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A High Prevalence of Functional Inactivation by Methylation Modification of *p16^{INK4A}/CDKN2/MTS1* Gene in Primary Urothelial Cancers

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We analyzed the genetic and epigenetic alterations of *p16^{INK4A}/CDKN2/MTS1* gene (*MTS1* gene) in 38 primary urothelial cancers. Genetic alterations of the *MTS1* gene consisted of one base substitution mutation in exon 2 (2.6%) and 6 homozygous deletions (16.2%). Hypermethylation of the 5′ CpG island in exon 1 of the *MTS1* gene was observed in 12 tumors (37.5%). Consequently, 19 of 38 tumors (50%) showed genetic alterations or epigenetic hypermethylation of the *MTS1* gene. Retention of hypermethylated *MTS1* gene(s) in 36% of the tumors showing loss of heterozygosity at the critical region indicates that the methylation modification could be an initial event followed by genomic rearrangements associated with total loss of *MTS1* gene function. Immunohistochemical analysis of *MTS1* expression revealed that all the tumors with genetic alterations of the *MTS1* gene and 9 of 12 highly methylated tumors displayed an absence of *MTS1* nuclear antigen. Genetic and epigenetic changes of the *MTS1* gene were not correlated with the grade and stage of tumors, indicating that these alterations are early events in urothelial carcinogenesis, in which functional inactivation by hypermethylation is a predominant mechanism.

Key words: Hypermethylation — *MTS1* — Urothelial cancer

Multiple genetic alterations are thought to be involved in the pathogenesis of urothelial cancers.[1] The genes involved include some well-characterized tumor suppressor genes such as retinoblastoma and *p53* genes, which are altered in about 30% and 50% of bladder cancers, respectively. The *RB* and *p53* alterations in bladder cancers are often associated with tumor progression.[1–6] In the context of the biological significance of multiple genetic alterations in the stepwise pathogenesis of urothelial cancers, combined alterations of both *RB* and *p53* genes are a very rare occurrence in bladder cancers.[4] This suggests that the alterations of the *RB* and *p53* genes represent either multiple pathways to urothelial carcinogenesis or later events associated with tumor progression, and the critical genetic alteration in urothelial carcinogenesis remains to be identified.

In bladder cancers, frequent allele loss has been demonstrated on chromosome 9p21–22,[7–9] which harbors the tumor suppressor genes *p16^{INK4A}/CDKN2/MTS1* and *p15^{INK4B}/MTS2* (herein referred to as *MTS1* and *MTS2* genes).[10,11] The *MTS1* gene product specifically inactivates cyclin-dependent kinases (CDK4 and CDK6), which interact with cyclin D1 and stimulate the transition of eukaryotic cells through the G1/S boundary of the cell cycle.[12] The *MTS2* gene product is also a negative regulator of cell cycle progression.[13] Homozygous deletions of the *MTS* genes have been described in a variety of human cancer cell lines including bladder cancer lines, but with a significantly lower frequency in primary cancers.[10,11,14,15] These observations suggested the possibility of *in vitro* selection for tumors with *MTS* gene loss or co-loss with other genes, and raised a question as to the significance and prevalence of *MTS* gene alterations *in vivo*. However, most recently, transcriptional silencing of the *MTS1* gene by aberrant DNA methylation has been demonstrated in lung cancers, breast cancers, gliomas, and head and neck tumors, as an alternative mechanism of inactivation.[16–18] Gonzalez-Zulueta *et al.*[19] have also shown a significant correlation between hypermethylation of the *MTS1* gene and its loss of expression in some bladder cancer cell lines and primary bladder cancers, suggesting that epigenetic alteration of the *MTS1* gene also plays a role in urothelial carcinogenesis. Identification of the inactivation pathways is particularly important in connection with developing a molecular strategy for cancer intervention and treatment, either by gene transfer or by demethylation of the critical gene. However, reports on the nature of the inactivation pathways, structural alteration or methylation modification, of the *MTS1* gene in urothelial cancers are conflicting; the reported prevalence of homozygous deletions ranges from 19%[15] to 44%.[20]

We have carried out a comprehensive analysis of the genetic and epigenetic alterations of the *MTS1* gene in 38

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The abbreviations used are: MTS, multiple tumor suppressor; RB, retinoblastoma; PCR-SSCP, polymerase chain reaction single-strand conformation polymorphism; LOH, loss of heterozygosity; IFNA, interferon α; IHC, immunohistochemistry.

primary urothelial cancers, including 30 bladder, 4 renal pelvic and 4 ureteral cancers. We suggest that inactivation of the *MTS1* gene is an early event in urothelial carcinogenesis, in which hypermethylation of the 5′ CpG island associated with the loss of transcription is the predominant mechanism.

MATERIALS AND METHODS

Tissue samples Fresh tumor tissue samples were obtained from 30 patients with bladder cancers, 4 patients with ureteral cancers, and 4 patients with renal pelvic cancers. Portions of each tumor specimen were fixed in 10% formalin and paraffin-embedded for histological examination and immunohistochemical analysis. Tumors graded according to the WHO classification[21] and staged according to the TNM pathological staging system[22] were divided into non-invasive (pTa) and invasive (pT1-4) groups. Tumor grade was evaluated as the highest grade observed. Kidney tissues, normal bladder mucosa or peripheral blood leukocytes from patients served as controls. DNA of high molecular weight was obtained by proteinase K digestion and phenol-chloroform extraction.

SSCP and sequence analysis We analyzed mutations in exon 1 and exon 2 of the *MTS1* gene by the PCR-SSCP method as described previously.[23] Briefly, the mixture containing [α-^{32}P]dCTP with Ampli Taq (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ) was amplified by 35 cycles of 1 min at 94°C, 1 min at 55–60°C, and 1 min at 72°C. The sense and antisense PCR primers were 5′-GGGAGCAGCATGGAGCCG-3′ and 5′-AGTCGCCCGCCATCCCCT-3′ for *MTS1* exon 1, and 5′-GGAAATTGGAAACTGGAAGC-3′ and 5′-TCTGAGCTTTGGAAGCTCT-3′ for *MTS1* exon 2. Similarly, PCR-SSCP analysis was carried out for exon 2 of the *MTS2* gene, with PCR primers 5′-TGAGTTTAACCTGAAGGTGG-3′ and 5′-GGGTGGGAAATTGGGTAAG-3′. The PCR products were heat-denatured, and then resolved by electrophoresis on 6% non-denaturing polyacrylamide gels with or without 10% glycerol. PCR fragments of exon 2 of the *MTS1* and *MTS2* genes were digested with *Sma* I before electrophoresis to reduce the size to <250 bp.

The PCR products were directly sequenced as described.[23] The products were combined with a ^{32}P-labeled primer and the sequencing reaction was done using a BcaBEST sequencing kit (Takara Co., Kyoto). The sequencing primers were: 5′-CCGGCCCCCACCCTGGCA-3′, 5′-ACACGCTGGTGGTGCTGC-3′, and 5′-TACAAATTCTCAGATCAT-3′. All the coding regions of the *MTS1* exon 2 can be analyzed using these primers.

LOH analysis We analyzed LOH at chromosome 9p21–22 using the following microsatellite primers: the *IFNA* locus, 5′-TGCGCGTTAAGTTAATTGGTT-3′ and 5′-AGTGGGGGTTTCCACCTTAC-3′, and the *D9S171* locus, 5′-AGCTAAGTGAACCTCATCTCTGTCT-3′ and 5′-ACCCTAGCACTGATGGTATAGTCT-3′.[24,25] PCR amplification in the presence of [α-^{32}P]dCTP consisted of 26 cycles of 40 s at 94°C, 40 s at 60°C, and 40 s at 72°C. The PCR products were heat-denatured and then resolved by electrophoresis on a 6% denaturing polyacrylamide gel.

Detection of homozygous deletions of the *MTS1* gene Southern blotting was conducted as described.[10] In brief, 5 μg of genomic DNA was digested with *EcoR* I (10U/μg), separated on 0.8% agarose gel, then transferred to nylon membrane (Hybond-N, Amersham Life Science, Bucks., UK). Each blot was hybridized with the probe for the PCR product containing exon 2 of the *MTS1* gene. It was also hybridized with the probe, *Mup* 7, on chromosome 9 as an internal control. The signal density of each band was measured by an UltroScan XL (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Methylation analysis in the *MTS1* gene PCR-based methylation analysis was performed as described with slight modifications.[19] We examined the methylation status of the upstream portion of the *MTS1* gene including the coding region of exon 1 that resides on the 5′CpG island. Genomic DNA (1 μg) was digested with one of two methylation-sensitive enzymes, *Hha* I and *Hpa* II (40 U/μg), or a methylation-insensitive enzyme, *Msp* I (40 U/μg), for 16 h. The restriction sites of these enzymes in the *MTS1* gene exon 1 are shown in Fig. 1. Digested or undigested DNA was subjected to PCR using primers: 5′-GGGAGCAGCATGGAGCCG-3′ and 5′-AGTCGCCCGCCATCCCCT-3′. PCR amplification with [α-^{32}P]dCTP consisted of 21 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The PCR products were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. Autoradiography was conducted for

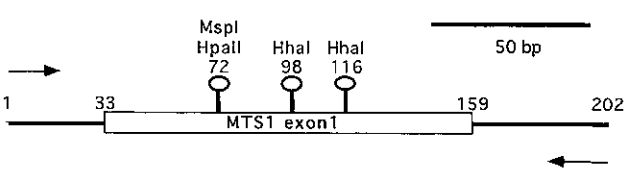

Fig. 1. Schematic representation of PCR-based methylation assay. After digestion with *Hha* I, *Hpa* II, or *Msp* I, PCR was performed under the appropriate conditions. Arrows indicate the PCR primers. PCR amplification produced a 202 bp product. The first base at the 5′ site of the PCR product was designated as number 1. The *MTS1* gene exon 1 that extends from position 33 to 159 was included in the PCR product. *Msp* I and *Hpa* II have the same restriction site at position 72. *Hha* I has restriction sites at positions 98 and 116.

24–48 h at room temperature. The density of each band was measured (UltroScan XL). Under the conditions described above, the PCR products of undigested samples showed obvious bands, whereas the products of samples digested with *Msp* I showed faint or no bands. The assay was in the linear range of amplification by PCR (data not shown). The negative control was the *β-actin* promoter region, which is not considered to undergo hypermethylation.

IHC The immunohistochemical staining of formalin-fixed and paraffin-embedded surgical sections were performed as described elsewhere.[6] Briefly, following depar-

Table I. Genetic Alterations and Hypermethylation of the *MTS1* Gene, Expression of the *MTS1* Gene, LOH at Chromosome 9p21–22 and Pathological Features in 38 Primary Urothelial Cancers

Sample no.	Origin	Grade	Stage	LOH[a] *IFNA*[b]	LOH *D9S171*	Mutation *MTS1*	Homozygous deletion *MTS1, 2*	Methylation *MTS1*	IHC[c] *MTS1*
1	bladder	2	pT2	−	NI	−	+, +	ND	−
2	bladder	2	pTa	−	NI	−	+, +	ND	−
3	bladder	2	pTa	NI	NI	−	−, −	−	+
4	bladder	3	pT3	+	NI	−	−, −	−	−
5	bladder	1	pTa	+	NI	−	+, +	ND	−
6	bladder	2	pT1	NI	NI	−	−, −	+	−
7	bladder	2	pT1	+	NI	−	+, −	ND	−
8	bladder	3	pT1	+	NI	−	−, −	−	−
9	bladder	2	pTa	NI	NI	−	−, −	−	+
10	bladder	3	pT3	−	−	−	−, −	−	+
11	bladder	2	pTa	−	NI	−	−, −	+	−
12	bladder	1	pTa	+	NI	−	−, −	+	+
13	bladder	2	pTa	−	NI	−	−, −	+	−
14	bladder	1	pTa	+	NI	−	−, −	−	+
15	bladder	1	pTa	NI	NI	−	−, −	−	+
16	bladder	2	pTa	NI	NI	−	−, −	+	+
17	bladder	2	pTa	NI	NI	−	−, −	+	−
18	bladder	1	pTa	+	NI	−	−, −	+	−
19	bladder	2	pTa	NI	NI	−	−, −	−	+
20	bladder	2	pT1	−	NI	−	−, −	−	+
21	bladder	3	pT2	−	NI	−	−, −	+	−
22	bladder	2	pTa	−	NI	−	−, −	+	−
23	bladder	3	pT1	+	NI	−	−, −	−	+
24	bladder	3	pT4	+	+	−	−, −	+	−
25	bladder	2	pT2	−	NI	−	−, −	−	−
26	bladder	3	pT3	−	NI	−	−, −	−	−
27	bladder	2	pT1	NI	+	−	+, +	ND	−
28	bladder	1	pTa	+	−	−	−, −	+	+
29	bladder	2	pT1	+	NI	−	−, −	−	+
30	bladder	3	pT1	ND	ND	−	ND, ND	−	+
31	renal pelvis	2	pT3	−	NI	−	−, −	−	−
32	renal pelvis	2	pT1	+	NI	−	+, +	ND	−
33	renal pelvis	2	pT1	NI	NI	−	−, −	−	−
34	renal pelvis	1	pTa	−	NI	−	−, −	−	+
35	uretrer	1	pTa	+	NI	−	−, −	−	−
36	uretrer	1	pTa	−	NI	−	−, −	−	+
37	uretrer	2	pT3	−	NI	−	−, −	+	−
38	uretrer	2	pTa	+	+	+	−, −	−	−
Frequency				14/28 (50%)	3/5 (60%)	1/38 (2.6%)	6/37–5/37 (16.2%–13.5%)	12/32 (37.5%)	23/38 (60.5%)

a) LOH, loss of heterozygosity.
b) *IFNA*, interferon α.
c) IHC, immunohistochemical staining for p16/MTS1 nuclear antigen.
LOH (+, loss of heterozygosity; −, retention of heterozygosity; NI, not informative; ND, not done), Mutation (+, presence of mutation; −, no mutation), Homozygous deletion (+, homozygously deleted; −, not homozygously deleted; ND, not done), Methylation (+, hypermethylated; −, not hypermethylated; ND, not done), IHC (+, positive staining; −, negative staining).

affinization and rehydration, a universal DAKO LSAB (labeled streptavidin biotinylated immunoperoxidase) kit (Dako Corp., Carpinteria, CA) was used. The sections were incubated overnight with a primary polyclonal rabbit anti-p16/MTS1 antibody (15126E, Pharmingen, San Diego, CA) at a dilution of 1 : 250. After incubation with the biotinylated antiserum and streptavidine peroxidase, the color was developed with diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA). The slides were examined under a light microscope. Tumors in which more than 10% of the neoplastic cells showed intense homogeneous nuclear staining were classified as positive for expression.

Statistical analysis Statistical analyses were performed by Fisher's exact test (two-sided). Differences between groups with $P<0.05$ were considered significant.

RESULTS

The genetic and epigenetic alterations of the *MTS1* gene, and IHC of p16/MTS1 proteins in 38 primary urothelial cancers are summarized in Table I.

Genetic alterations at the *MTS* locus LOH at the *IFNA* locus was detected in 14 of 28 informative samples, although at the *D9S171* locus, only 5 samples showed heterozygosity where 3 had LOH (data not shown). The relationships between LOH and pathological features (tumor grade and stage) are summarized in Table II. There were no statistically significant correlations between LOH and pathological features.

No mutations were detected by PCR-SSCP analysis in exon 1 of the *MTS1* gene or in exon 2 of the *MTS2* gene. Only one sample (2.6%; sample no. 38) had a mutation in exon 2 of the *MTS1* gene. This was a grade 2 and non-invasive (pTa) ureteral tumor. Sequencing revealed a G-to-T transversion at codon 81, which resulted in a missense mutation (Gly to Val) (data not shown). The tumor also showed LOH at both the *IFNA* and *D9S171* loci. We further directly sequenced exon 2 of the *MTS1* gene in 28 of the remaining tumor specimens, but no other mutation was found.

A tumor was defined as having a homozygous deletion when the intensity of the band at the *MTS* genes was reduced to less than 20% of that at *MUP 7* (Fig. 2). We found homozygous deletions of the *MTS1* in 6 tumors (16.2%), sample nos. 1, 2, 5, 7, 27 and 32. Sample no. 7 had a homozygous deletion of the *MTS1*, but retained the *MTS2* gene. The other 5 tumors had homozygous deletions of both the *MTS1* and the *MTS2* genes. There were no statistically significant correlations between homozygous deletion of the *MTS1* gene and pathological features (Table II).

Methylation modification and expression of the *MTS1* gene After measuring the densities of *Hha* I-, *Hpa* II-, or *Msp* I-digested and undigested samples, the methylation

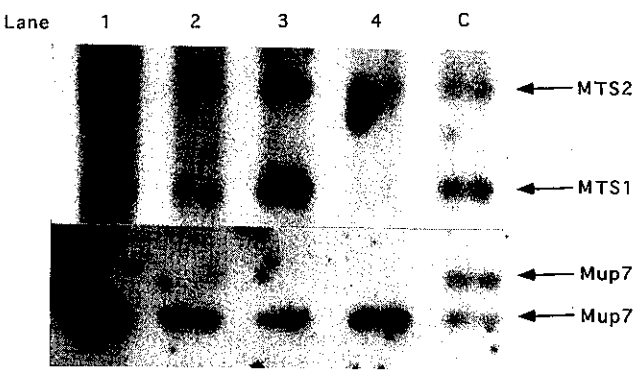

Fig. 2. Representative examples of Southern blotting for homozygous deletions of the *MTS* genes. Lanes 1 and 2 (sample nos. 2 and 5) show homozygous deletions of both the *MTS1* and the *MTS2* genes. Lane 4 (sample no. 7) shows a homozygous deletion of the *MTS1* gene, but not of the *MTS2* gene. C: control DNA from normal tissue.

Table II. LOH at Chromosome 9p21–22, Homozygous Deletions of the *MTS1* Gene, Hypermethylation of the *MTS1* Gene, and Pathological Features (%)

	LOH[a] (*IFNA*[b])	LOH (*D9S171*)	HD[c] (*MTS1*)	Hypermethylation
Non-invasive	7/13 (53.8%)	1/2 (50%)	2/19 (10.5%)	8/17 (47.1%)
Invasive	7/15 (46.7%)	2/3 (66.7%)	4/18 (22.2%)	4/15 (26.7%)
Total	14/28 (50%)	3/5 (60%)	6/37 (16.2%)	12/32 (37.5%)
Grade 1	6/8 (75%)	0/1 (0%)	1/9 (11.1%)	3/8 (37.5%)
Grade 2	4/13 (30.8%)	2/2 (100%)	5/21 (23.8%)	7/16 (43.8%)
Grade 3	4/7 (57.1%)	1/2 (50%)	0/7 (0%)	2/8 (25%)
Total	14/28 (50%)	3/5 (60%)	6/37 (16.2%)	12/32 (37.5%)

a) LOH, loss of heterozygosity.
b) *IFNA*, interferon α.
c) HD, homozygous deletion.

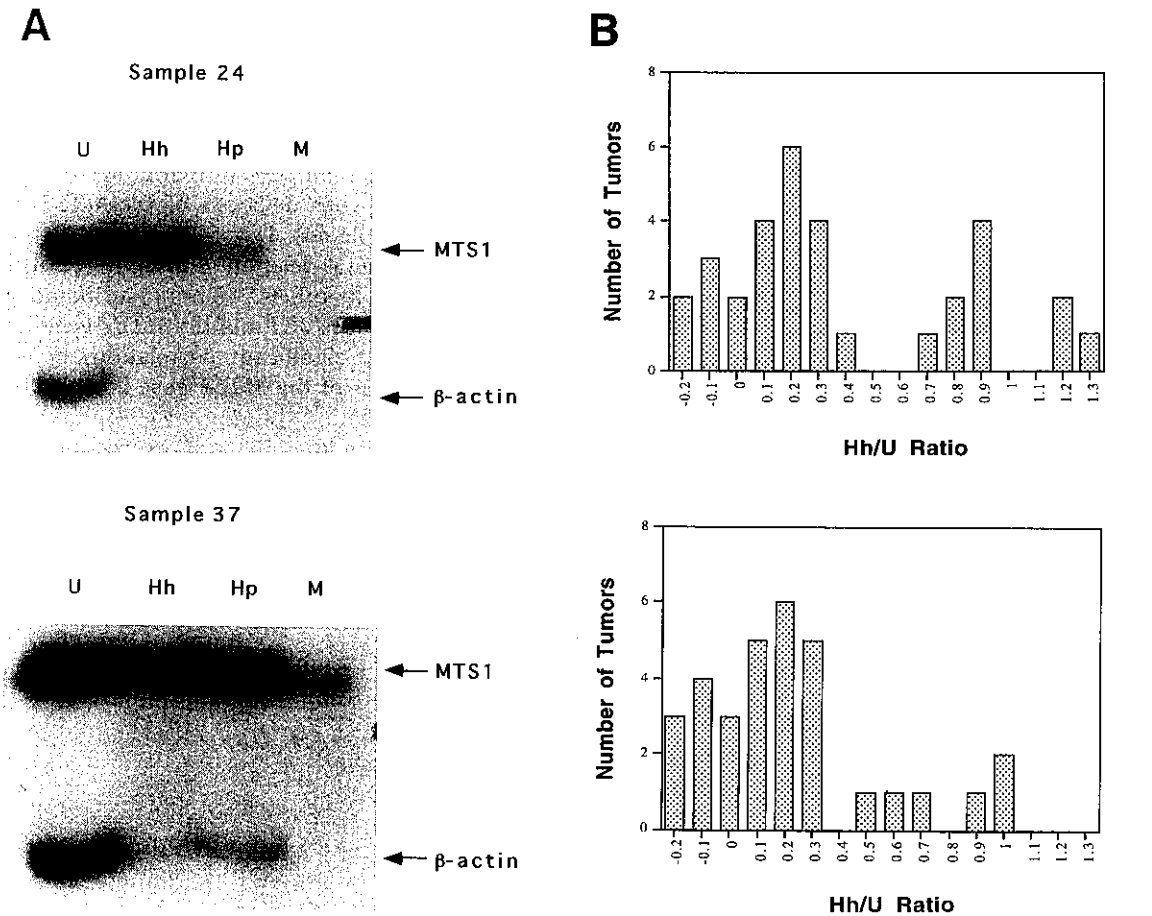

Fig. 3. A, Representative examples of PCR-based methylation analysis of primary cancers. Sample 24 is hypermethylated at the *Hha* I site. Sample 37 is hypermethylated at both the *Hha* I and *Hpa* II sites. The *β-actin* promoter region was used as the negative control. U, undigested; Hh, *Hha* I-digested; Hp, *Hpa* II-digested; M, *Msp* I-digested. B, The signal ratios of *Hha* I-digested *versus* undigested samples (Hh/U) and *Hpa* II-digested *versus* undigested samples (Hp/U) are plotted in a histogram. Since the histogram showed two peaks, the samples were divided into two groups. Samples with a ratio over 0.7 were defined as hypermethylated.

status of the *MTS1* gene was expressed in terms of their relative ratios as follows.

Hh/U or Hp/U ratio = [(density of *Hha* I- or *Hpa* II-digested samples) − (density of *Msp* I-digested samples)]/[(density of undigested samples) − (density of *Msp* I-digested samples)]

In separate experiments with five *MTS1*-positive bladder cancer cell lines, we confirmed that each cell line had characteristic Hh/U and Hp/U ratios, which were reproducible and inversely correlated with the expression of the *MTS1* gene as determined by western blotting (data not shown).

PCR-based methylation assay was performed in 32 primary urothelial cancers, excluding those with homozygous deletions of the *MTS1* gene (Fig. 3A). The Hh/U and Hp/U ratios thus obtained in the primary urothelial cancers are plotted in Fig. 3B. It is evident that the distributions of the ratios are bipartite, which is more clearly seen in the Hh/U ratio. The tumor sample was defined as hypermethylated when the Hh/U or Hp/U ratio was over 0.7. According to this criterion, 12 tumors (37.5%) were hypermethylated (Table I). There were no statistically significant correlations between the presence or absence of hypermethylation and pathological features. Hypermethylation was observed in 4 of 11 tumors showing LOH without any evidence of structural abnormalities in the retained *MTS1* gene.

The expression of the *MTS1* gene was further studied by immunohistochemical staining in the histological sec-

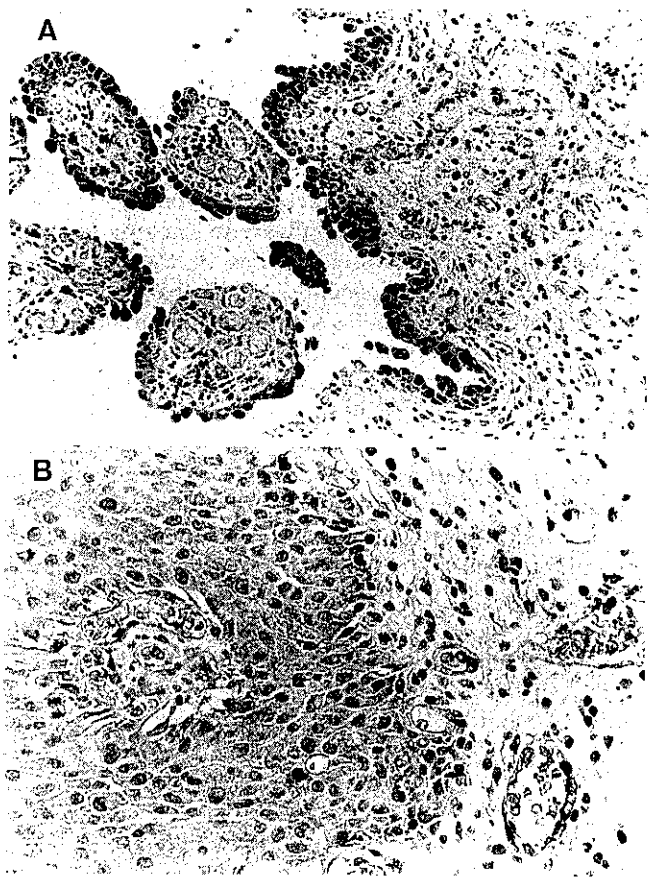

Fig. 4. IHC analysis for p16/MTS1 nuclear antigen in primary urothelial cancers. A, IHC-positive tumor. The adjacent carcinoma *in situ* region of sample no. 23 displays strong nuclear reactivity. Admixed stromal elements are also stained. ×100. B, IHC-negative tumor. The tumor nests exhibit nonreactivity of nuclei, while admixed stromal cells are stained (sample no.18). ×200.

tions (Fig. 4, A and B). The p16/MTS1 nuclear staining was negative in 23 of 38 urothelial cancers (60.5%). The p16/MTS1-negative tumors included all the tumors with genetic alterations (mutations and homozygous deletions) and 9 of 12 tumors with a hypermethylated *MTS1* gene.

DISCUSSION

We examined both genetic and epigenetic alterations of the *MTS1* gene in primary urothelial cancers. Genetic alterations of the *MTS1* gene were found in 7 cases; one case (2.6%) with a base substitution mutation and 6 cases (16.2%) with homozygous deletions. This is consistent with the observation of Spruck *et al.*[15], who reported homozygous deletions of the *MTS1* gene in 19% of primary bladder cancers. The characteristic feature of the genetic alterations of the *MTS1* gene in urothelial cancers is that the fraction of homozygous deletions is considerably higher than that of point mutations.[26,27] This is in contrast to other cancers such as pancreatic cancers[28] and esophageal cancers,[29] where point mutations or small intragenic deletions predominate.

In lung cancers, the *MTS* genes were more often mutated or deleted in metastatic tumors than in the primary tumors.[30] Kinoshita *et al.*[31] have also shown more frequent LOH at chromosome 9p21 in renal cell carcinomas with metastasis, and 3 of 5 metastatic tumors had hemi- or homozygous deletions of the *MTS* genes. These observations suggest that deletions of chromosome 9p21–22 including the *MTS1* gene are late events in these tumors and are related to the progression of these tumors. In contrast, deletions in chromosome 9p have been suggested to be an early event in bladder cancers.[7–9] Orlow *et al.*[32] found a statistically significant association between *MTS1* gene deletions and bladder cancers of low stage and grade. In the present study, LOH at chromosome 9p21–22, homozygous deletions of the *MTS1* gene and hypermethylation of the *MTS1* gene were not correlated with pathological features. This is consistent with the notion that alterations of the *MTS1* gene play a crucial role in the initiation of urothelial cancers.

Hypermethylation of the 5′ CpG island has been proposed as an alternative pathway for inactivating the *MTS1* gene.[16–19] In this study, hypermethylation of the *MTS1* gene was found in 12 out of 32 *MTS1*-positive tumors (37.5%), which is a much higher proportion than that of genetic alterations (mutations and homozygous deletions: 18.4%). An even higher frequency of hypermethylation of the *MTS1* gene (67% or 12 of 18 primary bladder cancers) has been reported by Gonzalez-Zulueta *et al.*[19] The discrepancy may be partly due to the differences in sample size, definition of the degree of methylation and/or number of methylation sites tested. They examined the methylation status at *FnuD* II, *Sac* II, and *Sma* I sites in addition to *Hha*I and *Hpa*II sites. An analysis of methylation status at multiple CpG sites might result in a higher frequency of hypermethylation. Moreover, in the case of *Hha* I sites, if either of two restriction sites between the PCR primer regions is demethylated, the methylated status of the other site is not detected. In other words, it is impossible to detect hypermethylation in *Hha* I unless both restriction sites are methylated. Therefore, the frequency of hypermethylation in this study may be underestimated. Indeed, the immunohistochemical analysis revealed the absence of p16/MTS1 nuclear staining in 17 of 31 *MTS1*-positive tumors (54.8%), among which 7 tumors displayed the absence of p16/MTS1 staining without any detectable

genetic or epigenetic changes in the *MTS1* gene. These tumors may harbor a loss-of-function mutation in some other region of the gene or may be methylated at other CpG sites. Hsieh[33] reported that in the *in vitro* CAT assay, transcription was dramatically repressed even when only 7% of the CpG sites were methylated. Thus, methylation of either the *Hha* I or the *Hpa* II site is likely to be enough to repress transcription.

However, a question still remains as to the discordant cases; 3 of 12 hypermethylated tumors showed the presence of p16/MTS1 proteins. Boyes and Bird[34] reported that the level of transcriptional repression is dependent on methylation density. These 3 tumors were all methylated at the *Hha* I site (Hh/U ratio; 1.29, 0.861, and 0.927, respectively), but not at the *Hpa* II site. The site of methylation or partial methylation may influence expression of the gene. Another possibility is regional heterogeneity within tumor tissue with respect to the methylation and/or expression of the gene, as indicated by Geradts and Wilson.[35] Although the relationship between aberrant expression of the *MTS1* gene and methylation status in some tumors remains to be elucidated, the present observations represent firm evidence that transcriptional inactivation of the *MTS1* gene by methylation modification is the predominant pathway in urothelial carcinogenesis. Moreover, the retention of a hypermethylated copy or copies of the *MTS1* gene in LOH-positive tumors (4 of 11, 36%) suggests that the methylation modification could be an initial event, followed by genomic rearrangements associated with total loss of function of the *MTS1* gene. Lack of correlation with the stage and grade of tumors indicates that the alterations of the *MTS1* gene are an early event and play a crucial role in the development of urothelial cancers.

Loss-of-function alteration of the *MTS1* gene in urothelial cancers is unique in that transcriptional silencing by methylation modification of the gene predominates. Bladder cancers and lung cancers are two major cancers among those associated with cigarette smoking as a risk factor. In these tumors, mutations of the *p53* tumor suppressor gene are frequently found, but they are of different types. G : C-to-T : A transversion predominates in lung cancers and G : C-to-A : T transition predominates in bladder cancers, where approximately half of the transition mutations occur at methylation sites, CpG dinucleotides.[36] The difference has been discussed in relation to the difference in the active intermediates in the two organs. The G : C-to-A : T transition mutation at CpG dinucleotides is formed via spontaneous hydrolytic deamination of 5-methylcytosine[37] as well as catalytically by the transmethylation process itself through an imbalance in the levels of DNA-(cytosine-5)-methyltransferase and methyl donor, such as *S*-adenosylmethionine.[38] An elevated level of methyltransferase and/or deprivation of methyl donor is known to increase the deamination rate of cytosine.[39] It is thus tempting to speculate that inactivation by hypermethylation of methylation-sensitive genes such as *MTS1* is closely correlated with G : C-to-A : T transition mutation at CpG dinucleotides and this may be the mechanism underlying urothelial carcinogenesis.

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