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.

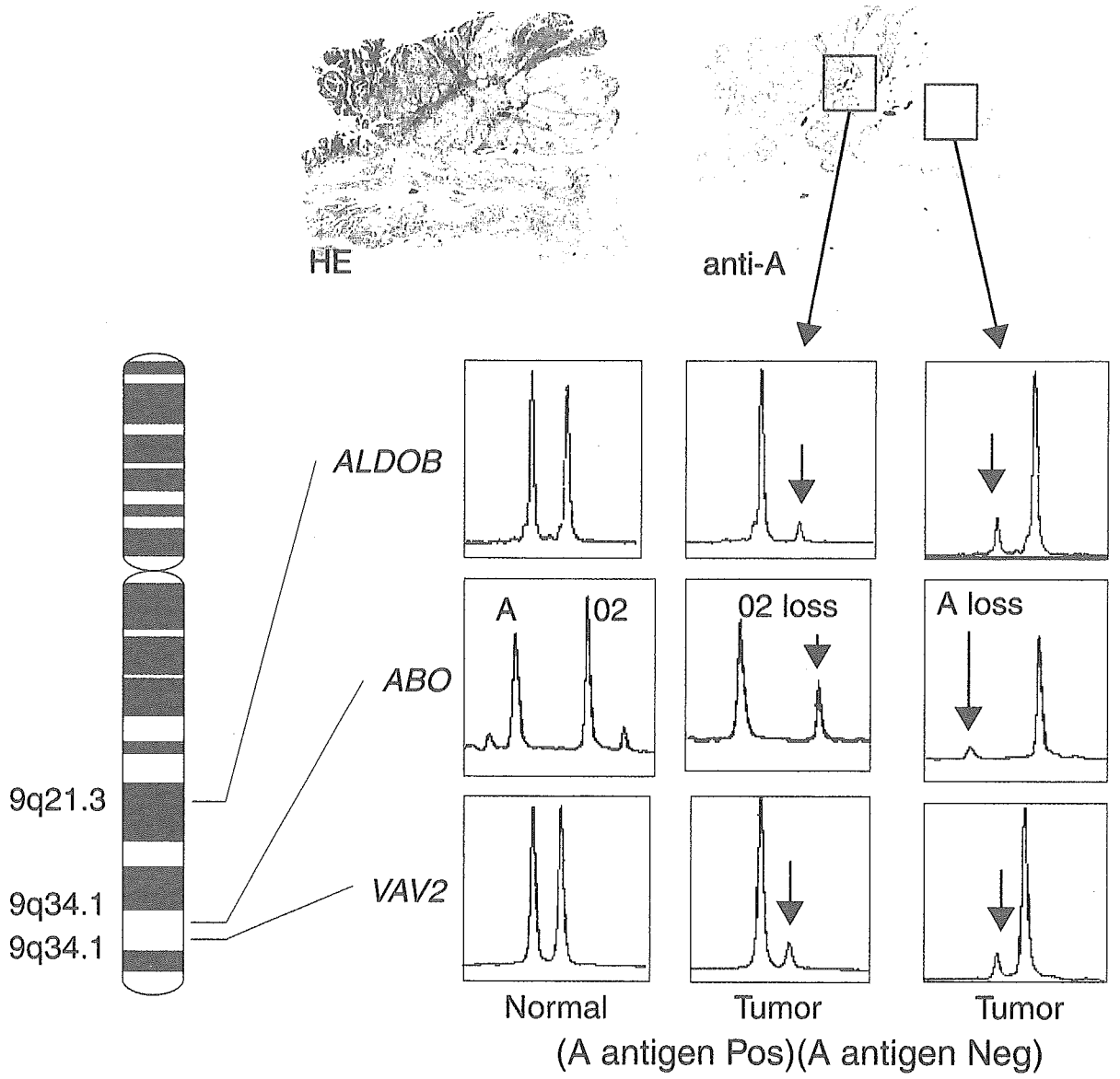

Figure 4 A case of bladder cancer showing chimeric expression of the A antigen. DNA was extracted from areas showing positive or negative A antigen expression and subjected to blunt-end SSCP analysis using three single nucleotide polymorphic markers (*ALDOB*, *ABO* and *VAV2*) on 9q. The patient's genotype 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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