RSPH9 methylation pattern as a prognostic indicator in patients with non-muscle invasive bladder cancer

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Abstract. DNA methylation is a frequent and early epigenetic event with potential application as a biomarker for cancer detection and an indicator of disease evolution. The aim of the present study was to identify novel methylation markers for the prediction of patient outcomes using microarray analysis of DNA methylation in samples from long-term follow-up patients with non-muscle invasive bladder cancer (NMIBC). Candidate methylation markers were selected from our previously published genome-wide methylation profiles. The clinical relevance of candidate methylation markers was determined by quantitative pyrosequencing analysis of 136 human bladder specimens (8 normal controls and 128 NMIBCs). The reversibility of DNA methylation was examined by 5-Aza-CdR treatment in human bladder cancer cell lines. The methylation patterns of candidate markers were significantly associated with aggressive clinicopathological features. In multivariate regression analysis, hypermethylation of radial spoke head 9 homolog (*RSPH9*) was an independent predictor of disease recurrence (hazard ratio, 3.02; P=0.001) and progression (hazard ratio, 8.25; P=0.028). The methylation level of *RSPH9* decreased with 5-Aza-CdR treatment and progressively increased in its absence in bladder cancer cell lines. *RSPH9* methylation is an independent prognostic indicator in NMIBC patients, and could be of value for the assessment of disease recurrence and progression and for clinical decision-making regarding treatment.

Introduction

Although the majority of newly diagnosed bladder cancer is non-muscle invasive bladder cancer (NMIBC), most patients with NMIBC relapse after adequate treatment, and some progress to muscle invasive disease (1,2). Furthermore, the potential of these tumors for recurrence and progression into muscle invasive disease is highly unpredictable. The detection of high-risk cases after transurethral resection (TUR) of the bladder tumor is essential and depends on efficient prognostic biomarkers for high-risk patients. Research efforts worldwide have focused on the identification of clinically helpful tumor markers or potentially valuable therapeutic targets to improve current diagnostic and management strategies for patients with bladder cancer (3).

Disease-specific aberrant DNA methylation is recognized as a hallmark of many cancers, which led to new opportunities for the understanding, detection, treatment, and prevention of diseases including bladder cancer (4-12). The use of DNA methylation as a biomarker has gained increasing interest in recent years, as aberrant DNA methylation is a major characteristic of bladder cancer and plays a crucial role in tumor initiation and progression (7-13). Although many of the different genetic or epigenetic changes that lead to aberrant gene expression in bladder cancer have been identified, the discovery of novel candidate methylation biomarkers was accelerated by the advent of high-throughput methylation profiling using normal and malignant cells (14).

NMIBC of similar morphology may behave differently, and it is difficult to predict clinical outcomes after initial treatment (1,2). Therefore, novel biomarkers predictive of disease outcomes, in addition to commonly used clinicopathological parameters, would be valuable for guiding appropriate management strategies (7). The aim of the present study was to identify novel methylation markers predictive of patient

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outcomes using microarray analysis of DNA methylation profiles in long-term follow-up NMIBC samples.

Materials and methods

Subjects and sample collection. A total of 136 human bladder specimens were used for pyrosequencing (PSQ) analyses, including 8 normal controls (NC) and 128 NMIBC samples (Table I). NMIBC specimens were obtained from 128 primary NMIBC patients who underwent TUR for histologically diagnosed transitional cell carcinomas between 1995 and 2010 at our institute. To exclude the possibility of incomplete resection or confounding factors that may unduly affect the analyses, patients followed-up for less than 6 months or those that experienced disease relapse within 6 months were excluded from the study. Samples of normal bladder urothelium obtained from individuals with benign prostate hyperplasia or bladder injury were used as controls.

All tumors were macro-dissected within 15 min of surgical resection. Each NMIBC specimen was confirmed by pathological analysis of a part of the tissue sample (i.e., sections were taken from TUR specimens and then snap-frozen in liquid nitrogen and stored at -80°C). The specimens were provided by the Chungbuk National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. The collection and analysis of all samples was approved by the Chungbuk National University Hospital Institutional Review Board (GR2010-12-010), and informed consent was obtained from each subject.

Tumors were staged according to the 2002 TNM classification and the 1973 WHO grading system (1,2). A second TUR was performed 2-4 weeks after the initial resection if a bladder cancer specimen did not include proper muscle, or if a high-grade tumor was detected. Patients with intermediate- or high-risk NMIBC received one cycle of intravesical therapy. Each patient was followed-up and managed according to the standard recommendations (1,2). Recurrence was defined as the recurrence of primary NMIBC at a lower or equivalent pathologic stage (Ta/T1), and progression was defined as muscular invasion (TNM stage T2 or higher) or metastatic disease.

DNA methylation profiling. Microarray methylation data of 24 human bladder specimens (NMIBC=18, NC=6) previously published by our group were used (15). Methylation patterns were assayed using the genome-wide Illumina Infinium HumanMethylation27 BeadChip array (Illumina Inc., San Diego, CA, USA), which enables interrogation of 27,578 CpG dinucleotides covering 14,495 genes. Methylation assays were carried out according to the manufacturer's protocol. Bisulfite conversion of genomic DNA was carried out using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). Fluorescence signals corresponding to C- or T-nucleotides were measured, and the data were used to assign a quantitative measure of methylation level (β-value). Each methylation data point represents the fluorescent signal from the methylated (M) and unmethylated (U) alleles. Background intensity was computed from a set of negative controls and subtracted from each analytic data point. The ratio of the fluorescent signals

Table I. Baseline characteristics of the study subjects.

Variables	NC (n=8)	NMIBC (n=128)
Age (years), mean	59.0±22.2	62.6±14.5
Gender, no. of patients (%)		
Male	6 (75.0)	103 (80.5)
Female	2 (25.0)	25 (19.5)
No. of tumors (%)		
Single	-	77 (60.2)
Multiple	-	51 (39.8)
Tumor size, no. of patients (%)		
<3 cm	-	74 (57.8)
≥3 cm	-	54 (42.2)
Grade, no. of patients (%)		
G1	-	40 (31.3)
G2	-	75 (58.6)
G3	-	13 (10.2)
T stage, no. of patients (%)		
Ta	-	50 (39.1)
T1	-	78 (60.9)
Median RFS, months (range)	-	30.6 (6.0-205.3)
Recurrence, no. of patients (%)		
No	-	74 (57.8)
Yes	-	54 (42.2)
Median PFS, months (range)	-	54.1 (6.4-205.3)
Progression, no. of patients (%)	-	
No		113 (88.3)
Yes		15 (11.7)

NC, normal control; NMIBC, non-muscle invasive bladder cancer; RFS, recurrence-free survival; PFS, progression-free survival.

from the 2 alleles was then computed. The β-value value represents a quantitative measure of the DNA methylation level of specific CpG islands, and ranges from 0 (completely unmethylated) to 1 (completely methylated).

PSQ analysis. The DNA methylation status of candidate NMIBC-specific hypermethylated CpG sites was assessed by PSQ using PyroMark Q96 ID (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. PSQ primers were designed to encompass the CpG sites assayed on the Illumina Infinium array. The primer sequences and amplification conditions are described in Table II.

Bladder cancer cell lines, culture, and drug treatment. The human bladder cancer cell lines T24 (KCLB 30004) and J82 (KCLB 30001) were purchased from the Korean Cell Line Bank (Seoul, Korea). All cells were maintained in RPMI-1640 medium supplemented with 10% FBS (both from Gibco-BRL, Grand Island, NY, USA) and L-glutamine, and cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Both cell lines were cultured until confluent and then treated

Table II. Primers used for pyrosequencing analysis.

Genes	Forward (5'-3')	Reverse (5'-3')	Sequencing primer (5'-3')	Annealing Tm (°C)	Amplicon location relative to TSS	Sequence to analyze	Product size (bp)
BARHL2	GGTTTTTTTATTG TTATTGTTATATGA	(Biotin)-ACAAATTACCACTTCCCAATTA	AAAATAAAATAAATATTAAATAATG	56	(-106)-(-94)	TTGTYGTYGTTT	184
RSPH9	GTTAAGGATGAGG TTTTAGAAGAGAA	(Biotin)-ACAACCTCCTACTATCTCT	AGGATGAGGTTTTAGAAGAGAAT	52	117-147	TYGATTATAGYGGTA GTYGYGTTTAATTAG	146
RAB37	(Biotin)-TGAATGAAATAA GTAGGGATTATTAGT	AACCAAAATTCTAAAATCCTATC	CTTAAAACTCCAAAATACTAAC	52	(-348)-(-327)	GTYGGAGGTAAAAACR CAAAATTAAATCCRACT	111

Primers were designed using NCBI Reference Sequences (NCBI Build 36.1). The PCR reaction contained 0.01 μM primers, Bioneer Taq (Bioneer, Daejeon, Korea), and 20 ng of bisulfite-treated DNA. The thermocycling parameters were as follows: denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 30 sec, annealing at 52-56°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 5 min. *BARHL2*, BarH-like homeobox 2; bp, base pairs; *RAB37*, member RAS oncogene family; *RSPH9*, radial spoke head 9 homolog; Tm, temperature; TSS, transcription start site.

with PBS or 0.3 μmol/l 5-Aza-CdR (both from Sigma-Aldrich, St. Louis, MO, USA). The medium was changed the following day, and cells were counted and harvested on days 3 and 7, and then once per week.

Statistical analysis. DNA methylation data were normalized using quantile normalization in the R language environment (version 2.10.0, available at http://www.r-project.org/). The detailed analytical methods have been described previously (16,17). To detect NMIBC-specific candidate methylation markers, genes whose methylation levels differed between NMIBC and NC by a β-value (Δβ-values) >0.5 were selected. To validate the genes identified in this study, our Δβ-values were compared with those of two microarray data sets obtained from Western populations (9,13): i), 32 bladder tissues (NMIBC =26, NC =6); and ii), 70 bladder tissues (NMIBC =64, NC =6). Continuous variables between groups were compared using a two-sample t-test or ANOVA trend analysis using polynomial contrasts. To minimize bias against arbitrary cut-off points, median values were applied to divide patients into subgroups (hypomethylation or hypermethylation), and the survival function of candidate genes was evaluated. The Kaplan-Meier method was used to estimate the time-to-recurrence or progression based on methylation status, and differences were assessed using log-rank statistics. For the multivariate Cox proportional hazards regression models, the prognostic value of methylation status was evaluated separately and adjusted for well-known clinicopathological (gender, age, tumor size, tumor number, intravesical therapy, grade, and stage) factors. Statistical analysis was performed using IBM SPSS Statistics ver. 21.0 (IBM Co., Armonk, NY, USA). P<0.05 was considered statistically significant.

Results

Baseline characteristics. The baseline characteristics of the NC and NMIBC patients are presented in Table I. Mean recurrence-free survival and progression-free survival in patients with NMIBC was 44.1±39.1 months (median, 30.6; range, 6.0-205.3) and 60.8±40.7 months (median, 54.1; range, 6.4-205.3), respectively.

Identification of differentially methylated and expressed genes in NMIBC and NC. Our previously published genome-wide methylation profiles obtained from 18 NMIBC patients were analyzed and compared with those from 6 NC (15). The complete sets of microarray data derived from the human bladder tissues are available online (http://www.ncbi.nlm.nih.gov/geo/) under the data series accession number GSE37817. To select NMIBC-specific methylation markers, a highly stringent selection criterion (Δβ-value >0.5) was applied. We identified 25 unique CpG island loci in 23 genes that were hypermethylated in NMIBC compared with NC, and these are listed in Table III. The validity of our candidate genes as methylation markers for NMIBC was evaluated using an independent set of Infinium microarray methylation data derived from two Western populations (9,13). Only partial validation was possible because of the limited data provided in both studies. The Δβ-values obtained in the present study were comparable to those of other studies (Table III). Of the

Table III. Comparison of β-value differences between tumors and normal controls with Infinium DNA methylation array data.

Target ID	Symbol	Annotation	Our study			Reinert et al (9)	Ibragimova et al (13)	CpG island location[b]
			Normal	Tumor	Δβ-value[a]	Δβ-value[a]	Δβ-value[a]	
cg02983451	KLF11	Kruppel-like factor 11	0.13	0.75	0.62	0.42	0.58	2:10099704-10102471
cg17241310	BARHL2	BarH-like homeobox 2	0.12	0.7	0.57	0.46	N/A	1:90954376-9095242
cg05899618	GDF7	Growth differentiation factor 7	0.12	0.69	0.57	0.39	N/A	2:20728362-20731136
cg04600618	RSPH9	Radial spoke head 9 homolog	0.14	0.71	0.57	0.44	0.59	6:43720593-4372185
cg12448933	RAB37	RAB37, member RAS oncogene family	0.2	0.76	0.56	0.49	N/A	17:70178644-70179471
cg12374721	PRAC	Prostate cancer susceptibility candidate	0.15	0.71	0.56	0.48	N/A	17:44154292-44154687
cg04396791	SHANK2	SH3 and multiple ankyrin repeat domains 2	0.25	0.81	0.56	0.29	N/A	11:70185287-70186268
cg24199834	POU4F2	POU class 4 homeobox 2	0.14	0.68	0.54	0.47	0.61	4:147778503-147781596
cg25802093	SPAG6	Sperm associated antigen 6	0.21	0.75	0.54	0.52	N/A	10:22673932-22675060
cg08536841	IZUMO1	Izumo sperm-egg fusion 1	0.21	0.73	0.53	0.4	N/A	19:53942196-53942451
cg00117172	RUNX3	Runt-related transcription factor 3	0.08	0.61	0.53	0.42	0.46	1:25127692-2513 1906
cg21790626	ZNF154	Zinc finger protein 154	0.09	0.61	0.53	0.52	0.59	19:62911404-62912681
cg23563234	PCDHGB7	Protocadherin γ subfamily B, 7	0.16	0.68	0.53	0.45	N/A	5:140777221-140777959
cg08260959	HIST1H4F	Histone cluster 1, H4f	0.09	0.61	0.52	0.45	0.58	6:26348554-2634 9101
cg21475402	BCAN	Brevican	0.18	0.7	0.52	N/A	N/A	1:154878094-154879230
cg05159188	HIST1H4F	Histone cluster 1, H4f	0.11	0.63	0.52	0.46	0.48	6:26348554-2634 9101
cg14456683	ZIC1	Zic family member 1	0.21	0.73	0.51	0.52	N/A	3:148609249-148612068
cg12874092	VIM	Vimentin	0.04	0.55	0.51	0.42	0.43	10:17310042-17312147
cg08668790	ZNF154	Zinc finger protein 154	0.11	0.62	0.51	0.5	0.61	19:62911404-62912681
cg23129478	ST8SIA5	ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 5	0.09	0.6	0.51	0.37	0.47	18:42589800-42592268
cg19352038	PAX3	Paired box 3	0.25	0.76	0.51	0.42	N/A	2:222872611-222873277
cg07778029	HOXA9	Homeobox A9	0.06	0.57	0.51	0.5	0.46	7:27170287-27173690
cg03975694	ZNF540	Zinc finger protein 540	0.18	0.68	0.51	0.39	N/A	19:42733846-42734771
cg01295203	PRDM14	PR domain containing 14	0.14	0.65	0.5	0.48	0.55	8:71144163-71147771
cg12111714	ATP8A2	ATPase, aminophospholipid transporter, class I, type 8A, member 2	0.32	0.82	0.5	0.42	N/A	13:24940557-24941659

[a]Δβ-values were calculated by subtracting the Infinium values for the normal tissue from those of the tumor; [b]NCBI Reference Sequences build version 36.1. N/A, not available.

Table IV. Association between methylation markers and clinicopathological characteristics.

Variables	BARHL2 Methylation level (%)	BARHL2 P-value	RSPH9 Methylation level (%)	RSPH9 P-value	RAB37 Methylation level (%)	RAB37 P-value
Normal vs. cancer		<0.001[a]		<0.001[a]		<0.001[a]
Normal	17.1±3.5		18.0±6.6		24.4±1.0	
Cancer	53.8±24.0		52.6±25.9		42.6±18.2	
No. of tumors		0.043[a]		0.030[a]		0.496[a]
Single	50.5±25.3		48.6±25.6		43.5±18.4	
Multiple	58.9±21.2		58.7±25.4		41.2±18.0	
Tumor size (cm)		0.461[a]		0.196[a]		0.386[a]
<3	52.4±25.2		55.1±25.0		41.4±17.8	
≥3	55.7±22.4		49.1±26.0		44.2±18.7	
Grade		0.002[b]		0.001[b]		0.753[b]
G1	46.7±23.4		45.6±22.8		45.7±23.4	
G2	54.7±23.0		52.1±27.0		39.6±17.6	
G3	70.8±23.5		76.6±11.5		49.6±20.2	
T stage		0.004[a]		0.042[a]		0.216[a]
Ta	46.2±24.1		47.7±23.1		45.1±20.0	
T1	58.7±22.7		57.8±27.2		41.0±17.1	
Recurrence		0.048[a]		0.005[a]		0.154[a]
No	50.5±24.4		47.1±24.3		40.5±16.1	
Yes	58.7±22.7		60.1±26.4		45.4±20.5	
Progression		0.022[a]		0.002[a]		0.554[a]
No	52.1±24.2		50.1±25.1		43.0±25.1	
Yes	67.1±18.6		71.5±24.4		40.0±23.5	

[a]P-value calculated using Student's t-test; [b]p-value calculated using ANOVA trend analyses test. *BARHL2*, BarH-like homeobox 2; *RAB37*, member RAS oncogene family; *RSPH9*, radial spoke head 9 homolog.

candidate markers identified, genes in the top fifth Δβ-value were selected for evaluation of their clinical relevance in the validation cohort.

PSQ analysis. To verify the clinical relevance of candidate methylation markers, PSQ analysis was performed using bisulfite-modified genomic DNA obtained from 136 human bladder specimens (NMIBC=128, NC=8). PSQ analysis was technically possible in three out of five candidate genes [BarH-like homeobox 2 (*BARHL2*), radial spoke head 9 homolog (*RSPH9*), and member RAS oncogene family (*RAB37*)], and these genes were analyzed by PSQ in the present study.

Association between methylation levels and clinicopathological variables. As shown in Table IV, the methylation levels of candidate genes were significantly higher in NMIBC patient samples than in normal samples (P<0.001). To evaluate the relationship between methylation patterns and clinicopathological factors, methylation levels were examined in correlation with well-known prognostic factors such as tumor number, tumor size, and tumor grade and stage. The results showed that high levels of methylation of *BARHL2* and *RSPH9* were significantly associated with tumor size, grade, and stage.

Methylation status as a predictor of prognosis. The methylation levels of *BARHL2* and *RSPH9* were significantly higher in the poor prognosis group (recurrence or progression) than in the favorable prognosis group (Table IV). Stratification of NMIBC patients into three prognostic groups (non-recurrence and non-progression, recurrence only, and progression) showed that the methylation of *BARHL2* and *RSPH9* was positively correlated with poor prognosis (Fig. 1). To further determine the relevance of candidate gene methylation status as a predictive indicator, the methylation levels of each gene were dichotomized (hypomethylation or hypermethylation) using median cut-off points. Kaplan-Meier estimates identified significant differences in time-to-recurrence or progression according to methylation status of *BARHL2* and *RSPH9* (Fig. 2, log-rank test, each P<0.05). In univariate and multivariate Cox regression analyses, *RSPH9* methylation status was an independent predictor of recurrence [hazard ratio (HR), 3.02; P=0.001] and progression (HR, 8.25; P=0.028, Table V) in primary NMIBC patients. However, differences in *BARHL2* methylation did not reach statistical significance for the prediction of prognosis (P>0.05).

Reversibility of RSPH9 methylation with AZA treatment. To investigate the potential reversibility of *RSPH9* methylation,

I. Normal
II. Non-recurrence and non-progression
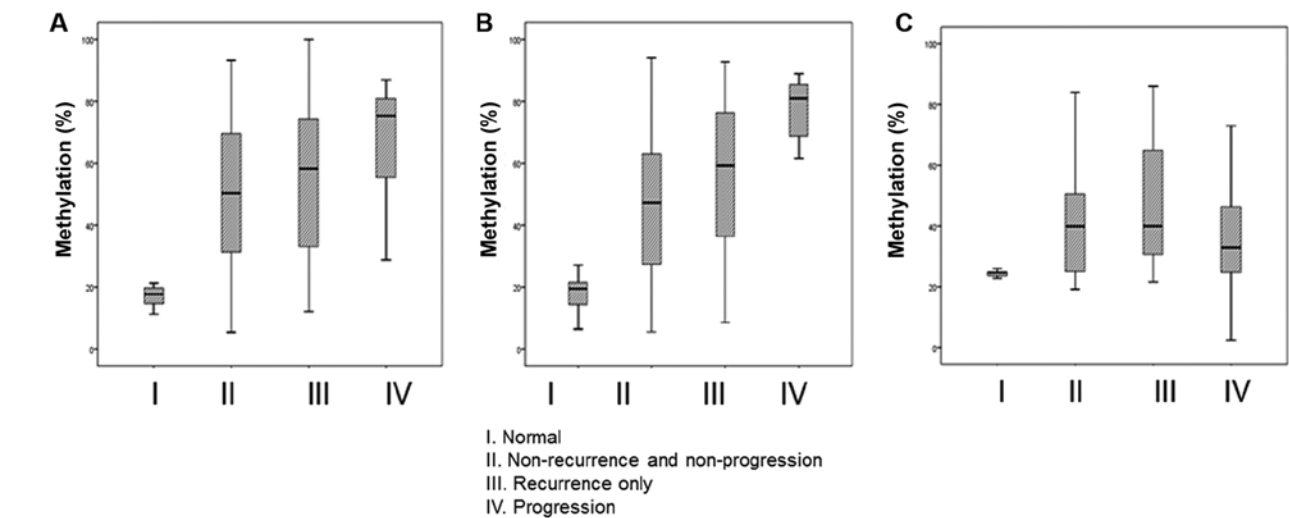III. Recurrence only
IV. Progression

Figure 1. Methylation level of BarH-like homeobox 2 (*BARHL2*) (A), radial spoke head 9 homolog (*RSPH9*) (B), and member RAS oncogene family (*RAB37*) (C) in bladder tissues according to the prognosis of the patients.
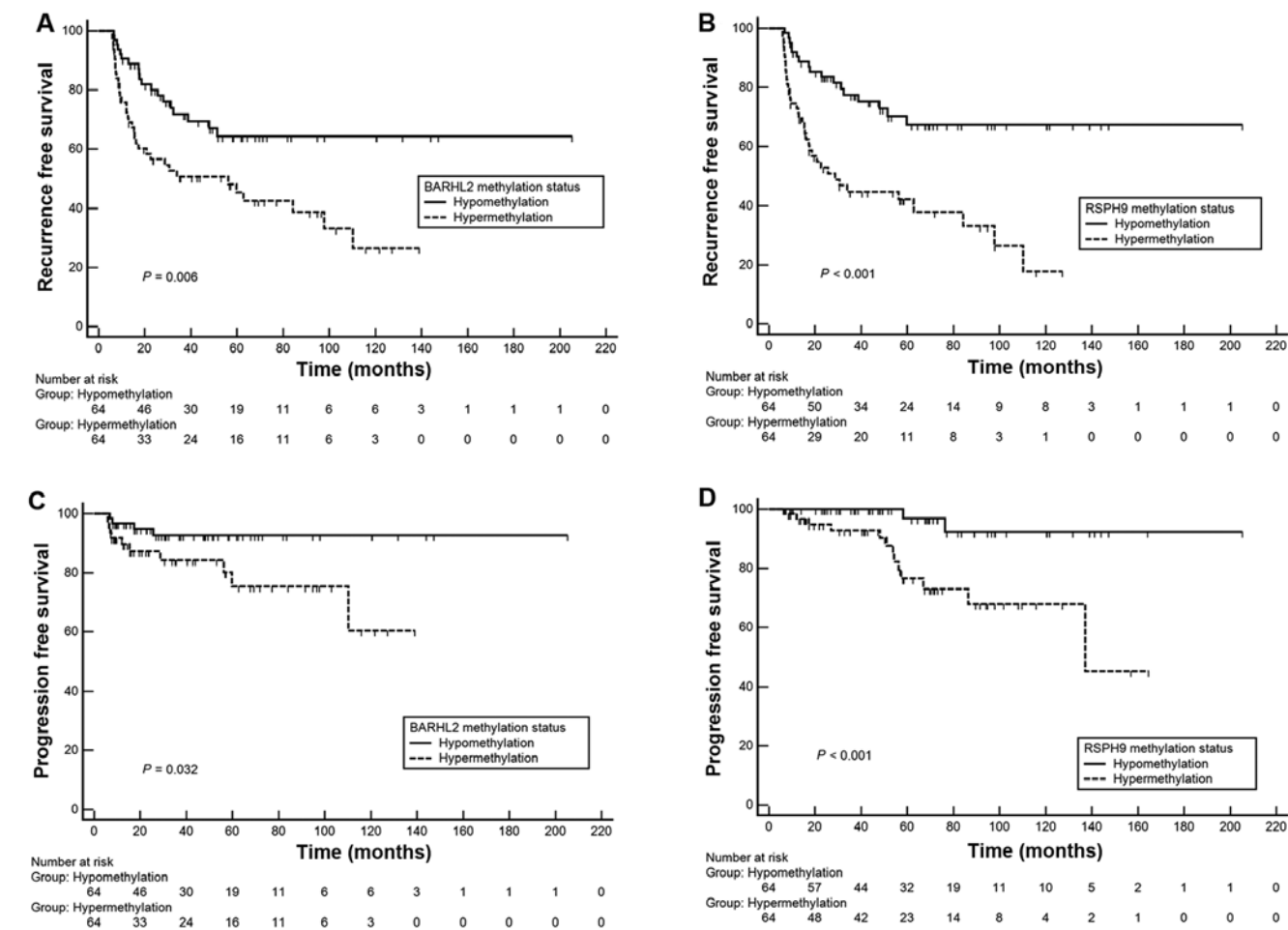

Figure 2. Kaplan-Meier curves for the prediction of prognosis according to the methylation status of each gene in patients with non-muscle invasive bladder cancer (NMIBC). Recurrence-free survival (A and B) and progression-free survival (C and D) according to the methylation status of *BARHL2* and *RSPH9*.

cells from two bladder cancer lines (T24 and J82) were treated with 0.3 μM 5-Aza-CdR for 24 h, and sequential changes in cell number and *RSPH9* methylation level were evaluated in each cell line. Compared to the untreated cells, a decrease in cell count was detected on day 1 after 5-Aza-CdR treatment, reaching a maximal level on day 3 in the J82 and T24 cells, followed by a gradual increase in cell number starting 1 week after 5-Aza-CdR treatment, reaching constant values after 6 weeks in both cell lines. Consistent with the cell-count changes, the methylation level of *RSPH9* decreased on day 3

Table V. Multivariate Cox regression analysis of disease outcomes according to *RHPH9* methylation in non-muscle invasive bladder cancer (n=128).

Variables	Recurrence		Progression	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (<66 vs. ≥66 years)	0.99 (0.57-1.76)	0.973	2.31 (0.69-7.73)	0.175
Gender (male vs. female)	0.56 (0.25-1.26)	0.158	0.18 (0.20-1.55)	0.118
No. of tumors (single vs. multiple)	1.14 (0.62-2.08)	0.672	6.91 (1.70-28.10)	0.007
Tumor size (<3 vs. ≥3 cm)	1.27 (0.69-2.23)	0.158	2.92 (0.85-9.96)	0.088
Stage (Ta vs. T1)	0.69 (0.33-1.43)	0.322	1.17 (0.12-11.87)	0.896
Grade				0.021
G1	1	-	1	-
G2	1.37 (0.65-2.90)	0.408	3.04 (0.23-40.06)	0.399
G3	1.20 (0.41-3.53)	0.744	17.49 (1.11-276.29)	0.042
Intravesical therapy (no vs. yes)	1.41 (0.70-2.83)	0.331	0.84 (0.14-4.91)	0.835
RSPH9 (hypomethylation vs. hypermethylation)	3.02 (1.61-5.67)	0.001	8.25 (1.26-54.09)	0.028

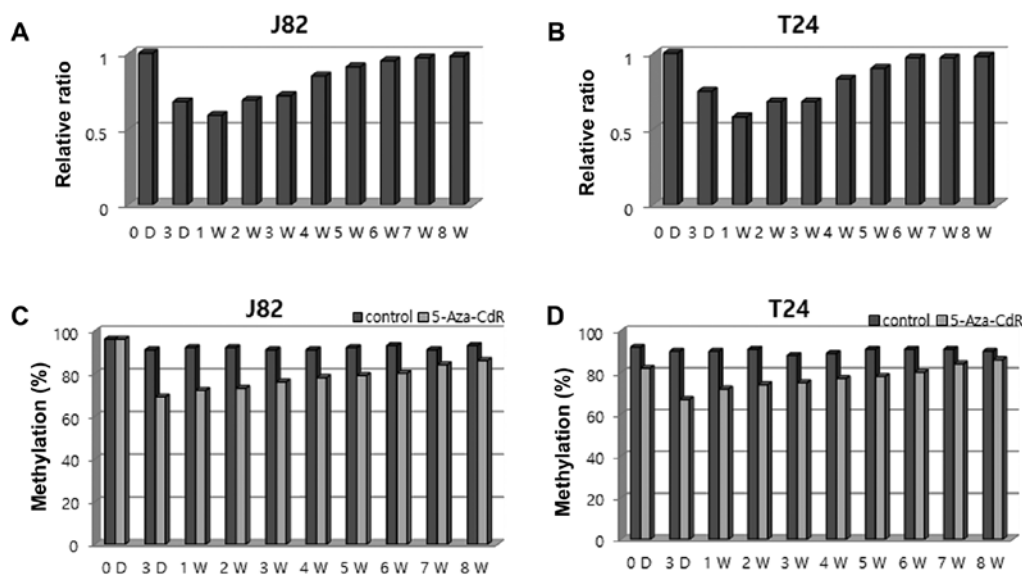*RSPH9*, radial spoke head 9 homolog.

Figure 3. Changes of tumor cell count and *RSPH9* methylation level in bladder cancer cell lines treated with 5-Aza-CdR. Tumor cell count decreased initially in response to 5-Aza-CdR treatment and increased gradually over time without Aza-CdR in J82 (A) and T24 (B) cells. The methylation level of *RSHP9* changed in correlation with cell-count changes in the J82 (C) and T24 (D) cell lines. Cell count was represented as the relative ratio of the number of treated to untreated cells.

and progressively increased 1 week after treatment in the J82 and T24 cell lines (Fig. 3).

Discussion

Similar to other human cancers, bladder cancer is a molecular disease driven by multiple genetic, epigenetic, and environmental factors (6,7). DNA methylation, which is the most common and best characterized epigenetic change in bladder cancer, inactivates tumor-suppressor genes and may be used as potential biomarkers (6,7). In the present study, we used microarray-based profiling to discover novel epigenetic markers relevant to NMIBC. Among the candidate methylation markers identified, the *RSPH9* methylation pattern showed close associations with aggressive NMIBC characteristics, including advanced stage and high tumor grade. The methylation status of *RSPH9* was identified as an independent predictive indicator of prognosis.

The evolution of classic single-gene DNA methylation detection assays to genome-wide microarray-based analyses enabled a better understanding of the role of DNA methylation in cancer (6,14). However, the identification of bona fide candidate methylation markers of clinical relevance requires appropriate selection criteria, validation with external data sets,

confirmation of the methylation status using highly targeted locus-specific assays of human tissues, and comparison with clinicopathological parameters or disease outcomes (4,18). Considering these criteria, the results of the present study are promising. Candidate methylation markers were selected by genome-wide microarray profiling, and their relevance was validated with two microarray data sets obtained from Western populations with NMIBC (9,13). Furthermore, the association between the methylation status of *RSPH9* and prognostic outcomes was verified in long-term follow-up NMIBC patients. The results suggested that the novel methylation marker identified is specific to NMIBC and appropriate for predicting prognosis.

Little information is available concerning the function of the *RSPH9* gene (19-21). It is located on chromosome 6p21.1 and encodes radial spoke head protein 9. Previous studies suggested that *RSPH9* is mutated in primary ciliary dyskinesia patients with microtubule defects (19-21). To the best of our knowledge, the present study is the first to identify *RSPH9* as a cancer-related methylation marker. Despite the prognostic significance of *RSPH9* in NMIBC identified in the present study, these findings do not indicate that it plays a crucial role in bladder tumor initiation or progression. The lack of a clear association between these candidate markers and bladder cancer is a limitation of the present study, and this issue will be addressed in future studies. However, the objective of the present study was the identification of disease markers; we focused on the association between methylation changes of specific methylation markers and disease phenotype rather than analyzing the effect of methylation status on gene transcription and function (4).

DNA methylation is a reversible modification, which makes it a potential therapeutic target. Drugs that target epigenetic alterations, such as DNA methylation inhibitors, restore the activity of genes by targeting aberrant heterochromatic regions, ultimately leading to the reactivation of tumor-suppressor genes and/or other genes that are crucial for normal cellular function (5). In the present study, we showed that the methylation level of *RSPH9* in human bladder cancer cell lines decreased in response to 5-Aza-CdR treatment and increased progressively in its absence. The reversibility of *RSPH9* methylation shown in the present study confirms its value as a candidate therapeutic target. Large-scale validation studies using human samples, as well as functional analyses and a gene ontologic approach to the study of *RSPH9*, may provide additional knowledge about its biological mechanism and clinical relevance.

From a clinical point of view, the most promising applications for epigenetic markers are early detection, prediction of response to treatment, and indication of disease prognosis. The results presented herein are promising because the candidate methylation markers were selected from a genome-wide analysis and validated in a relatively large number of human tissue samples obtained from long-term follow-up patients. In addition, the selected methylation markers are independent predictors of disease outcome. An accurate prediction of prognosis made using these candidate methylation markers would aid clinicians in terms of patient counseling, determining the frequency and extent of monitoring, and whether more aggressive therapy is needed. However, despite these promising results, further validation studies are necessary to reduce false prediction rates and achieve reliable clinical relevance. It may also lead to new therapies that target specific molecular defects, thereby significantly lowering the morbidity associated with NMIBC.

In conclusion, our findings suggest that the novel methylation marker *RSPH9* is an independent indicator of prognosis in NMIBC patients. This prognostic marker may constitute a promising tool for assessing the recurrence and progression of NMIBC and may facilitate the design of individualized therapeutic modalities.

Acknowledgements

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