The Korean Journal of Pathology 2010; 44: 623-30
DOI: 10.4132/KoreanJPathol.2010.44.6.623

Prognostic Significance of Methylation Profiles in Urothelial Carcinomas of the Bladder

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Received : August 4, 2010
Accepted : September 27, 2010

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Background : Study on epigenetics of urothelial carcinomas has expanded and allowed better understanding of their correlation with clinicopathologic features. The aim of this study was to determine reliable predictive epigenetic markers for patients with urothelial carcinoma of urinary bladder. **Methods :** In 64 urothelial carcinomas of the urinary bladder, methylation-specific polymerase chain reaction with RAS association domain family 1A (*RASSF1A*), adenomatous polyposis coli (*APC*), death-associated protein-kinase (*DAPK*), runt-related transcription factor 3 (*RUNX3*), *p14, p16* and *MGMT* was performed and correlated the results with *p53* mutations, DNA ploidy, clinicopathologic parameters and recurrences. **Results :** Hypermethyation of *RASSF1A*, *APC*, *DAPK, RUNX3, p14, p16* and *MGMT* promoters was observed in 35 (54.7%), 29 (45.3%), 18 (28.1%), 18 (28.1%), 9 (14.1%), 2 (3.1%), and 6 (9.4%) cases, respectively. Hypermethylation of *RUNX3* and *APC* was significantly associated with high histologic grades and aneuploidy. Methylation of *DAPK* was significantly associated with muscle invasion. Methylation of *DAPK* and *RUNX3* genes was significantly associated with recurrence. In survival analyses, methylation of *RUNX3* gene and methylation-high (methylation at two or more loci) phenotype was significantly associated with poor recurrence-free survival. **Conclusions :** Methylation of *RUNX3* gene and methylation-high phenotype are significant indicator of recurrence.

Key Words : Methylation; Urothelial carcinoma; Genes, tumor suppressor; Prognosis

Urinary bladder cancer is the fifth most common cancer in the Western world and is responsible for about 3% of all cancer-related deaths.[1] Urothelial (transitional cell) carcinoma, the most common carcinoma of the urinary tract, is a disease of the entire urothelium and is characterized by multiplicity. Although urothelial carcinoma can be removed by transurethral resection, more than 50% of cases will experience recurrence.[2]

Hypermethylation in CpG islands of growth-regulating gene promoter regions is now recognized as a common alternative mechanism for gene inactivation in human cancer, an event as important as somatic mutations in the coding regions of tumor suppressor genes.[3,4] Hypermethylation of CpG islands has also been detected in urothelial carcinoma of the bladder.[5-9] In urothelial carcinomas, hypermethylation of CpG islands around the promoter region and decreased expression of tumor suppressor genes including death-associated protein-kinase (*DAPK*), RAS association domain family 1A (*RASSF1A*), adenomatous polyposis coli (*APC*), runt-related transcription factor 3 (*RUNX3*), *p16*, retinoic acid receptor beta, and *E-cadherin* have been reported.[6,8,10,11] Inactivation of *RUNX3* through promoter hypermethylation is associated with invasion, progression, and poor prognosis.[8] Methylation of the *DAPK* promoter has been found in 4% to 58.2% of urinary bladder carcinoma cases and is associated with higher tumor stages, higher grades, and muscle invasiveness.[6,12-15] *APC* hypermethylation is associated with malignant behavior and worse outcomes.[16,17] Promoter hypermethylations of *RASSF1A* and *p14* appear to be initial events in urinary bladder carcinogenesis, which are maintained during tumor progression.[3,18]

p53 is a key player in tumor suppression, as it promotes growth arrest, apoptosis, and cellular senescence and blocks angiogenesis.[19] *p53* mutations provide precancerous cells with a selective

advantage for unregulated growth and the acquisition of genetic instability.[20] Alterations in tumor suppressor *p53* in bladder cancer, which are known to decrease genetic stability, are associated with higher grades, later stages, and worse patient outcomes.[21]

Aberrant promoter methylation has been described for several genes in various malignant diseases and is associated with tumor progression. Further, each tumor type may have its own distinct pattern of methylation.[6] Selection of appropriate clinically significant methylation markers will help predict the aggressiveness of bladder carcinomas. Although hypermethylation of specific genes has been extensively investigated in urothelial carcinomas of the bladder,[5,8,10,13,14,22] a multiple gene methylation profile is lacking and has never been studied in Korean populations.[11,13,23] Establishing DNA methylation profiles for bladder carcinoma might provide valuable markers for predicting prognosis.

For this purpose, we selected seven genes frequently silenced by aberrant methylation in a number of tumor types to investigate the methylation profile of bladder cancer. We correlated our findings with *p53* mutations, DNA ploidy, and clinicopathological parameters in patients with bladder carcinoma.

MATERIALS AND METHODS

Patient selection

We randomly selected 64 patients diagnosed with urothelial carcinoma of the urinary bladder at Samsung Medical Center, Seoul, Korea between 1998 and 2003, who had undergone clinical follow-up. All patients had a histological diagnosis of urothelial carcinoma. A complete transurethral resection of the bladder tumor was performed in cases of superficial bladder tumors. Patients with superficial bladder tumors received one cycle of intravesical treatment (bacillus Calmette-Guerin or mitomycin). A radical cystectomy and complete pelvic lymph node dissection were performed for invasive bladder tumors.

The median age at the time of diagnosis was 65 years (range, 36 to 81 years); seven (10.9%) patients were female and 57 (89.1%) were male. All tumors were diagnosed according to American Joint Committee on Cancer staging and were divided into low grade (n = 23) and high grade (n = 41).[24] The depth of invasion was categorized as noninvasive (pTa, n = 25), superficial (pT1, n = 24), or deeply invasive (pT2 to T4, n = 15) carcinoma (Table 1).

Table 1. The clinical and pathological characteristics of 64 patients with urothelial carcinoma of the bladder

Parameter	No. of cases (%)	Cases with recurrence (%)	p-value
Gender			0.82
Female	7 (10.9)	4 (57.1)	
Male	57 (89.1)	35 (61.4)	
Age (yr)			0.62
≤ 65	28 (43.8)	18 (64.3)	
> 65	36 (56.2)	21 (58.3)	
Depth of invasion			0.48
Noninvasive	25 (39.1)	15 (60.0)	
Superficial invasive	24 (37.5)	13 (54.2)	
Deep invasive	15 (23.4)	11 (73.3)	
DNA ploidy			0.31
Diploid	36 (56.2)	20 (55.6)	
Aneuploid	28 (43.8)	19 (67.9)	
p53			0.68
Wild	25 (39.1)	16 (64.0)	
Mutation	39 (60.9)	23 (59.0)	
Histologic grade			0.04
Low grade	23 (35.9)	12 (52.2)	
High grade	41 (64.1)	27 (65.9)	

Follow-up was available in all cases. Recurrence of disease was observed in 29 patients (45.3%) and distant metastasis was observed in ten cases (15.6%). The median follow-up was 62.0 months (range, 6.0 to 141.0 months).

Bisulfite modification and methylation-specific polymerase chain reaction

Genomic DNA was extracted from paraffin-embedded tumor tissue using manual microdissection, standard proteinase K digestion, and a QIAamp DNA mini kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). We used the EZ DNA Methylation kit for bisulfite modification, according to the manufacturer's protocol (Zymo Research Corp., San Diego, CA, USA). The modified DNA was subject to methylation-specific polymerase chain reaction (MS-PCR) using primers. Primer sequences, annealing temperatures, and the expected product size are listed in Table 2. Bisulfate-modified DNA (2 μL) was amplified in a total volume of 10 μL containing 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), 2 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphate, 1 μM of each primer, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems) at 95˚C for 10 minutes; 40 cycles of 95˚C for 30 seconds, the specific annealing temperature for 30 seconds, and 72˚C for 40 seconds; followed by a final extension at 72˚C for 10 minutes. Commercial methylated DNA (Chemicon, Temec-

Table 2. Primer sequences and polymerase chain reaction (PCR) conditions for methylation-specific PCR and *p53* mutation analysis

Primer name (function of gene)		Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	Product size (bp)	Annealing temp (℃)
DAPK	U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	106	60
(proapoptotic death-associated protein kinase)	M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60
RUNX3	U	ATAATAGTGGTTGTTAGGGTGTTG	ACTTCTACTTTCCCACTTCTCACA	115	65
(tumor suppressor gene)	M	ATAATAGCGGTCGTTAGGGCGTCG	GCTTCTACTTTCCCGCTTCTCGCG	115	65
APC	U	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	108	60
(Wnt-signaling pathway)	M	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	98	55
RASSF1A	U	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	105	60
(tumor suppressor with cell cycle arrest)	M	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	93	60
MGMT	U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59
(DNA repair)	M	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	59
p16^{INK4A}	U	TTATTAGAGCTGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60
(cell cycle regulation)	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65
p14^{ARF}	U	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	63
(cell cycle regulation)	M	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	63
p53 mutation					
Exon 5		CTCTTCCTACAGTACTCCCCTGC	GCCCCAGCTGCTCACCATCGCTA	184	55
Exon 6		GATTGCTCTTAGGTCTGGCCCCTC	GGCCACTGACAACCACCCTTAACC	113	68
Exon 7		GTGTTATCTCCTAGGTTGGCTCTG	CAAGTGGCTCCTGACCTGGAGTC	110	68
Exon 8		ACCTGATTTCCTTACTGCCTCTGGC	GTCCTGCTTGCTTACCTCGCTTAGT	137	55

U, unmethylated; M, methylated; *DAPK*, death-associated protein-kinase; *RUNX3*, runt-related transcription factor 3; *APC*, adenomatous polyposis coli; *RASSF1A*, RAS association domain family 1A.

ula, CA, USA) was used as a positive control for methylation, and water was used as the negative control. PCR products (10 μL) were loaded onto nondenaturing 8% polyacrylamide gels that were then stained with SYBR green (Lonza, Rockland, ME, USA) and visualized under UV illumination.

p53 mutation and DNA ploidy

Exons 5, 6, 7, and 8 of the *p53* gene were examined for mutations using genomic DNA obtained from tumor tissue by PCR-direct sequencing methods (Table 2). Genomic DNA (100 μg) was amplified in a total volume of 50 μL containing 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphate, 1 μM of each primer, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems) at 95℃ for 10 minutes; 35 cycles of 95℃ for 30 seconds, the specific annealing temperature for 30 seconds, and 72℃ for 40 seconds; followed by a final extension at 72℃ for 10 minutes. The purified PCR products (Bionics, Seoul, Korea) were directly sequenced, according to a dual DNA purification kit, using a BigDye Terminator v3.1 Cycle Sequencing kit, followed by analysis with an Applied Biosystems 3700 automated sequencer (Applied Biosystems).

Flow cytometric analyses were performed as previously described.[25,26] Briefly, sections (5 μm) from each paraffin block were deparaffinized, according to the modified Hedley's method. Then, 1 mL of 0.5% prewarmed pepsin in 0.9% saline at pH 1.5 was added to each sample, followed by a 45-60 minute incubation at 36℃, with vortexing at intervals of 10 minutes. All residual solid tissue was then removed, and the remaining nuclear suspension was filtered through a 50 μm nylon mesh. The suspension was then centrifuged at 800 × g for 5 minutes, and the supernatant was decanted. The pellet was resuspended in 1 mL of Hanks' solution, centrifuged as before, and the supernatant was decanted. After disaggregation, the samples were stained with propidium iodide and analyzed using a BD FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The diploid tumors had a DNA index range of 0.95-1.05 to allow for a 5% instrument error.

Statistical analysis

The statistical analysis was performed with the SPSS ver. 16 (SPSS Inc., Chicago, IL, USA). The association between parameters was assessed using the χ^2 test or Fisher's exact test. The recurrence-free interval and survival were analyzed according to the Kaplan-Meier method, and differences in their distribution models were evaluated by means of the log-rank test. A p-value

equal to or less than 0.05 was considered statistically significant.

RESULTS

The methylation statuses of seven loci were examined using MS-PCR in 64 bladder carcinoma samples. Hypermethylation of the *RASSF1A, APC, DAPK, RUNX3, p14, p16,* and *MGMT* promoters was observed in 35 (54.7%), 29 (45.3%), 18 (28.1%), 18 (28.1%), nine (14.1%), two (3.1%), and six (9.4%) cases, respectively. Fig. 1 shows the representative results of MS-PCR with the bladder carcinoma samples. Although the hypermethylation statuses of most genes were not related, methylation of the APC gene was significantly associated with methylation of the *RASSF1A* gene ($p < 0.001$).

The relationships between promoter hypermethylation and clinicopathological features were examined in urothelial carcinomas (Table 3). Methylation of *RUNX3* and *APC* was significantly associated with higher histological grade and aneuploidy ($p < 0.05$). Methylation of the *DAPK* and *RUNX3* genes was more frequently observed in patients with recurrence or metastasis than in patients without recurrence (33.3% vs 20% and 30.8% vs 24.0%, respectively; $p = 0.04$). The *DAPK* gene promoter was significantly more methylated in carcinomas with muscle invasion than in carcinomas with superficial invasion or without invasion (60.0% vs 18.4%, respectively; $p = 0.005$).

Although *MGMT* methylation was rare, it was found solely and significantly in patients older than 65-years ($p = 0.02$). No significant differences were observed between *p14, p16*, and *RASSF1A* methylation and clinicopathological parameters, in-

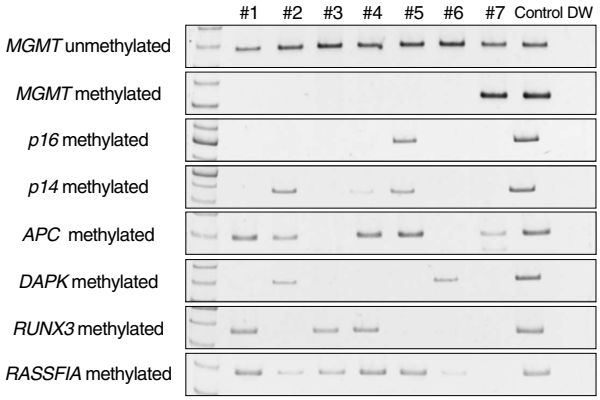

Fig. 1. Representative results of the methylation-specific polymerase chain reaction in urothelial carcinomas of the urinary bladder. *APC*, adenomatous polyposis coli; *DAPK*, death-associated protein-kinase; *RUNX3*, runt-related transcription factor 3; *RASSF1A*, RAS association domain family 1A; DW, distilled water.

Table 3. Correlations between *DAPK, APC*, and *RUNX3* methylation and clinicopathological factors

Parameter	No. of cases	*DAPK*		*RUNX3*		*APC*	
		n (%)	p-value	n (%)	p-value	n (%)	p-value
Gender			0.35		0.35		0.50
Female	7	3 (42.9)		3 (42.9)		4 (57.1)	
Male	57	15 (26.3)		15 (26.3)		25 (43.9)	
Age (yr)			0.52		0.10		0.50
≤ 65	28	9 (32.1)		5 (17.9)		14 (50.0)	
> 65	36	9 (25.0)		13 (36.1)		15 (41.7)	
Histologic grade			0.78		0.002		0.02
Low grade	23	6 (26.1)		1 (4.3)		6 (26.1)	
High grade	41	12 (29.3)		17 (41.5)		23 (56.1)	
Depth of invasion			0.005		0.07		0.23
Noninvasive	25	6 (24.0)		3 (12.0)		8 (32.0)	
Superficial invasive	24	3 (12.5)		9 (37.5)		13 (54.2)	
Deep invasive	15	9 (60.0)		6 (40.0)		8 (53.3)	
p53			0.08		0.98		0.49
Wild type	25	10 (40.0)		7 (28.0)		10 (40.0)	
Mutation	39	8 (20.5)		11 (28.2)		19 (48.7)	
DNA ploidy			0.52		0.02		0.02
Diploid	36	9 (25.0)		6 (16.7)		12 (33.3)	
Aneuploid	28	9 (32.1)		12 (42.9)		17 (60.7)	
Recurrence or metastasis			0.04		0.04		0.39
None	25	5 (20.0)		6 (24.0)		9 (36.0)	
Recur	29	7 (24.1)		6 (20.7)		14 (48.3)	
Metastasis	10	6 (60.0)		6 (60.0)		6 (60.0)	

DAPK, death-associated protein-kinase; *APC*, adenomatous polyposis coli; *RUNX3*, runt-related transcription factor 3.

cluding gender, age, *p53* mutation, DNA ploidy, or disease recurrence. As the methylation status of *p14, p16*, and *RASSF1A* was not related to any clinicopathological features and *MGMT* methylation was so rare, those markers were excluded from further statistical analyses. Therefore, the methylation status of *APC, DAPK*, and *RUNX3* was scrutinized. A methylation-low phenotype (methylation at one locus or none) was found in 49 cases (76.6%), and a methylation-high phenotype (methylation at two or more loci) was observed in 15 cases (23.4%). Recurrence or metastasis of disease was observed in 28 cases (57%) with methylation-low phenotypes and in 11 cases (73%) with methylation-high phenotypes. The methylation-high phenotype was significantly associated with higher histological grade, tumors with deeper invasion, aneuploidy, and recurrence or metastasis of disease (p = 0.006, 0.02, 0.04, and 0.001, respectively) (Table 4).

Table 4. Clinical significance of methylation phenotypes with the *RUNX3, APC*, and the *DAPK* genes

Parameter	No. of cases	Methylation-low phenotype (%)	Methylation-high phenotype (%)	p-value
Histologic grade				0.007
Low grade	23	22 (95.7)	1 (4.3)	
High grade	41	27 (65.9)	14 (34.1)	
Depth of invasion				0.02
Noninvasive	25	23 (92.0)	2 (8.0)	
Superficial invasive	24	18 (75.0)	6 (25.0)	
Deep invasive	15	8 (53.3)	7 (46.7)	
DNA ploidy				0.04
Diploid	36	31 (86.1)	5 (13.9)	
Aneuploid	28	18 (64.3)	10 (35.7)	
Recurrence or Metastasis				0.001
None	25	21 (84.0)	4 (16.0)	
Recur	29	25 (86.2)	4 (13.8)	
Metastasis	10	3 (30.0)	7 (70.0)	

RUNX3, runt-related transcription factor 3; APC, adenomatous polyposis coli; *DAPK*, death-associated protein-kinase.

Aneuploidy with chromosome abnormalities was observed in 28 of 64 patients (43.7%) and was closely associated with higher histological grade (0% vs 68.3%, respectively; p < 0.001) and deeper invasion (16.0% vs 61.5%, respectively; p = 0.001). Of 64 patients, 39 harbored *p53* mutations (60.9%), although this was not related to histological grade, depth of invasion, or disease recurrence (p > 0.05). Promoter *APC* and *RUNX3* hypermethylation was significantly associated with DNA ploidy and histological grade (p = 0.003 and p = 0.004, respectively). Moreover, cases with both *RASSF1A* methylation and *p53* mutation were closely associated with DNA aneuploidy and higher histological grades (p = 0.01 and p = 0.04, respectively) (Table 5).

The univariate Kaplan-Meier analysis showed that pathological grade (p < 0.001) and depth of invasion (p = 0.03) were significantly associated with disease recurrence (Fig. 2), and that *RUNX3* gene methylation was significantly associated with reduced recurrence-free survival (p = 0.05). However, methylation of *APC* (p = 0.12) and *DAPK* (p = 0.80) was not associated with recurrence-free survival. When methylation of the three genes was grouped into methylation-low and methylation-high phenotypes, the methylation-high group showed a worse disease-free survival than that of the methylation-low phenotype group (p = 0.01) (Fig. 3).

DISCUSSION

In our comprehensive study of primary bladder urothelial carcinomas, the methylation profiles of tumor suppressor genes, including *DAPK, RASSF1A, APC, RUNX3, p14, p16,* and *MGMT* were investigated, and their associations with clinico-

Table 5. Correlations between molecular parameters and clinicopathological features (n = 64)

Parameter	*APC* and *RUNX3* methylation			*p53* mutation and *RASSF1A* methylation		
	Negative	Positive	p-value	Negative	Positive	p-value
DNA ploidy			0.003			0.01
Diploid	21 (58.3)	15 (41.7)		28 (77.8)	8 (22.2)	
Aneuploid	6 (21.4)	22 (78.6)		13 (46.4)	15 (53.6)	
Histologic grade			0.001			0.02
Low grade	16 (59.3)	7 (18.9)		19 (82.6)	4 (17.4)	
High grade	11 (26.8)	30 (73.2)		22 (53.7)	19 (46.3)	
Depth of invasion			0.06			0.26
Noninvasive	15 (55.6)	10 (27.0)		19 (46.3)	6 (26.1)	
Superficial invasive	8 (29.6)	16 (43,2)		13 (31.7)	11 (47.8)	
Deep invasive	4 (14.8)	11 (19.7)		9 (22.0)	6 (26.1)	

APC, adenomatous polyposis coli; *RUNX3*, runt-related transcription factor 3; *RASSF1A,* RAS association domain family 1A.

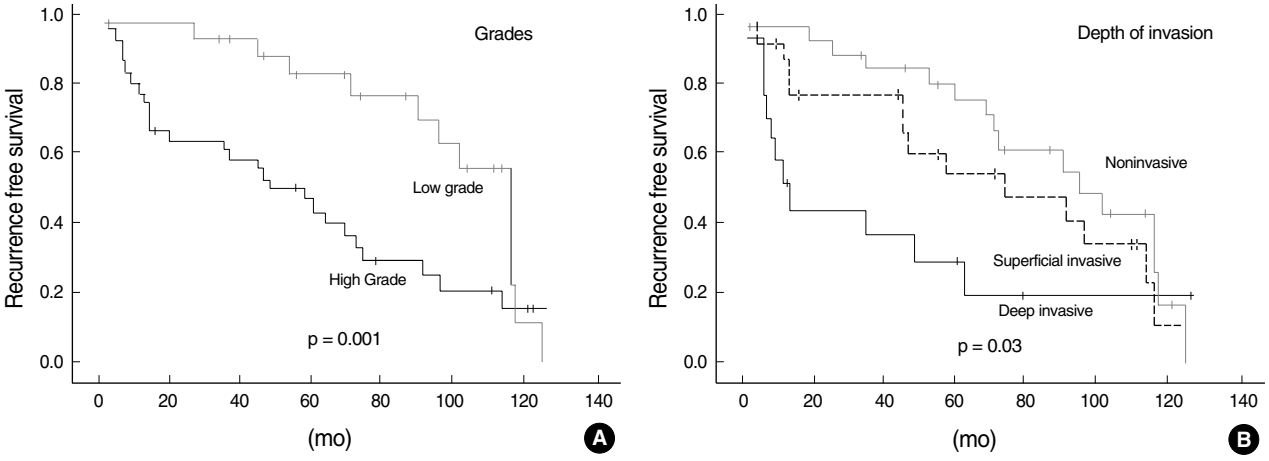

Fig. 2. Kaplan-Meier survival curves show that pathological grade and depth of invasion are significantly associated with disease recurrence.

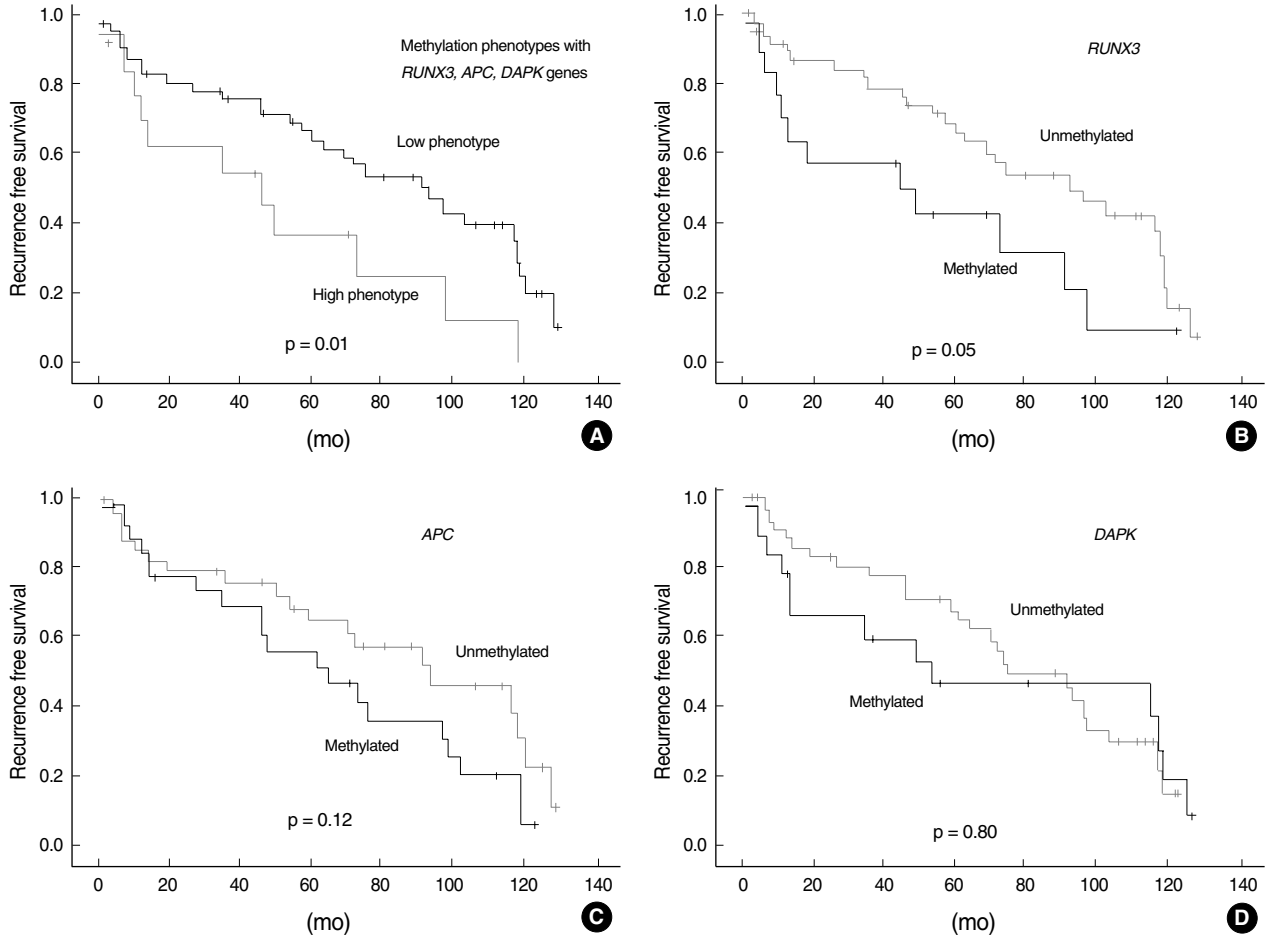

Fig. 3. Kaplan-Meier survival curves. (A) The methylation-high group with three tumor suppressor gene loci (runt-related transcription factor 3 [*RUNX3*], adenomatous polyposis coli [*APC*], death-associated protein-kinase [*DAPK*] genes) show poor recurrence-free survival compared to that of the methylation-low phenotype group. (B) *RUNX3* gene methylation is significantly associated with a shorter recurrence-free survival. (C) Methylation of *APC* and (D) *DAPK* are not associated with recurrence-free survival.

pathological parameters and disease recurrence were studied. We found that *RUNX3* and *APC* gene methylation was significantly associated with higher histological grade and aneuploidy, *DAPK* methylation was significantly associated with muscle invasion, and *DAPK* and *RUNX3* gene methylation was significantly associated with recurrence. In survival analyses, *RUNX3* gene methylation and the methylation-high phenotype were significantly associated with reduced recurrence-free survival.

Inactivation of *DAPK* occurs largely through transcriptional silencing via DNA hypermethylation and is involved in the process of bladder cancer development or invasion.[13] The frequency of *DAPK* methylation in urothelial carcinomas varies from 6% to 74%.[6,7,11,13,14] We observed *DAPK* methylation in 28.1% of cases, and this epigenetic alteration was closely associated with recurrence or metastasis. Our results confirmed the significance of *DAPK* methylation as one of the prognostic indicators for progression of bladder carcinomas.

RUNX3 methylation is an early event in bladder tumorigenesis, and its frequency increases over time.[27] In previous studies with Korean populations, *RUNX3* methylation was observed in 71.2% and 73% of bladder urothelial carcinomas and was associated with invasive tumors, tumor progression, and poor survival.[5,8] In our study, *RUNX3* methylation was observed in 28.1% of cases. In previous studies in which fresh tissue was used (we used 8 to 12-year-old formalin-fixed paraffin-embedded tissue), the percentages of invasive tumors were 37.1% and 39.8% compared to the 23.4% observed in our study. This difference may be related to our lower frequency of *RUNX3* methylation, which, although being lower than that in a previous report, was significantly associated with higher histological grade and DNA aneuploidy in our study. Maruyama *et al*.[6] reported that methylation of the *APC* gene was observed in 35% of bladder carcinomas and was correlated with high tumor grade and muscle invasion. In our results, *APC* methylation was observed in 45.3% of cases, similar to the frequency previously reported, and was associated with high histological grade and DNA aneuploidy. The lineage-specific tumor suppressor *RUNX3* and the ubiquitous p53 protein are both principal responders of the *p14^{ARF}-MDM2* cell surveillance pathway, which prevents pathological consequences due to abnormal oncogene activation.[28] Inactivation of cell-cycle regulation genes is associated with abnormal DNA ploidy. In our results, cell-cycle regulation genes such as *APC* and *RUNX3* were more frequently methylated in patients with abnormal DNA ploidy. Furthermore, patients with aneuploidy were strongly associated with high histological grade, deep tumor invasion, and a subsequent poor prognosis. Our results suggest that methylation of *APC* or *RUNX3* dysregulates cell-cycle arrest, generates aneuploidy, and may lead to tumor progression in human urothelial carcinoma. Densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones.[29] As these epigenetic events are reversible, they may be potential targets for agents that inhibit DNA methylation or histone deacetylation.[30] Both 5-azadeoxycytidine and histone deacetylase inhibitors have shown promising activity in clinical trials as single agents in patients with cancer. The future progress of this form of epigenetic cancer therapy will be followed with great interest.[30]

Hypermethylation in the promoter regions of tumor suppressor genes is an early and frequent event associated with transcriptional silencing of regulatory genes during carcinogenesis.[3,4] The pathogenesis of cancer is attributable to multiple molecular changes, including gene mutations, changes in gene expression, protein functions, and other cellular processes.

As *p53* is directly targeted in approximately 50% of human malignancies,[19] it has been suggested that other mechanisms are required to shutdown the *p53* pathway in tumors with wild-type *p53*. A small but substantial proportion of bladder carcinomas demonstrate *p53* nuclear reactivity but do not show detectable mutations in the *p53* gene.[21] Recently, it was reported that inactivating the *p53* pathway may contribute to increased promoter hypermethylation as a response to the increasing genomic instability and recombination events leading to alterations in chromatin structure and, thus, DNA methylation.[20] The *p53* pathway plays an important role limiting the propagation of cultured aneuploid human cells to preserve the diploid karyotype of the population. *RASSF1A* can mediate cell-cycle arrest and senescence in human cancer cells through p53-independent regulation of *p21$^{Cip\ 1/Waf}$*.[30] In our study, *p53* mutation and *RASSF1A* methylation were not associated with clinicopathological features or recurrence, in agreement with previous reports.[6,14,30] However, aneuploidy was significantly more frequent in patients harboring *RASSF1A* methylation and a *p53* mutation, indicating that these genetic and epigenetic alterations may generate abnormal DNA ploidy and may involve progression of urothelial carcinoma.

In conclusion, *RUNX3* gene methylation and a methylation-high phenotype were significant indicators of poor recurrence-free survival. A *p53* mutation and *RASSF1A* methylation may be involved in abnormal DNA ploidy and tumor progression in urothelial carcinoma of the bladder.

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