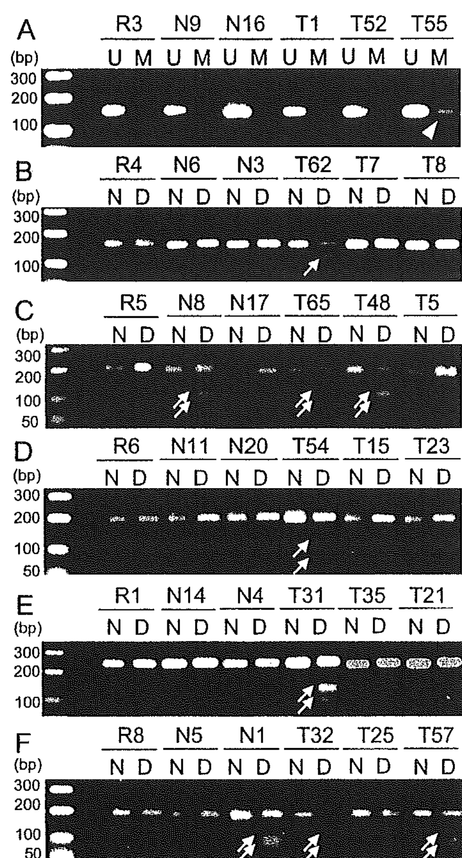

FIG. 2. Examples of PCR products from DNA methylation analyses of multiple CpG islands in patients with or without bladder cancer. DNA methylation status on CpG islands of *p16* gene (*A*) was evaluated by MSP. In this analysis PCR products generated by primer sets reflected presence of methylated (*M*, arrowhead) and unmethylated (*U*) genes. DNA methylation on CpG islands of *DAPK* (*B*) gene, and MINT-2, 12, 25 and 31 clones (*C* to *F*, respectively) was evaluated by COBRA. In this analysis only methylated genes (arrows) were digested by restriction enzymes. *R*, normal urothelium obtained from patients who underwent total pelvic exenteration for rectal cancer. *N*, noncancerous urothelium showing no remarkable histological changes obtained from patients with bladder cancer. *T*, TCC. *N*, non-digestion with restriction enzyme. *D*, digestion with restriction enzyme.

which 5 or all 6 CpG islands could be evaluated. However, we examined DNA methylation status on only 4 or fewer CpG islands in the remaining 18 specimens because of a shortage of DNA extracted from the microdissected samples. Concurrent DNA methylation on 3 or more CpG islands was detected in 0 of the 8 normal urothelium samples (0%), 6 of the 16 NBCs (38%) and 37 of the 63 TCCs (59%). Such TCCs were considered CIMP positive based on described criteria.[4] The incidence of concurrent DNA hypermethylation on 3 or more CpG islands increased progressively from normal urothelium to NBCs and then to TCCs (chi-square test p = 0.0043). Even in NBCs it was significantly higher than in normal urothelium (chi-square test p = 0.0455).

Correlation between DNA methylation status on multiple CpG islands and clinicopathological parameters in TCC 23 (52%). Of the 44 specimens of superficial carcinoma (pTa, pTis and pT1) and 14 (74%) of 19 of invasive carcinoma (pT2 to pT4) were CIMP positive. Invasion depth (pTa, pTis and pT1 vs pT2 to pT4) did not significantly correlate with CIMP (chi-square test p = 0.1131). Ten of 25 specimens (40%) of papillary carcinoma (pTa) and 27 of 38 (71%) of nonpapillary carcinoma (flat CIS or pTis and invasive carcinoma pT1 or greater) were CIMP positive. The incidence of CIMP was

Specimen	Number of methylated CpG islands	Sample name	pT	DNMT1 protein expression	p16	DAPK	MINT-2	MINT-12	MINT-25	MINT-31
Normal urothelia obtained from patients with rectal cancer	<3	R1		−						■
		R2		−			■			
		R3		−		NE	■			
		R4		−			■			
		R5		+					■	■
		R6		+			■			■
		R7		−		■				■
		R8		−			■		■	
Non-cancerous urothelia showing no remarkable histological changes obtained from patients with bladder cancer	<3	N1		−						■
		N2		−					NE	■
		N3		+						■
		N4		+		NE				■
		N5		−		NE	■			
		N6		+			■			
		N7		−			■			■
		N8		−			■			■
		N9		+			■		NE	■
		N10		−			■		■	
	≥3	N11		−		■	■		■	
		N12		−	■		■		■	
		N13		+	■	■	■			
		N14		+	■	■	■			
		N15		−		■	■	■		■
		N16		+		■	■		■	■
Transitional cell carcinomas	<3 (CIMP-negative)	T1	pTa	−						
		T2	pTa	−					NE	
		T3	pTa	−						
		T4	pTa	−						■
		T5	pTa	−						■
		T6	pT2-3	++				NE		■
		T7	pTa	++						■
		T8	pTis	++						■
		T9	pTis	++					NE	■
		T10	pTa	+			■			NE
		T11	pT2-3	+					■	■
		T12	pTis	++					■	■
		T13	pTa	−			■	NE		■
		T14	pTa	−			■			■
		T15	pTa	−			■			■
		T16	pTa	−			■			■
		T17	pT1	+			■			■
		T18	pT2-3	+			■			■
		T19	pT2-3	+			■			■
		T20	pTis	++			■			■
		T21	pTis	++			■			■
		T22	pTa	−	NE		■			■
		T23	pTa	−			■		■	
		T24	pTa	−			■		■	
		T25	pT2-3	−			■		■	
		T26	pTa	−		■	■			
	≥3 (CIMP-positive)	T27	pTa	−			■		■	■
		T28	pT2-3	+			■		■	■
		T29	pT2-3	+		NE	■		■	■
		T30	pT1	++			■		■	■
		T31	pTis	++			■		■	■
		T32	pT2-3	NE	NE		■		■	■
		T33	pTa	+	NE	■			■	■
		T34	pT2-3	−			■	■		■
		T35	pTa	−			■	■		■
		T36	pT2-3	+			■	■		■
		T37	pTa	+			■	■		■
		T38	pTis	+		NE	■	■		■
		T39	pT1	++		NE	■	■		■
		T40	pT2-3	−		■	■			■
		T41	pT1	+		■	■			■
		T42	pTis	++		■	■			■
		T43	pT2-3	++	■		■			■
		T44	pTis	++	■		■			■
		T45	pT2-3	+	■	■				■
		T46	pTa	+			■	■	■	
		T47	pT1	+		■	■	■		
		T48	pTis	++		■	■	■		
		T49	pTa	−	■		■	■		
		T50	pT2-3	+	■	■	■			
		T51	pT2-3	++			■	■	■	■
		T52	pT2-3	−		■	■		■	■
		T53	pTa	−		■	■		■	■
		T54	pT1	−		■	■	■		■
		T55	pTa	−	■	NE	■	■		■
		T56	pTa	−	■		■	■		■
		T57	pTa	−	■		■	■		■
		T58	pT2-3	++	■	NE	■	■		■
		T59	pT1	++	■		■	■	■	
		T60	pTis	+		■	■	■	■	■
		T61	pT2-3	+	■		■	■	■	■
		T62	pTis	++	■	■		■	■	■
		T63	pT2-3	+	■	■	■		■	■

FIG. 3. DNA methylation profiles for CpG islands and protein expression levels of DNMT1 in specimens in which 5 or all 6 CpG islands could be evaluated. DNA methylation status was examined by MSP or COBRA (fig. 2). DNMT1 protein expression levels were defined as described. Vertical columns indicate specimen number, invasion depth in TCC specimens and protein expression of DNMT1. Top row indicates CpG islands. Filled box indicates methylated. Open box indicates unmethylated. *NE*, not evaluable.

significantly higher in nonpapillary than in papillary carcinomas (chi-square test p = 0.0143). Among nonpapillary carcinomas there was no significant difference in the incidence of CIMP between flat CIS (pTis in 7 or 12 specimens or 58%) and invasive carcinomas (pT1 or greater in 20 of 26 or 77%) (chi-square test p = 0.2402).

Correlation between DNA methylation status on multiple CpG islands and DNMT1 protein expression during multistage urothelial carcinogenesis. We have previously reported

the results of immunohistochemical examination for DNMT1 in 89 of the current 105 specimens.[7] We subjected the remaining 16 specimens to the same immunohistochemical examination for DNMT1. DNMT1 immunoreactivity of a tissue sample was considered positive (+) if more than 30% of cells showed the same nuclear staining intensity as positive internal control lymphocytes, and strongly positive (++) if more than 30% of cells showed stronger intensity, as described previously.[7] Figure 3 shows the intensity of DNMT1 immunoreactivity in the 86 specimens for which DNA methylation status on 5 or all 6 CpG islands could be evaluated. In 86 specimens of normal urothelium, NBC and TCC concurrent DNA hypermethylation on 3 or more CpG islands significantly correlated with increased (+ or ++) DNMT1 protein expression (chi-square test $p = 0.0167$).

DISCUSSION

The incidence of aberrant DNA hypermethylation, such as concurrent DNA methylation on 3 or more CpG islands, was significantly higher in NBCs than in normal urothelium. TCCs are notorious for their clinical features of multicentricity and tendency toward recurrence. Synchronously or metachronously multifocal TCCs often develop in certain patients. Although multifocal development of TCCs may be partly attributable to intraluminal seeding, a possible mechanism for multiplicity is the field effect, whereby carcinogenic agents in urine cause malignant transformation of multiple urothelial cells.[12] Even noncancerous urothelium showing no remarkable histological changes can be considered precancerous, because they may be exposed to carcinogens in the urine. Our data suggest that aberrant DNA hypermethylation on multiple CpG islands may participate even in precancerous conditions during multistage urothelial carcinogenesis.

CIMP did not correlate with TCC aggressiveness (eg depth of invasion) but it significantly correlated with morphological structure (papillary vs nonpapillary). Bladder carcinomas are classified as papillary or nodular according to their macroscopic configurations. Papillary carcinomas usually remain noninvasive, although patients must undergo repeat cystoscopic resection because of recurrences.[13] In contrast, the clinical outcome of nodular invasive carcinomas is poor.[13] Flat CIS, which frequently spreads widely and is sometimes scattered over the bladder, is associated with nodular invasive carcinomas. Frequent *p53* gene mutations[14] and loss of heterozygosity on chromosome 14q[15] indicated a common background for flat CIS and invasive carcinomas and, therefore, flat CIS is considered a precursor of nodular invasive carcinomas of the bladder. In this study we successfully examined DNA methylation status even in flat CIS, which was macroscopically indistinguishable from noncancerous urothelium, using microdissection techniques. Our results suggest that CIMP is particularly associated with the development of flat CIS and nodular invasive carcinomas with a poorer prognosis.

DNMT1 targets replication foci, where DNA methylation patterns are copied from the mother strand, by binding to PCNA.[16] However, excessive amounts of DNMT1, which cannot target replication foci, may participate in de novo methylation of CpG islands that are not methylated in normal cells. In addition, targeting of substrate DNA by DNMT1 may be disrupted by mechanisms, such as dysfunction of p21WAF1,[17] which competes with DNMT1 for binding to PCNA, in cancer cells.[16] Moreover, it was recently suggested that DNMT1 is capable of de novo methylating activity as well as having a maintenance function.[18,19] Therefore, it is feasible that in cancers DNMT1 participates in regional DNA hypermethylation on CpG islands. The incidence of concurrent DNA hypermethylation on 3 or more CpG islands significantly correlated with increased DNMT1 protein expression in all examined specimens of normal urothelium, NBC and TCC, suggesting the possibility that the previously proven DNMT1 over expression actually resulted in frequent regional DNA hypermethylation during urothelial carcinogenesis.

DNMT1 mRNA is expressed mainly during the S-phase.[1] Because tumor tissue presumably contains a greater proportion of dividing cells than normal tissue does, it has been debatable whether increased DNMT1 expression is due to an increase in the proportion of dividing cells or to an acute increase in DNMT1 expression per individual cell.[20] However, we have previously reported that DNMT1 expression levels are already increased in NBCs in which the PCNA labeling index has not yet increased.[7] Increased DNMT1 expression did not result entirely from increased numbers of dividing cells in the tissues examined, but rather it clearly preceded increased cell division.[7] In our current study the incidence of concurrent DNA methylation on 3 or more CpG islands was significantly higher in NBCs than in normal urothelium, in parallel with the previously proven DNMT1 over expression. Moreover, the frequent regional DNA hypermethylation observed in our current study and the previously proven DNMT1 over expression were associated with the pathway of development of CIS and nodular invasive carcinomas. These data further support the concept that the previously proven DNMT1 over expression resulted in frequent regional DNA hypermethylation during urothelial carcinogenesis.

In our previous study in nonpapillary carcinomas DNMT1 protein expression was significantly higher in flat CIS than in invasive carcinomas.[7] On the other hand, in our current study there was no difference in the incidence of CIMP between flat CIS and invasive carcinomas. After markedly over expressed DNMT1 induces de novo DNA hypermethylation on multiple CpG islands at the stage of flat CIS, aberrant DNA methylation status may be maintained successfully even if DNMT1 expression is decreased to some extent in invasive carcinomas.

Although DNMT1 is a major DNA methyltransferase in humans, to date 2 other enzymes, namely DNMT3a and DNMT3b, have also been shown to possess DNA methyltransferase activity.[1] Genomic methylation patterns may be established through cooperation among these 3 enzymes even in cancer cells.[19] Further studies of how cooperation between DNMT1 and other components of the DNA methylation machinery affects DNA methylation status in tissue specimens may increase our understanding of the basis of regional DNA hypermethylation during urothelial carcinogenesis.

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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.
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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
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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	

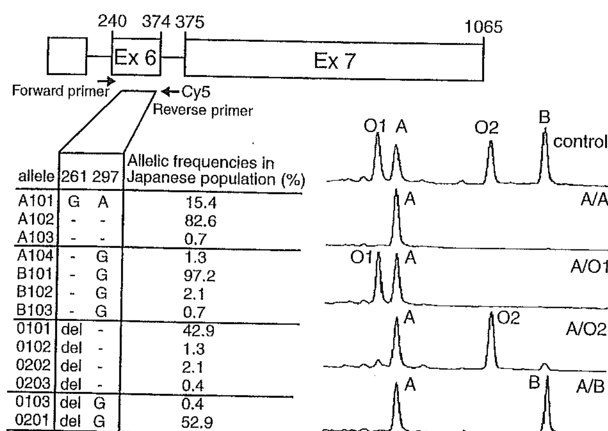
history, the proportion of cases with advanced stage or high-grade tumors was significantly higher in those who underwent radical cystectomy than those who underwent TUR ($P<0.01$). In 44 patients who underwent TUR, DNA was extracted from fresh specimens and normal DNA was extracted from peripheral blood 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



allele	261	297	Allelic frequencies in Japanese population (%)
A101	G	A	15.4
A102	-	-	82.6
A103	-	-	0.7
A104	-	G	1.3
B101	-	G	97.2
B102	-	G	2.1
B103	-	G	0.7
0101	del	-	42.9
0102	del	-	1.3
0202	del	-	2.1
0203	del	-	0.4
0103	del	G	0.4
0201	del	G	52.9

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

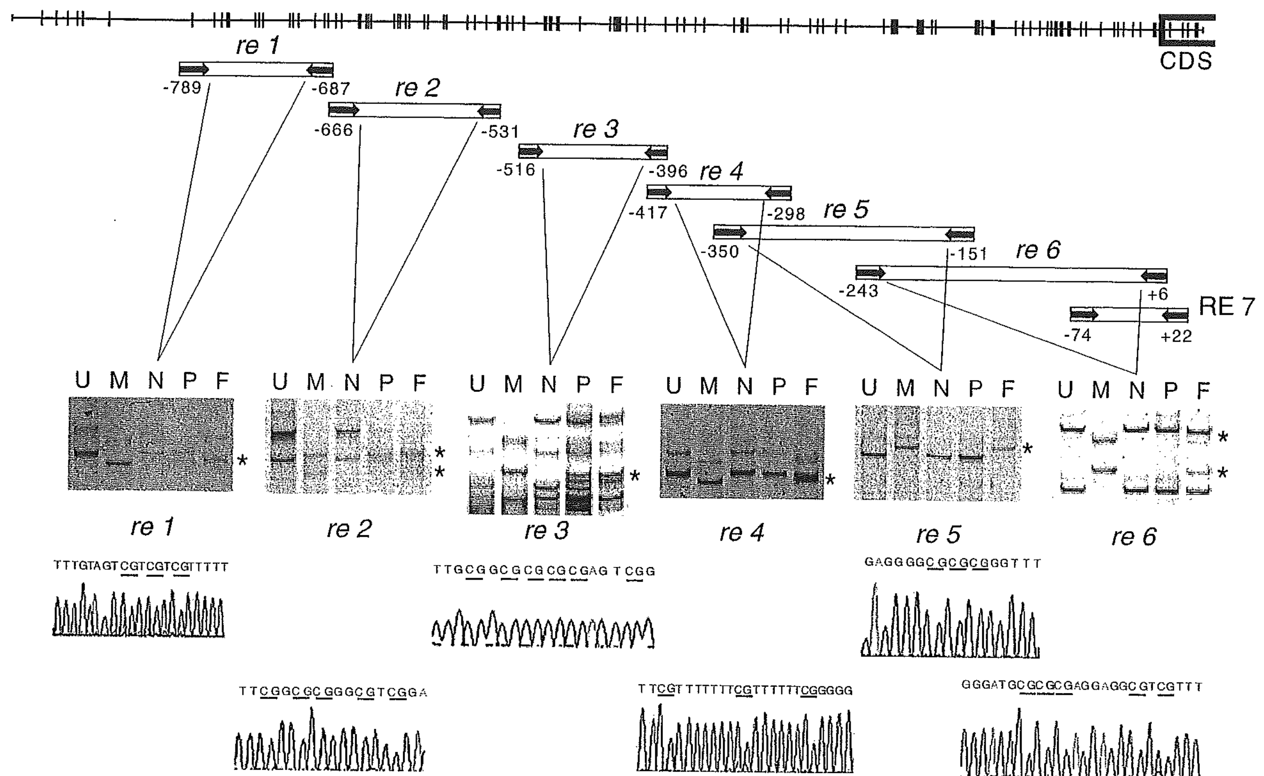


Figure 2 Map of the 5′ CpG island of the *ABO* gene and result of BiPS analysis. (Top) CpG sites in the promoter region of the *ABO* gene are indicated by vertical lines. (Middle) The amplified DNA fragments from regions 1 to 7 are indicated. PCR primer set of each region was indicated by arrows. (Bottom) 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′-CGAATCCCCAAAAAACCCCTACTAA-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

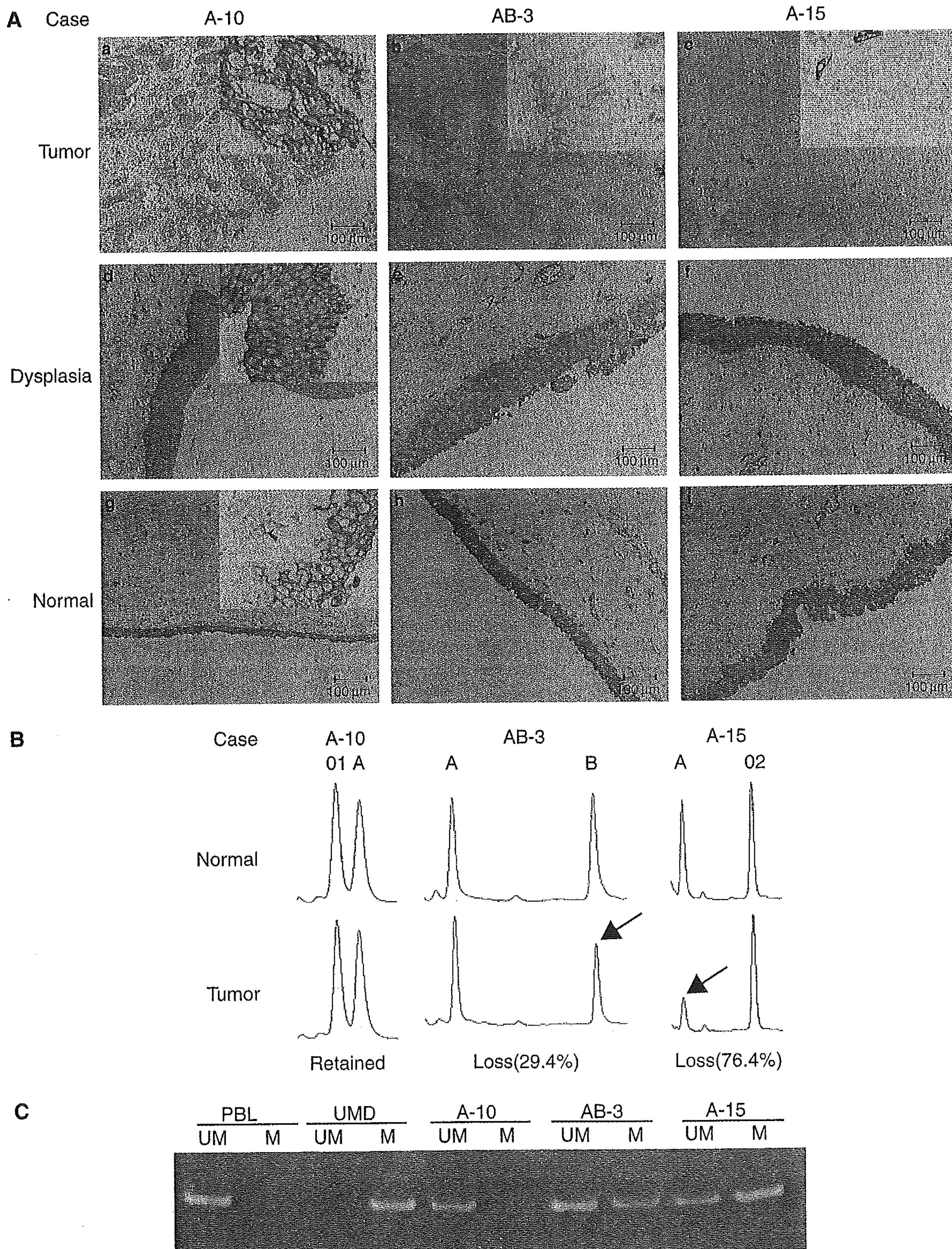
Statistical analysis was performed using a likelihood χ^2 analysis or Fisher's exact test. Probability (*P*) values of <0.05 were considered to be significant.

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.

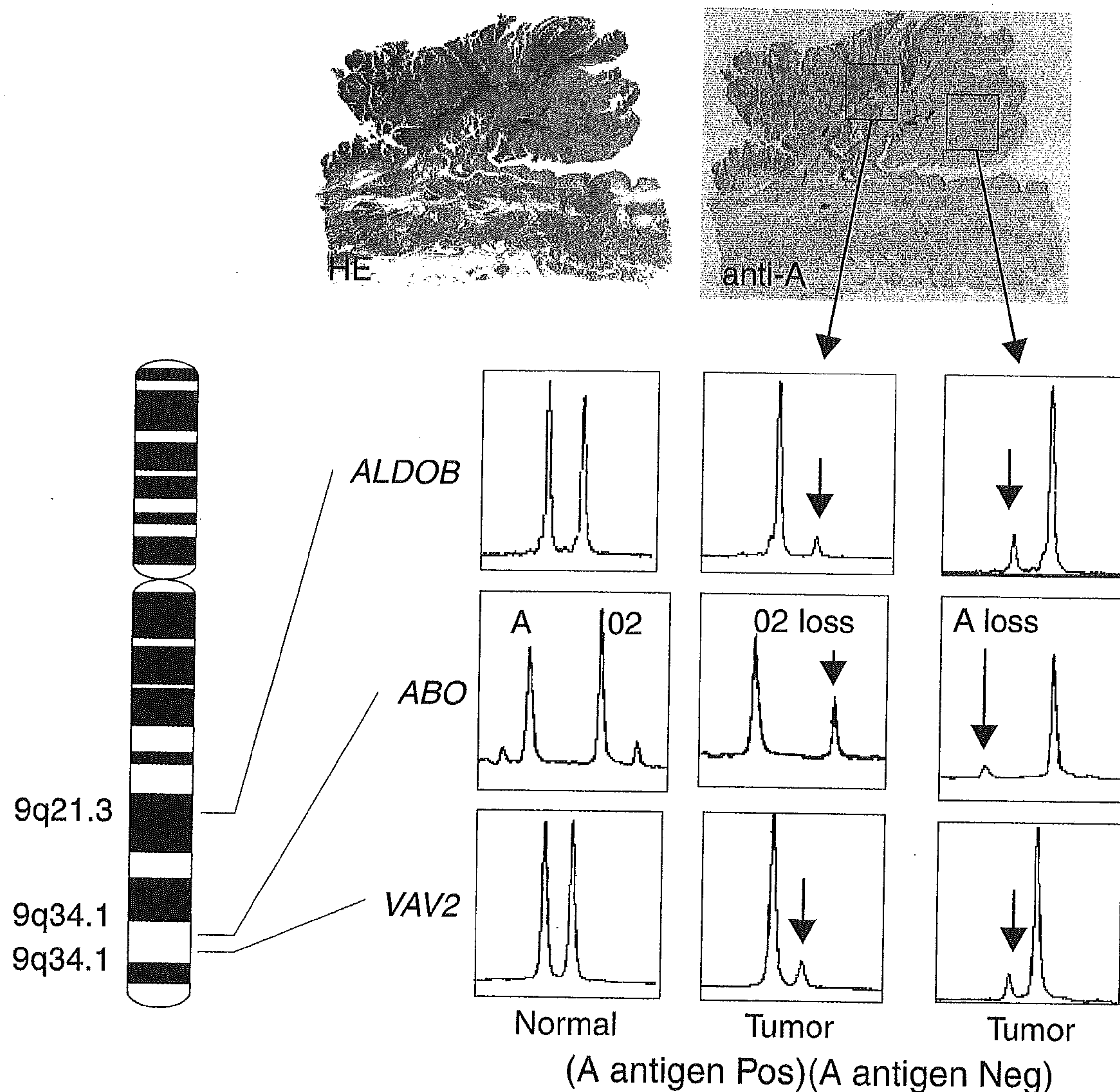


Figure 4 A case of bladder cancer showing chimeric expression of the A antigen. DNA was extracted from areas showing positive or negative A antigen expression and subjected to blunt-end SSCP analysis using three single nucleotide polymorphic markers (*ALDOB*, *ABO* and *VAV2*) on 9q. The patient's genotype was A/O2. A allele was lost in the sample taken from the area showing negative A antigen expression, while O2 allele was lost in the sample taken from the area showing positive A antigen expression. Note that two polymorphic loci (*ALDOB*, 9q21.3 centromeric to the *ABO* locus and *VAV2*, 9q34.1 telomeric to the *ABO* locus) also showed LOH and suggested a large regional chromosome deletion, while the parental origin of the lost allele in these two loci was different between areas showing A-antigen positive or negative expression.

which six cases showed negative and three cases showed heterogenous expression of the A antigen (Table 5). Cases homozygous for A allele were regarded as retaining at least one copy of the *ABO* gene. No statistical association was found between the expression level of the A antigen and A allelic loss ($P=0.26$). In BiPS analysis, expression of the A antigen was negative in all of the four cases with full methylation and statistical association was shown between the expression of the A antigen and methylation status ($P=0.035$). Taking A allelic loss or full methylation in combination, 76.9% (10/13) cases with A allelic loss and/or full methylation showed negative A antigen expression, while the expression of the A antigen was negative in 38.7% (12/31) of cases that retained A allele and showed partial or no methylation. Cases with A allelic loss and/or full methylation showed significant correlation with negative A antigen expression ($P=0.02$) (Table 5). In analysis of 37 cases that underwent radical cystectomy, A allelic loss was observed in seven cases and they all showed negative A antigen expression in the tumor (Table 6). Compared with 30 cases that retained the A allele (including A/A

Table 3 Methylation status in the *ABO* gene promoter region and expression of A antigen

No.	*Case*	*Genotype*	*LOH*[a]	*Methylation status*[b]	*Methylation status (%)*[c]						*RE 7*[d]	*Expression of A antigen*
					re 1	*re 2*	*re 3*	*re 4*	*re 5*	*re 6*		
1	37	A/O2	O2	Full	+(100)	+(100)	+(100)	+(100)	+(100)	+(71)	+	–
2	65	A/B	B	Full	+(100)	+(100)	+(100)	+(86)	+(100)	+(97)	+	–
3	72	A/O1	O1	Full	+(70)	+(62)	+(85)	+(57)	+(87)	+(61)	+	–
4	228	A/B	Ret	Full	+(100)	+(100)	+(100)	+(100)	+(100)	+(100)	+	–
5	85	A/O1	O1	Partial	–	+(85)	+(69)	–	+(100)	+(100)	+	–
6	235	A/O1	O1	Partial	–	+(85)	+(100)	–	–	–	+	–
7	10	A/O2	Ret	Partial	–	–	+(23)	–	–	–	–	–
8	186	A/O2	Ret	Partial	–	+(62)	+(38)	–	–	–	–	–
9	220	A/O1	O1	Partial	–	–	+(62)	–	–	–	–	–
10	229	A/O1	O1	Partial	–	–	+(46)	–	–	–	–	–
11	226	A/O2	Ret	Partial	–	–	+(62)	–	–	–	–	+/–
12	40	A/O1	Ret	Partial	+(100)	+(100)	+(100)	–	–	–	–	+
13	141	A/O2	O2	Partial	+(100)	+(100)	+(100)	–	–	–	–	+
14	5	A/O2	O2	No	–	–	–	–	–	–	–	–
15	43	A/O1	O1	No	–	–	–	–	–	–	–	–
16	77	A/O2	O2	No	–	–	–	–	–	–	–	–
17	97	A/O2	O2	No	–	–	–	–	–	–	–	–
18	195	A/O1	Ret	No	–	–	–	–	–	–	–	–
19	7	A/A	NI	No	–	–	–	–	–	–	–	–
20	71	A/B	B	No	–	–	–	–	–	–	+	+/–
21	184	A/O2	O2	No	–	–	–	–	–	–	+	+/–
22	183	A/B	Ret	No	–	–	–	–	–	–	+	+/–
23	212	A/O2	Ret	No	–	–	–	–	–	–	–	+/–
24	225	A/B	Ret	No	–	–	–	–	–	–	–	+/–
25	3	A/A	NI	No	–	–	–	–	–	–	–	+/–
26	98	A/A	NI	No	–	–	–	–	–	–	–	+/–
27	78	A/O2	Ret	No	–	–	–	–	–	–	–	+
28	79	A/O2	Ret	No	–	–	–	–	–	–	–	+
29	94	A/O1	Ret	No	–	–	–	–	–	–	–	+
30	185	A/O2	Ret	No	–	–	–	–	–	–	–	+
31	193	A/B	Ret	No	–	–	–	–	–	–	–	+
32	221	A/O1	Ret	No	–	–	–	–	–	–	–	+
33	222	A/O2	Ret	No	–	–	–	–	–	–	–	+
34	45	A/A	NI	No	–	–	–	–	–	–	–	+
35	80	A/A	NI	No	–	–	–	–	–	–	–	+

[a]The cases in which A allele was retained were shown.
[b]Full methylation indicates all the regions were methylated, Partial; at least one regions were methylated, No; all the regions were ummethylated by SSCP analysis.
[c]Numbers in parentheses indicate the proportion of CpG sites methylated in the amplified DNA fragments.
[d]Methylation was analyzed using MSP.

Table 4 Correlation of the expression of A antigen with methylation status in 35 cases underwent TUR

Expression of A antigen	*Each locus (Nos. methylated/nos. unmethylated)*						*All loci*			P
	re 1	*re 2*	*re 3*	*re 4*	*re 5*	*re 6*	*Full*	*Partial*	*None*	
Positive/Hetero	2/17	2/17	3/16	0/16	0/16	0/16	0	3	16	0.0093
Negative	4/12	8/8	9/7	4/12	5/11	4/12	4	6	6	

Among 44 cases that underwent TUR, nine cases showing loss of A allele were not included in Table 4.
Hetero: heterogenous expression.

homozygotes), the frequency of A antigen expression was significantly low in those showing A allelic loss ($P=0.003$) (Table 6). MSP of *RE 7* showed methylation in seven cases (18.9%) in which the expression of the A antigen was negative in six cases. Methylation status was significantly correlated with negative expression of the A antigen ($P=0.03$). Taking A allelic loss and methylation in combination, 91% (10/11) of cases with A allelic loss and/or methylation were negative for the A antigen expression, while the expression of the A antigen was negative in 23.8% (5/21) of cases

Table 5 Correlation of the expression of A antigen with A alleleic loss and hypermethylation of the *ABO* gene promoter region in 44 cases that underwent TUR

Expression of A antigen	A allele		P	Full methylation	Partial or no methylation	P	A loss and/or full methylation[a]	A retained and partial/ no methylation	P
	Loss	Retain							
Positive/ Hetero	3	19	0.26	0	22	0.035	3	19	0.02
Negative	6	16		4	18		10	12	

[a]The cases that showed loss of A allele and/or full and partial methylation.
Hetero: heterogenous expression.

Table 6 Correlation of the expression of A antigen with A allele loss and/or hypermethylation of the *ABO* gene promoter region in 37 cases that underwent radical cystectomy

Expression of A antigen	A allele		P	MSP (RE 7)		P	A loss and/or methylated	A retain and unmethylated	P
	Loss	retain		M	UM				
Positive/heterogenous	0	19	0.003	1	16[a]	0.03	1	16	0.0005
Negative	7	11		6	9[b]		10	5	

[a]Two cases were not available.
[b]Three cases were not available.
M, methylated; UM, unmethylated.

showing retained A allele and no methylation. A allelic loss and methylation were significantly correlated with the expression level of the A antigen ($P=0.0005$) (Table 6). In one case, the expression of the A antigen was chimeric and the tumor was divided into areas showing positive or negative expression (Figure 4). This case was an A/O2 heterozygote, and the allelic status was determined from the dissected specimen. O2 allele was lost in the area showing positive staining, while the A allele was lost in the area showing negative staining. Allelic status was also examined in the *ALDOB* and *VAV2* loci, where the parental origin of the lost allele was different between positively and negatively stained areas, indicating that allelic loss in the tumor involved large chromosomal region between 9q21.3 and 9q34.1.

Expression of the A Antigen in Dysplasia and Normal Urothelium

A total of 23 cases that underwent radical cystectomy were examined for expression of the A antigen in concomitant dysplastic lesions and normal urothelium (Table 7). In analysis of 13 cases showing positive A antigen expression in the tumor, A allele was retained in all cases and only one case showed hypermethylation together with normal expression of the A antigen in the dysplasia specimen. In analysis of 10 cases showing negative expression of the A antigen in the tumor, eight showed A allelic loss and/or methylation. Abnormal expression of the A antigen was observed only in one case (A-9), in which dysplasia specimen showed heterogeneous expression but A allelic loss and methylation were not observed in the tumor.

Discussion

Previously, we reported that LOH on chromosome 9 was a frequent genetic event in TCCs of the bladder and its detection in urine samples would be an useful indicator for tumor recurrence in patients with TCC that underwent TUR.[4] Frequencies of LOH of the *ABO* locus examined in this study seems higher than those reported previously.[17,18] In previous studies, allelic status of the *ABO* gene was examined by PCR/RFLP; however, LOH is barely detectable by PCR/RFLP if the proportion of tumor cells in the sample is below 60%, due to the formation of heteroduplex dimers that are resistant to the restriction enzyme digestion.[30] Blunt-end SSCP analysis is a sensitive method to detect an LOH from clinical samples, of which the proportion of tumor cells is as low as 10–20%.[26] However, LOH study from small lesions such as concomitant dysplasia was still difficult due to technical problems. Slebos *et al*[31] reported that the lower the amount of DNA in the PCR, the greater the risk for allele ratios that were abnormal due to a chance distribution of alleles in the reaction and the DNA equivalent of a minimum of about 100 cells is required for a full representation of both alleles in the analysis. Furthermore, DNAs extracted from formalin-fixed paraffin-embedded sections often harbor degradation and fail in the PCR amplifica-

Table 7 Correlation of the expression of A antigen in the tumor, dysplasia and normal urothelium specimens with the genetic and epigenetic changes in the primary tumor

Case	*Genotype*	*Tumor*			*Dysplasia expression*	*Urothelium expression*
		Expression	*LOH*	*Methylation status*		
A-22	A/O1	Positive	O1	M	Positive	Positive
A-1	A/O2	Positive	O2	UM	Positive	Positive
A-10	A/O2	Positive	O2	UM	Positive	Positive
A-3	A/O2	Positive	O2	UM	Positive	Positive
A-46	A/O2	Positive	O2	UM	Positive	Positive
A-5	A/O1	Positive	Ret	UM	Positive	Positive
A-43	A/O1	Positive	Ret	UM	Positive	Positive
AB-5	A/B	Positive	Ret	UM	Positive	Positive
A-5	A/O1	Positive	Ret	UM	Positive	Positive
A-18	A/O1	Positive	Ret	NA	Positive	Positive
A-29	A/A	Positive	NI	UM	Positive	Positive
A-47	A/A	Positive	NI	UM	Positive	Positive
A-14	A/A	Positive	NI	NA	Positive	Positive
A-15	A/O2	Negative	A	M	Positive	Positive
A-16	A/O2	Negative	A	M	Positive	Positive
A-48	A/O2	Negative	A	M	Positive	Positive
A-2	A/O2	Negative	A	UM	Positive	Positive
A-31	A/O1	Negative	A	UM	Positive	Positive
A-6	A/O1	Negative	A	NA	Positive	Positive
A-13	A/O1	Negative	O1	M	Positive	Positive
A-9	A/O1	Negative	O1	UM	Hetero	Positive
A-33	A/O2	Negative	O2	NA	Positive	Positive
AB-2	A/B	Negative	B	M	Positive	Positive

UM and M indicate whether the *RE 7* sequences were unmethylated and methylated, respectively;
Hetero: heterogenous expression; NA: not applicable.

tion, suggesting potential difficulty in assessing the allelic status of small lesions from archival materials. The aim of the present study was to elucidate the underlying mechanisms of reduced expression of the histo-blood group A antigen in bladder cancer, and to determine if IHC of the A antigen expression could be available as a hallmark to determine the allelic loss and/or epigenetic alterations of the *ABO* gene on a cell-to-cell basis.

In cases with radical cystectomy, allelic status was examined using DNAs extracted from histological slides and directly comparable with the A antigen expression in the same specimen and expression of the A antigen was negative in all cases showing A allelic loss. In cases that underwent TUR, three cases showed heterogenous expression of the A antigen, regardless of A allelic loss in the sample. In TUR cases, DNAs were extracted from fresh frozen samples obtained by cold-cup biopsies, while the expression of the A antigen was examined in formalin-fixed paraffin-embedded sections of the resected tumors. Discrepancies between A allelic loss and A antigen expression in TUR cases may be explained by the difference of materials subjected to analysis. As we indicated in Figure 4, some tumors show polyclonal development as to the allelic loss of chromosome 9 and direct comparison between biopsies and resected specimen may be difficult in such cases. In BiPS analysis, full methylation was observed in four cases and they all showed negative expression of the A antigen (Table 5). CpG islands were densely methylated in full methylation and they were closely correlated with the transcriptional silencing of the *ABO* gene. In cases with partial methylation, A antigen expression was also negative in 66.7% (6/9) of cases. Although partial methylation may play some role in transcriptional silencing, we used full methylation as an indicator of methylation in this study. As methylation extended to the most downstream of the *ABO* gene promoter region (*re 6*) in full methylation, we designed a primer set for MSP spanning region 7, which overlapped the downstream of region 6. The size of the amplified DNA fragment in MSP was as short as 96 bp and we used it as an indicator of full methylation in analysis of DNAs extracted from formalin-fixed paraffin-embedded sections. As MSP amplifies methylated DNA sequences selectively, its sensitivity is much higher than that of BiPS analysis and may have a risk of overestimation. In fact, MSP showed methylation in three cases that showed no methylation in BiPS analysis and the expression of the A antigen in these three cases were heterogenous. This may indicate the heterogeneity of the methylation status, suggesting only small number of cells harbored methylation (Table 3). In cases that underwent TUR, negative A antigen expression was signifi-

cantly correlated with full methylation ($P=0.035$), but not with A allelic loss ($P=0.26$) (Table 5). In cases that underwent radical cystectomy, both methylation and A allelic loss were significantly correlated with the expression of the A antigen ($P=0.003$ for A allelic loss, $P=0.03$ for MSP, respectively). Using these two indices in combination, 29.5% (13/44) of the cases that underwent TUR and 29.7% (11/37) of cases that underwent radical cystectomy showed loss of the A allele and/or hypermethylation of the *ABO* gene. They were significantly correlated with the expression of the A antigen ($P=0.02$ for TUR cases, $P=0.0005$ for radical cystectomy cases) (Tables 5 and 6). Negative A antigen expression was observed in 50.0% (22/44) in TUR cases and 48.6% (18/37) in cases that underwent radical cystectomy, which was attributable to genomic deletion and/or hypermethylation of the *ABO* gene in at least 45% (10/22) of cases that underwent TUR and 66.7% (10/15) of cases that underwent radical cystectomy. It is apparent that A allelic loss and/or hypermethylation of the *ABO* gene could not be the sole cause for negative A antigen expression. As the antigenic determinant of the A antigen is the terminal structure of the carbohydrate chains, incomplete synthesis of carbohydrate chains associated with oncogenesis may also be concerned with the reduced expression of the A antigen. Methylation seems to be more predominant than loss of the A allele in cases that underwent TUR. This might be explained by the observation that superficial papillary tumors such as pTa or pT1 stages comprised most of the TUR cases, while more than 70% of them were invasive cancers above Stage pT2 in cases that underwent radical cystectomy. In our previous study, frequencies of LOH on chromosome 9 were 67% in pTa, 71% in pT1 and 80% in tumors ≧pT2 stages.[4] As for the putative tumor suppressors found on chromosome 9, p16 and p14^{ARF} are located on 9p21.[32,33] And an area on 9q31–34 is most prone to be deleted in TCC of the bladder,[34,35] which is also a candidate locus for a putative tumor suppressor gene. Reportedly, deletion of chromosome 9 is an early genetic event in the development of bladder cancers.[1] However, there is not enough evidence to support this hypothesis regarding the occurrence of chromosome 9 deletion in preneoplastic lesions. In a few studies using microsatellite markers from microdissected specimens, allelic loss on chromosome 9 was observed in bladder dysplasia.[3,7] We studied the expression of the A antigen on the dysplasia specimens by IHC, aiming at screening genetic alterations in precancerous lesions of the bladder. Expression of the A antigen was examined in 23 cases of bladder cancer comprising dysplasia, among which the numbers of tumors showing positive or negative expression were 13 and 10, respectively. All of the cases showing positive expression retained the A allele in the tumor and only one case showed hypermethylation, while the expression of the A antigen was preserved in dysplasia and normal urothelium in all cases. In the analysis of 10 cases showing negative A antigen expression in the tumor, loss of the A allele and/or the hypermethylation was observed in eight cases. Expression of the A antigen was preserved in normal urothelium and dysplasia in all but one case showing heterogenous expression in the dysplasia. This case did not exhibit LOH or hypermethylation in the tumor. These results suggested that LOH and/or hypermethylation of the *ABO* gene were infrequent genetic and epigenetic alterations in dysplasia and normal urothelium of the bladder bearing TCC. Furthermore, one case showed chimeric expression of the A antigen in the tumor, among which the expression of the A antigen coincided with loss or retention of the A allele (Figure 4). Analysis of two polymorphic markers in the vicinity of *ABO* gene locus also showed LOHs and the parental origin of the lost allele in these two loci was opposite as was shown in analysis of the *ABO* gene locus. Previously, we reported loss of chromosome 9 was observed in 71% of TCCs of the bladder and nearly 50% of them involved both 9p and 9q, suggesting monosomy or uniparental aneusomy of chromosome 9.[4] Thus, the deletion was considered to involve large chromosomal regions at least between 9q21.3 and 9q34.1 and possibly on the same allele. This finding may suggest the idea that the tumor showed polyclonal development as to the deletion of the 9q allele and that the loss of chromosome 9 might not be an early genetic event associated with tumorigenesis.

In conclusion, reduced expression of the A antigen in bladder cancer reflects allelic loss of the *ABO* gene assigned to 9q34.1 and/or hypermethylation of its promoter region, which is a specific marker for genetic and epigenetic alterations in bladder cancer but not in dysplasia.

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Randomized trial of dietary fiber and *Lactobacillus casei* administration for prevention of colorectal tumors

Hideki Ishikawa[1*], Ikuko Akedo[2], Toru Otani[3], Takaichiro Suzuki[4], Tomiyo Nakamura[1], Ikuko Takeyama[1], Shingo Ishiguro[5], Etsuo Miyaoka[6], Tomotaka Sobue[7] and Tadao Kakizoe[8]

[1]*Laboratory of Hereditary Tumors, Institute for Advanced Medical Sciences, Hyogo College of Medicine, Osaka, Japan*
[2]*Internal Medicine, Osaka Central Hospital, Osaka, Japan*
[3]*Department of Gastroenterology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[4]*Department of Cancer Epidemiology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[5]*Department of Pathology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[6]*Department of Mathematics, Tokyo University of Science, Tokyo, Japan*
[7]*Cancer Information and Epidemiology Division, Research Institute, National Cancer Center, Tokyo, Japan*
[8]*President, National Cancer Center, Tokyo, Japan*

The epidemiologic evidence that dietary fiber protects against colorectal cancer is equivocal. No large-scale clinical study of the administration of *Lactobacillus casei* has been reported. We examined whether dietary fiber and *L. casei* prevented the occurrence of colorectal tumors. Subjects were 398 men and women presently free from tumor who had had at least 2 colorectal tumors removed. Subjects were randomly assigned to 4 groups administered wheat bran, *L. casei,* both or neither. The primary end point was the presence or absence of new colorectal tumor(s) diagnosed by colonoscopy after 2 and 4 years. Among 380 subjects who completed the study, 95, 96, 96 and 93 were assigned to the wheat bran, *L. casei,* both and no treatment groups, respectively. Multivariate adjusted ORs for occurrence of tumors were 1.31 (95% CI 0.87–1.98) in the wheat bran group and 0.76 (0.50–1.15) in the *L. casei* group compared to the control group. There was a significantly higher number of large tumors after 4 years in the wheat bran group. The occurrence rate of tumors with a grade of moderate atypia or higher was significantly lower in the group administered *L. casei.* No significant difference in the development of new colorectal tumors was observed with administration of either wheat bran or *L. casei.* However, our results suggest that *L. casei* prevented atypia of colorectal tumors.
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Key words: colorectal cancer; *Lactobacillus casei*; dietary fiber; probiotic

The incidence of colon cancer is rapidly increasing in Japan.[1] It has been suggested that this trend is caused by the high-fat, low-dietary fiber diet resulting from Westernization of the lifestyle among Japanese. Indeed, intake of dietary fiber by the Japanese has decreased significantly over the past 10 years.[2]

Since Burkitt[3] proposed that a diet high in dietary fiber prevented colorectal cancer, basic studies have suggested the possibility of prevention of colorectal cancer by dietary fiber, through actions including absorption of carcinogens by insoluble dietary fiber[4] and dilution of bile acids and decrease of mutagenicity due to the increase in stool volume.[5,6]

A large number of case-control studies have suggested that dietary fiber may prevent the development of colorectal cancers.[7] However, reports of large-scale cohort studies have failed to show a preventive effect of dietary fiber against colorectal cancer, causing controversy.[8–11]

Randomized clinical trials have been conducted in Western countries[12–16] to evaluate the effectiveness of dietary fiber, using the development of colorectal adenoma as an end point. Many of these studies failed to prove that dietary fiber prevented the development of colorectal adenoma. No intervention study on dietary fiber has been reported in Asians.

It has been shown that *Lactobacillus casei* strain Shirota reduces DNA damage induced by chemical carcinogens in laboratory studies[17] and prevents carcinogenesis in animal experiments.[18,19] In addition, it has been reported, in humans, that lactobacilli reduce the level of mutagens in stool.[20] Furthermore, oral administration of *L. casei* strain Shirota preparation decreased the recurrence of superficial bladder cancer after transurethral resection,[21,22] and habitual intake of a fermented product with *L. casei* strain Shirota reduced the risk of bladder cancer in an epidemiologic study.[23] Thus, we decided to use a *L. casei* strain Shirota preparation in the present study. It has been suggested that high intake of yogurt and fermented milk is responsible for the low incidence of colon cancer in Finland, where consumption of fat is higher than in other countries.[24] Two case-control studies have shown that yogurt[25] and fermented milk[26] prevent colon cancer. In the Netherlands Cohort Study, it was reported that fermented milk intake showed an inverse relationship with the development of colon cancer, although there was no statistical significance.[27]

In 1993, we initiated a randomized clinical trial to determine whether dietary fiber from wheat bran and *L. casei* prevented the occurrence of colorectal tumors.

Material and methods

Study design and subjects

Part of the study design and methods have been previously described in detail.[28] Subjects were recruited at the Osaka Medical Center for Cancer and Cardiovascular Diseases between June 1993 and September 1997. The study protocol was approved by the Ethics Committee of the Osaka Medical Center for Cancer and Cardiovascular Diseases. Written informed consent was obtained from all subjects.

Inclusion criteria were men and women aged 40–65 years who had had at least 2 colorectal tumors (adenomas and/or early cancers) removed endoscopically within 3 months before recruitment. Endoscopic examination had been conducted twice, to detect and resect polyps, respectively. It must have been performed on the entire large intestine, and the subjects must have had an adequate nutritional status. Excluded were subjects with other malignant tumors, a history of intestinal or gastric resection (except appendectomy), familial adenomatous polyposis and severe illness.

Four regimens were incorporated for prevention of colorectal cancer: A, dietary instruction and regular intake of wheat bran biscuits; B, dietary instruction and regular intake of *L. casei* preparation; C, dietary instruction and regular intake of wheat bran biscuits and *L. casei* preparation; and D, dietary instruction alone.

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***Correspondence to:** Laboratory of Hereditary Tumors, Institute for Advanced Medical Sciences, Hyogo College of Medicine, 3-1-2F Kyomachibori Nishi-ku 2-chome, Osaka 550-0003, Japan. Fax: +81-6-6445-5586. E-mail: cancer@gol.com
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One of the 4 regimens was assigned randomly in advance each week. Physicians recruited subjects according to the regimen decided at the beginning of the week. Patients received consultation, including histologic diagnosis of the resected polyp, from group physicians as soon as possible from 1 week following endoscopic treatment. Since the regimen assignment could not be changed by the physicians or participants or arbitrarily manipulated by the authors, it was regarded as random. Trial physicians recruited all outpatients who met the inclusion criteria.

The amount of wheat bran biscuits and *L. casei* preparation to be consumed in 1 month was given to the participant at the start of the trial, and thereafter the amount for 3 months was given. The trial was started after confirming that the subject understood the procedure of the regimen. During the trial, consultation was performed every 3 months to check the participant's physical condition and to confirm the intake of wheat bran biscuits and the *L. casei* preparation. The regimen was continued for 4 years. Participants' compliance with taking wheat bran biscuits and/or the *L. casei* preparation was assessed at the consultations performed every 3 months. At each consultation, the numbers of unconsumed biscuit packages and silver packages of *L. casei* preparation were estimated by verbal inquiry of the patients, and the amounts of wheat bran biscuits and *L. casei* preparation taken in the past 3 months were calculated as the percentage of the target intake. Consultations were performed 16 times, every 3 months for 4 years, and the average at those 16 consultations was taken as the compliance over 4 years.

The target number of subjects was 100 in each group, for a total of 400 subjects. As the incidence of tumors in the control group can be estimated to be about 60%, a significant difference would be obtained if the occurrence rate of tumors could be reduced to 50% (suppression rate 17%) by administration of wheat bran or *L. casei* preparation. No midpoint analysis was performed.

Tumors discovered by colonoscopy performed at the end of the 2nd and 4th years were included in the analysis. The analysis included subjects with poor compliance with the regimen for intake of wheat bran biscuits and *L. casei* preparation on an intention-to-treat basis. For patients with early colorectal cancers resected during colonoscopy before entry in the trial, colonoscopy was performed 6 months after entry (35, 29, 25 and 24 subjects of groups A, B, C and D, respectively). Tumors discovered by colonoscopy performed at 6 months were excluded from analysis. Subjects who refused participation and dropouts were excluded from analysis.

Dietary instruction

The core purpose of the dietary instruction was to restrict fat intake so that the energy from fat constituted 18–22% of total energy intake. Subjects were asked to record, on a diet record form, the contents of their meals for the 3 days before consultation; and nutritionists calculated, from these records, the total energy intake and intake of fat and oil. Compliance with the dietary instructions on the restriction of fat intake was evaluated at dietary checkups 3 months and 4 years after beginning the regimen, and, when necessary, instruction was given again.

Wheat bran biscuits

Biscuits containing wheat bran at 30% of dry weight were prepared.[29] Patients were instructed to eat 25 g/day wheat bran biscuits (7.5 g as wheat bran) before each meal. Biscuits were developed by Ezaki Glico (Osaka, Japan) and Horii Pharmaceutical Industry (Osaka, Japan). The components and contents of the wheat bran biscuits were as follows: energy, 454 kcal/day; protein, 2.9 g/day; lipid, 3.3 g/day; and nonfibrous carbohydrate, 17.5 g/day.

L. casei *preparation*

The *L. casei* strain Shirota preparation was a powder containing approximately 10^{10} viable cells/g. It was stored in a refrigerator, and 1 g was taken after every meal. The *L. casei* preparation was provided by Yakult Honsha (Tokyo, Japan). The viable cell count of *L. casei* and absence of bacterial contamination were confirmed for all lots every 6 months during the 2-year storage period. To confirm the viable cell count of *L. casei*, MRS agar medium for detection of *L. casei* was used. It has been confirmed in previous studies that the number of bacteria per 1 g of *L. casei* preparation remained in the range of 1.5×10^9 to 2.1×10^{10} during 24 months when stored in a cool place (15°C). In addition, the average number of bacteria is 8.0×10^9 after 24 months.

Colonoscopy

The main end point of the trial was the presence or absence of new colorectal tumor(s). Colonoscopy was performed 2 and 4 years after the start of the regimen. The entire large intestine, from the anus to the cecum, was examined. Examinations for detection of new lesions were performed by 2 physicians. All lesions, except hyperplastic polyps clearly evaluated by colonoscopy, were examined histologically on the basis of the guidelines of the Japanese Society for Cancer of the Colon and Rectum.[30] All histologic diagnoses (inflammatory polyp; hyperplastic polyp; adenoma with mild atypia, with moderate atypia, with severe atypia; early cancer) were performed blindly without identification of the participant's dietary regimen.

In patients with early colorectal cancer, which was diagnosed from tumor tissue resected by colonoscopy before entry in the trial, colonoscopy was performed to detect local recurrence after 6 months of participation. All colorectal tumors discovered with this procedure were resected.

Statistical analysis

All colorectal tumors discovered at the end of the 2nd and 4th years were defined as "new". Analyses at years 2 and 4 were performed separately, and 2×2 contingency table analysis was performed. Comparison of baseline characteristics of subjects with or without wheat bran biscuits or *L. casei* intake was performed by appropriate tests such as *t*-test and the χ^2 test. Logistic regression models were used to estimate the odds ratio (OR) adjusted for covariates such as age and sex. Confidence intervals (CIs) based on Wald statistics were used to assess significance.

Results

Enrollment and randomization

The number of patients who met the inclusion criteria during the screening period was 470 (Fig. 1). All were invited to participate in the trial, but 60 patients (13%) declined. Of 410 patients who agreed to participate, 12 were excluded because of incompatibility with the protocol, including detection of cholangiocarcinoma and gastric cancer in 4, history of gastrectomy in 3, colectomy in one, familial adenomatous polyposis in one, advanced age in one, young age in one and more than 3 months after endoscopic treatment in one. Thus, 398 patients were assigned to the 4 groups.

Baseline characteristics of subjects

Table I shows the baseline characteristics of the 398 patients randomly assigned and the number of dropouts. There was no difference in baseline characteristics of subjects such as dietary content among the 4 groups. A total of 18 patients (4.5%) did not complete endoscopic examinations. The reasons for not receiving endoscopic examinations were death in 2 patients (from lung cancer and cerebral hemorrhage), serious illness in 5 patients and trial discontinuation in 11 patients. There was no difference in the rate of dropouts among all groups. Excluding 18 dropouts, 380 patients were included in the analysis.

Colonoscopy

Colonoscopic examination was possible throughout the length of the large intestine, up to the cecum, in all cases. There was no difference in the intervention period among groups (Table II).

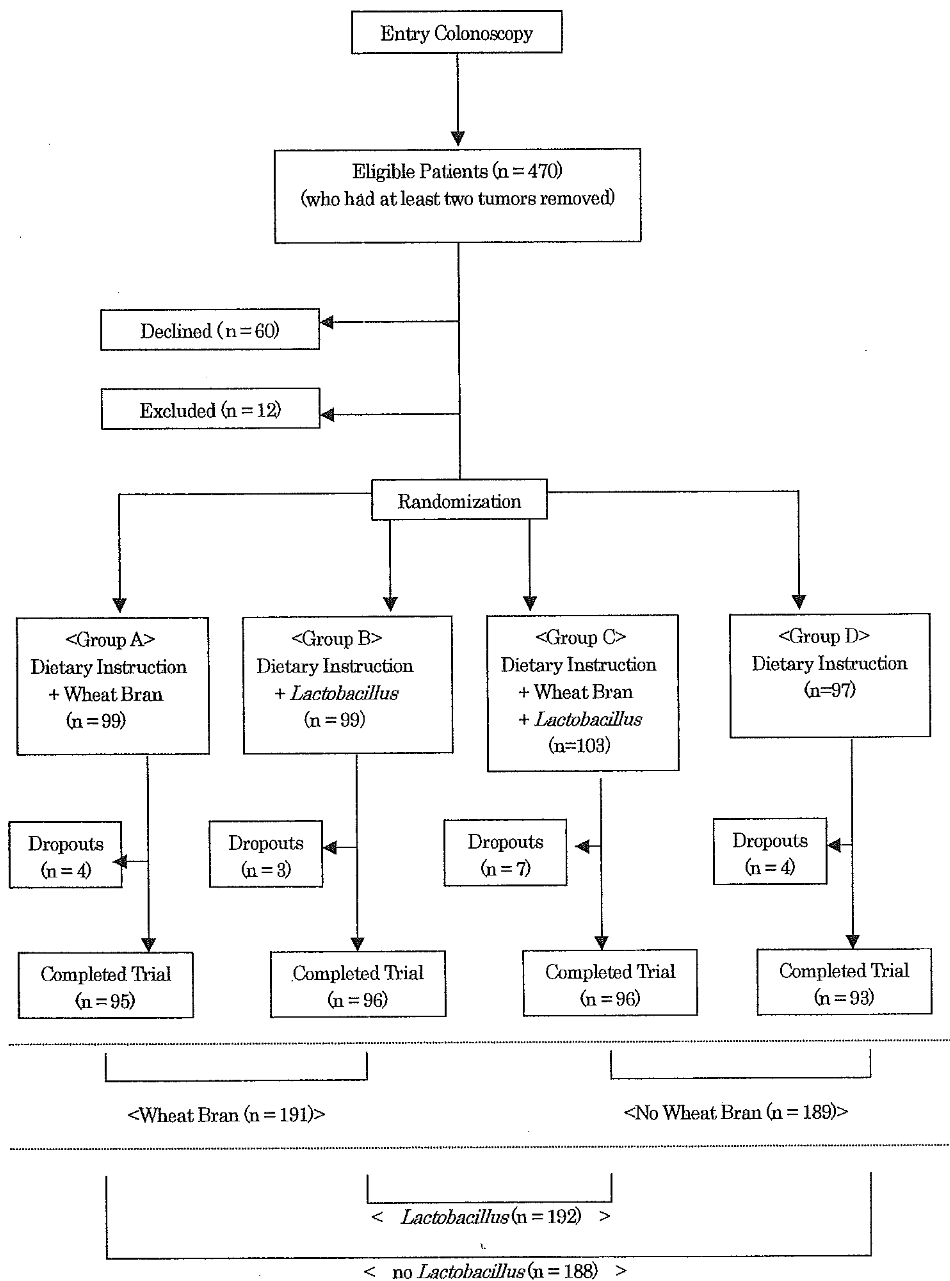

FIGURE 1 – Study participant data.

There was no difference in the time required for insertion into and removal from the cecum in all groups. No difference was found in the proportion of nonneoplastic lesions (inflammatory polyps and hyperplastic polyps).

Compliance

Compliance with intake of wheat bran biscuits was over 90% in 77 persons (40%) and over 70% in 135 persons (71%) for the entire 4 years. Compliance with intake of the *L. casei* preparation was over 90% in 130 persons (68%) and over 70% in 168 persons (88%).

Occurrence of tumors

The results after intake of wheat bran biscuits are shown in Table III. The wheat bran biscuit administration group included 191 persons, while the nonadministration group included 189 persons. The adjusted OR of developing at least one tumor was 1.31 (95% CI 0.87–1.98) after 2 years in the administration group compared to the nonadministration group. It was 1.31 (95% CI 0.87–1.97) after 4 years, showing some increase in adjusted OR, although not statistically significant. The adjusted OR for developing tumors larger than 3 mm was 1.14 (95% CI 0.76–1.72) in the administration group compared to the nonadministration group after 2 years and 1.57 (95% CI 1.04–2.37) after 4 years, showing a significant increase. In particular, the occurrence of tumors larger than 10 mm after 2 years showed no difference between the wheat bran administration and nonadministration groups. However, after 4 years, these tumors did not occur in the nonadministration group while they occurred in 7 patients (3.7%) in the administration group, showing a significant increase. There was no difference in the occurrence of more than one or more than 3 tumors with moderate or severe atypia.

Table IV shows the results after *L. casei* administration. The *L. casei* administration group included 192 persons and the nonad-

TABLE I – BASELINE CHARACTERISTICS OF SUBJECTS[1]

	Group A: wheat bran (n = 99)	Group B: *Lactobacillus* (n = 99)	Group C: wheat bran + *Lactobacillus* (n = 103)	Group D: no treatment (n = 97)
Age (years)	54.7±6.1	54.8±6.5	54.9±6.2	55.5±6.2
Male sex, number (%)	83 (83.8)	79 (79.8)	80 (77.7)	83 (85.6)
Height (cm)	164.6±8.4	164.6±7.3	163.0±7.1	164.1±7.6
Weight (kg)	66.1±10.5	64.6±10.5	62.7±8.7	63.2±9.4
Dietary intake				
Energy (kcal/day)	2,075±368	2,066±411	2,058±402	2,163±404
Total fat (g/day)	54.6±15.2	53.0±13.5	52.8±16.7	56.6±14.9
Dietary fiber (g/day)	15.1±3.9	14.5±3.9	15.4±4.7	15.5±4.0
Calcium (mg/day)	635.4±237.1	638.7±218.4	636.6±246.7	661.4±247.7
Alcohol drinking every day, number (%)	50 (50.5)	37 (37.4)	49 (47.6)	48 (49.5)
Current smoker, number (%)	47 (47.5)	41 (41.4)	43 (41.7)	44 (45.4)
Tumors before recruitment				
Total tumors	5.9±4.3	5.8±5.6	5.2±3.6	5.0±3.4
Adenomas with mild atypia	2.8±2.9	2.7±3.9	2.5±2.6	2.0±2.7
Adenomas with moderate atypia	2.1±2.2	2.4±2.1	1.9±1.8	2.0±1.9
Adenomas with severe atypia	0.6±0.8	0.4±0.7	0.5±1.1	2.0±1.9
Early cancers, number (%)	37 (37.4)	31 (31.3)	29 (28.2)	28 (28.9)
History of colorectal cancer in one parent or sibling, number (%)	15 (15.2)	8 (8.1)	15 (14.6)	11 (11.3)
Dropped out, number (%)	4 (4.0)	3 (3.0)	7 (6.8)	4 (4.1)

[1]Values are means ± SD.

TABLE II – INTERVENTION PERIOD OF COLONOSCOPY

Intervention period	Group A: wheat bran (n = 95)	Group B: *Lactobacillus* (n = 96)	Group C: wheat bran + *Lactobacillus* (n = 96)	Group D: no treatment (n = 93)
Period of 2nd year from entry (days)				
Mean ± SD	679.4±60.8	674.2±31.0	672.1±27.6	680.3±56.9
Maximum	1,009	827	778	925
Minimum	568	617	600	617
Period of 4th year from entry (days)				
Mean ± SD	1,339.6±46.9	1,339.7±51.1	1,338.1±40.5	1,367.4±120.4
Maximum	1,611	1,660	1,617	2,129
Minimum	1,275	1,275	1,233	1,201

ministration group, 188 persons. The adjusted OR of developing at least one tumor was 0.76 (95% CI 0.50–1.15) in the administration group compared to the nonadministration group after 2 years. After 4 years, it was 0.85 (95% CI 0.56–1.27), showing a decrease after both 2 and 4 years, although not statistically significant. For the occurrence of tumors with moderate or severe atypia, the adjusted OR was 0.80 (95% CI 0.52–1.22) in the administration group compared to the nonadministration group after 2 years and 0.65 (95% CI 0.43–0.98) after 4 years, showing a significant decrease after 4 years. There was no difference in the size and number of new tumors that developed.

When the results were examined separately for the different levels of compliance, they were similar to those described above.

Synergistic effects

Tumor occurrence in the group administered both wheat bran and *L. casei* was higher than that in the groups administered wheat bran or *L. casei* and lower than that in the nonadministered group (data not shown). No notable synergistic effects between the treatments were observed.

Adverse events

During the study period, colorectal cancers were discovered in 4 persons by endoscopy, including one person each in groups B, C and D after 2 years and one person in group B after 4 years. There was no bias in their occurrence among the groups. All were cancer invasion of mucosa and were completely resected endoscopically, not requiring colectomy. During the study period, 2 patients died, one of lung cancer in group A and one of cerebral hemorrhage in group C. One person each in groups A and C underwent surgery for peritonitis resulting from acute appendicitis. There was no other serious adverse event.

Discussion

It was found that *L. casei* intake appeared to suppress the development of colorectal tumors; in particular, it prevented, with statistical significance, the development of tumors with moderate and severe atypia. This large-scale randomized clinical study shows that an *L. casei* preparation prevented the development of colorectal tumors.

Since our study was performed at one hospital, the evaluations of endoscopic findings were thought to be consistent. All patients who satisfied the conditions for participation were asked to participate, and the rate of consent to participation was extremely high at 88%, supporting the high validity of the results. The reasons for the high rate of consent could be that a special organization was instituted in this hospital for this trial and that all participants were offered dietary instruction. In addition, the dropout rate was low at 4.5% and compliance was high, indicating that the results were highly reliable. Endoscopic examination was conducted twice before entry so that we could minimize oversights.

In our previous prevention studies, subjects were patients with at least one tumor, whereas the present study included patients with at least 2 tumors. It is known that patients with at least 2 tumors in the large intestine have a higher risk of colon cancer than those with only one tumor. It is difficult to apply the results of our clinical study to the general population. Many of the patients participating in this trial, different from other reports in the past, had a larger number of colorectal tumors together with a history of cancer. This difference appears to have resulted from the background of the population, who had a higher risk of colorectal cancer than those participating in previous clinical trials. Accordingly, our results should be discussed not on the basis of the general population but on the basis of a population with a high risk of colorectal cancer. Nonetheless, our study included patients