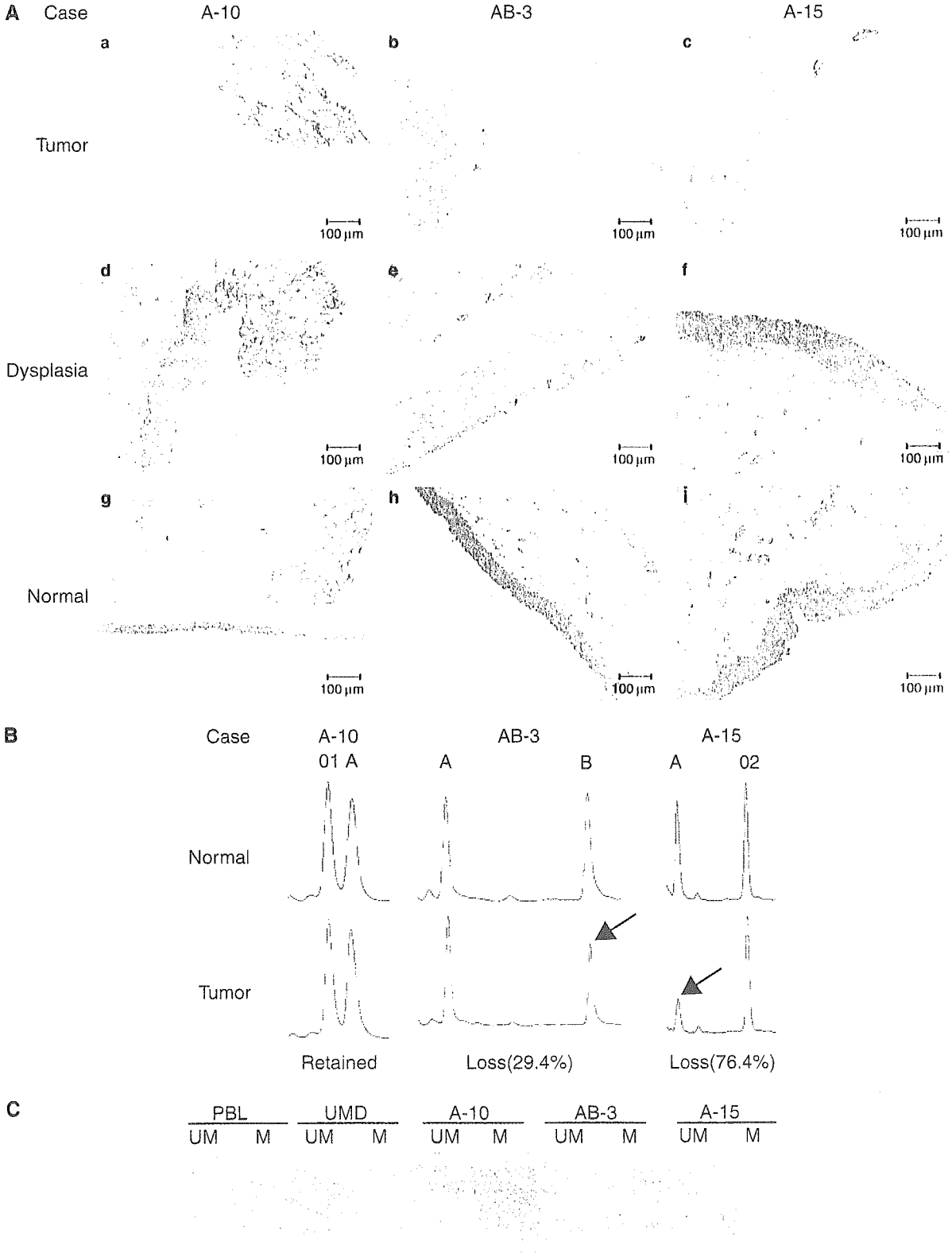
Statistical analysis was performed using a likelihood χ^2 analysis or Fisher's exact test. Probability (P) values of <0.05 were considered to be significant.

Results

Expression of the A Antigen in TCC of the Bladder by IHC

Expression of the A antigen in tumor and normal urothelium was examined by IHC (Figure 3A). The corresponding staining of A antigen on the normal

urothelium from histo-blood-group B or O donors resulted in background levels only (data not shown). All of the normal urothelium from blood-group A individuals stained positively. The numbers of cases showing positive, heterogeneous and negative staining were 11 (25.0%), 11 (25.0%) and 22 (50.0%) in 44 tumor specimens that underwent TUR, while they were 14 (37.8%), 5 (13.5%) and 18 (48.6%) in 37 tumor specimens that underwent radical cystectomy. The overall frequencies of negative A antigen



expression were 35.7% (5/14) for pTa, 58.3% (21/36) for pT1, 25.0% (3/12) for pT2, 40.0% (4/10) for pT3 and 77.8% (7/9) for pT4 stages, and 71.4% (5/7), 43.5% (10/23) and 49.0% (25/51) for Grade 1, 2 and 3 tumors, respectively. There were no significant differences between A antigen expression and tumor stages or histological grades.

LOH on 9q in TCC of the Bladder

Allelic status of the *ABO* gene and neighboring loci were analyzed by blunt-end SSCP analysis using three polymorphic markers (*ABO* (9q34.1), *ALDOB* (9q21.3-22.2), *VAV2* (9q34.1)) (Figure 4). Heterozygosity of each locus was 87.7% (71/81) for *ABO*, 52.6% (41/78) for *ALDOB* and 48.1% (38/79) for *VAV2*, respectively. As all samples were derived from patients with an A or AB blood group, heterozygosity at the *ABO* locus was highest of all the loci examined. Genotypes of the *ABO* gene were classified into four groups, that is, A/A ($n=10$), A/O1 ($n=34$), A/O2 ($n=26$) and A/B ($n=9$). The cutoff value for tumor cellularity in each genotype was defined as the mean + 3s.d. of the normal DNA samples: 20% for A/O1, 22% for A/O2, 26% for A/B, respectively. In 44 cases that underwent TUR, frequencies of LOH were 53.7% (22/41) for *ABO*, 43.5% (10/23) for *ALDOB* and 50.0% (10/20) for *VAV2*, respectively. Frequencies of allelic loss at the *ABO* locus were 23.1% (9/39), 33.4% (6/18), 33.3% (5/15) and 33.3% (2/6) for A, O1, O2 and B allele, respectively. In 37 cases that underwent radical cystectomy, frequencies of LOH were 76.7% (23/30) for *ABO*, 77.8% (14/18) for *ALDOB* and 83.3% (15/18) for *VAV2*, respectively. Frequencies of allelic loss in the *ABO* locus was 23.3% (7/30), 50.0% (8/16), 54.5% (6/11) and 66.7% (2/3) for A, O1, O2 and B allele, respectively. There were no significant differences as to the frequencies of LOH between three markers and between four alleles of the *ABO* gene. Frequencies of LOH were higher in cases that underwent radical cystectomy as compared to the TUR cases, that is, 76.7% (23/30) *vs* 53.7% (22/41) for *ABO* ($P=0.08$), 77.8% (14/18) *vs* 43.5% (10/23) for *ALDOB* ($P=0.054$) and 83.3% (15/18) *vs* 50.0% (10/20) for *VAV2* ($P=0.043$), among which VAV2 locus showed statistical significance.

Methylation Status of the *ABO* Gene Promoter Region

CpG island of the *ABO* gene extends from 0.7 kb upstream to 0.6kb downstream from the translation start site in exon 1. Reportedly, the promoter region of the *ABO* gene is located between −117 and +31 from the translation start site, of which hypermethylation regulates gene expression.[19,20] In the present study, we divided CpG island spanning −789 to +6 into six regions and examined the methylation status by BiPS analysis (Figure 2). In the preliminary experiment, methylated DNA could be identified as the extra band, if more than 25% of the template DNA was methylated (data not shown). Methylation patterns were defined as follows: full methylation if all regions showed methylation, partial methylation if at least one region showed methylation and no methylation. A total of 44 TUR cases were analyzed, and we assessed the correlation between methylation status and expression levels of the A antigen using a panel of 35 cases, for nine cases showing LOH of the A allele were not included in the first assessment (Tables 3 and 4). Frequencies of methylation in *re 1* through *re 6* were 17.1% (6/35), 28.6% (10/35), 34.3% (12/35), 11.4% (4/35), 14.3% (5/35) and 11.4% (4/35), respectively (Table 4). In *re 4*, *re 5* and *re 6*, methylation was not detected in all cases showing positive or heterogenous expression and expression of the A antigen was negative in four cases showing full methylation. Frequencies of cases showing negative A antigen expression were 100% (4/4) in full methylation, 66.7% (6/9) in partial methylation and 27.3% (6/22) in no methylation and significant association was observed between methylation status (full, partial and no methylation) and expression of the A antigen ($P=0.0093$) (Table 4). In analysis using MSP, methylation of *RE 7* was observed in nine cases, of which six cases showed full or partial methylation in BiPS analysis and the expression of the A antigen was negative in these six cases (Table 3). Discrepancies between MSP and BiPS analysis were shown in three cases, which showed methylation only in MSP and heterogeneous expression of the A antigen. Positive expression of the A antigen was found in 11 cases, in which two cases showed methylation of regions 1 through 3 by BiPS analysis and no cases showed methylation of *RE 7* by MSP (Table 3).

Correlation of the Expression of A Antigen with A Allelic Loss and Hypermethylation of the *ABO* Gene Promoter Region

In analysis of 44 cases that underwent TUR, loss of the A allele was observed in nine cases, among

Figure 3 Expression of the blood-group A antigen, allelic status of the *ABO* gene and MSP of region 7 in cases that underwent radical cystectomy. (**A**) Immunostaining of A antigen in tumor (a, b, c), dysplasia (d, e, f), and corresponding normal urothelium (g, h, I) from cases A-10, AB-3 and A-15, respectively. A-10 showed positive staining in tumor (a), dysplasia (d) and normal urothelium (g), while the tumor section showed heterogeneous staining for the case AB-3 (b), and negative staining for the case A-15 (c). Normal urothelium from cases A-10 (g), AB-3 (h) and A-15 (i) stained positively. Reduced from × 100. High magnification view (× 400) was shown as inset. (**B, C**) Analysis of LOH of the *ABO* gene locus using blunt-end SSCP and methylation status by MSP (*RE 7*). A-10 showed the expression of the A antigen in tumor tissue, no allelic loss and unmethylated CpG sites. AB-3 showed heterogenous expression of the A antigen and methylation of the *ABO* gene, while the A allele was retained. A-15 showed negative expression of the A antigen, loss of A alleles and methylation of the *ABO* gene.