A novel tumor suppressing gene, *ARHGAP9*, is an independent prognostic biomarker for bladder cancer

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Abstract. Screening for genes or markers relevant to bladder cancer (BC) tumorigenesis and progression is of vital clinical significance. The present study used reverse-transcription quantitative PCR reaction assays to examine the expression of mRNA encoding Rho GTPase-activating protein 9 (ARHGAP9) in BC tissue samples and to determine whether *ARHGAP9* is an independent prognostic biomarker for non-muscle invasive BC (NMIBC) and muscle invasive BC (MIBC). The results revealed that the downregulation of *ARHGAP9* expression in the tissue of patients with NMIBC or MIBC was significantly associated with a poor prognosis. In patients with NMIBC, a high expression of *ARHGAP9* was significantly associated with prolonged recurrence-free survival, whereas in MIBC patients, it was significantly associated with an increased progression-free and cancer-specific survival. The risk of cancer-specific death was 2.923 times higher (95% confidence interval, 1.192-7.163) when *ARHGAP9* levels were decreased. In conclusion, lower expressions of *ARHGAP9* correlated with BC prognosis, indicating that it may be a useful marker for guiding treatment application.

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Abbreviations: ARHGAP9, Rho GTPase-activating protein 9; BC, Bladder cancer; CI, confidence interval; CIS, carcinoma *in situ*; CSS, cancer-specific survival; EGFR, epidermal growth factor receptor; Gli1, glioma-associated oncogene homolog 1; HR, hazard ratio; MAPK1, mitogen-activated protein kinase 1 (also known as ERK2); MAPK14, mitogen-activated protein kinase 14 (also known as p38α); MIBC, muscle invasive BC; MKK3, mitogen-activated protein kinase kinase 3; MKK6, mitogen-activated protein kinase kinase 6; NGS, next generation sequencing; NMIBC, non-muscle invasive BC; PFS, progression-free survival; Real-time PCR, real-time polymerase chain reaction; RFS, recurrence-free survival; T1HG, T1 high grade

Key words: Rho GTPase-activating protein 9, non-muscle invasive bladder cancer, muscle invasive bladder cancer, recurrence, progression

Introduction

Bladder cancer (BC), one of the most common malignancies worldwide, is classified into two subtypes based on cancer cell infiltration into the muscle layer of the bladder. Non-muscle invasive BC (NMIBC) is less aggressive but has a high recurrence rate, whereas muscle invasive BC (MIBC) tends to metastasize and has a relatively poor prognosis (1-3). High throughput techniques such as microarray analysis and next generation sequencing, which are used commonly in the fields of genetics and epigenetics, have identified several genes involved in cancer pathogenesis, and have led to identification of cancer biomarkers and to development of novel effective gene targeted therapies (4). In a previous study, we used next generation sequencing and miRNA microarray assays to identify several miRNAs and their target genes that are differentially expressed in BC (5). We found that a novel gene, Rho GTPase-activating protein 9 (*ARHGAP9*), is down-regulated in BC. In addition, hsa-miR-3620, which interacts with *ARHGAP9*, is up-regulated.

Rho GTPases are key regulators of the actin cytoskeleton, which plays an important role in cell adhesion and migration.

The switch mechanism of Rho GTPases is controlled by binding to GTP or GDP (6-8). ARHGAP9 contains a diverse combination of functional protein domains, including the RhoGAP, SH3, WW, and PH domains (9). Binding of the RhoGAP domain to GTP-bound Rho proteins accelerates GTPase activity, and defective Rho GTPase signaling is implicated in tumorigenesis and metastasis (10,11). Silencing ARHGAP9 inhibits proliferation, migration, and invasion of breast cancer cells (12). Activated ARHGAP9 inhibits adhesion of a human leukemia cell line, KG-1, to fibronectin and collagen through activation of cdc42 and Rac1 but not RhoA (6).

Here, we asked whether *ARHGAP9* is a novel prognostic biomarker for BC. We used real-time polymerase chain reaction (PCR) to compare expression of *ARHGAP9* mRNA in human BC and control tissues (the latter comprised normal tissue surrounding BC and normal bladder mucosa); and analyzed its ability to predict prognosis of NMIBC and MIBC. ARHGAP9, known as a MAP kinase docking protein, was encoded by *ARHGAP9* gene, which shares 16 bases with *Gli1* in their 3' ends (9,13). Accordingly, we asked whether *ARHGAP9* plays a role in the MAPK and Hedgehog signaling pathways.

Materials and methods

Patients and tissue samples. The biospecimens used in the present study 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 study was approved by the Institutional Review Board at Chungbuk National University (GR2010-12-010), and the experiments were undertaken with the informed written consents of all participants. The study methodologies conformed with the standards set by the Declaration of Helsinki. The baseline characteristics of the case subjects (n=237 bladder tissue samples) are shown in Table I. Among these, 140 samples were from primary BC patients and were histologically verified as transitional cell carcinomas; the remaining 97 samples used as the control set comprised normal bladder mucosa or normal tissues from the area surrounding BC. To reduce the chances of confounding factors affecting the analyses, patients diagnosed with concomitant carcinoma in situ or carcinoma in situ lesions alone were excluded. Voided urine cytology was tested before surgical treatment to assist BC diagnosis and/or prognosis. Fresh-frozen specimens were obtained during surgical resection of transitional cell carcinoma at Chungbuk National University Hospital. All tumors were macro-dissected, typically within 15 min of surgical resection. Each specimen was confirmed by pathological analysis of a part of fresh-frozen specimens obtained from radical cystectomy and transurethral resection of bladder tumor (TURBT). Tumors were staged (2002 TNM Classification) and graded (2004 WHO Classification), according to standard criteria (14). Clinically metastatic disease and non-cystectomy cases were not excluded from the study. Each patient was followed and managed suggested management according to standard recommendations (15-17). Surveillance was performed by cystoscopic examination and upper urinary tract imaging in accordance with European Association of Urology guidelines (16). Recurrence was defined as relapse of primary NMIBC of the same pathologic stage, and progression of NMIBC and MIBC was defined as TNM stage progression after disease recurrence. The mean follow-up period for NMIBC patients was 72.95 months (range, 3.2-172.2). The mean follow-up period for MIBC patients was 36.18 months (range, 3.0-141.4).

RNA extraction. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen), as described previously (18), and stored at -80˚C. Next, cDNA was synthesized from 1 μg of total RNA using a First Strand cDNA Synthesis kit (Clontech, TAKARA), according to the manufacturer's protocol.

Microarray analysis. Five hundred nanograms of total RNA was used for labeling and hybridization prior to analysis, according to the manufacturer's protocols (Illumina). After the bead chips were scanned with an Illumina Bead Array Reader, the Robust Multiarray Average in R package was used to perform global correction, quantile normalization, and median polish summarization of the microarray data. P-values (t test) were calculated from bead mRNA signal intensities (19-21). The full set of microarray data set are available online at http://www.ncbi.nlm.nih.gov/geo/under data series accession number GSE13507 (21).

mRNA sequencing. Total sequencing reads were subjected to preprocessing as follows: Adapter trimming was performed using cutadapt with default parameters, and quality trimming (Q30) was performed using FastQC with default parameters. Processed reads were mapped to the human reference genome [Ensembl 72 (GRCh37: hg19)] using tophat and cufflink with default parameters (22). Fragments Per Kilobase of exon per million fragments Mapped (FPKM) values were normalized and quantitated using R package Tag Count Comparison (TCC) (23) to determine statistical significance (e.g., P and Q values) and differential expression (e.g., -fold changes).

Quantitative PCR analysis. Tissue mRNAs were amplified by quantitative PCR performed using a Rotor Gene 6000 instrument (Qiagen) and quantified using the $2^{-\Delta\Delta cq}$ method (24). QuantitativePCR reactions were carried out using the SYBR Premix Ex Taq II (Clontech, TAKARA). The following primers were used to amplify candidate genes: *ARHGAP9* (Gene ID: ENSG00000123329), sense, 5'-CAGAGCAGTGCC TCTCTC-3' (18 bp, Tm 58˚C); antisense, 5'-CTGCTGGGT CAGATGTCTC-3' (19 bp, Tm 58˚C) and the amplicon size was 179 bp. The control *GAPDH* (Gene ID: ENSG00000111640) primers were as follows: sense, 5'-CATGTTCGTCATGGG TGTGA-3' (20 bp, Tm 60˚C); antisense, 5'-ATGGCATGG ACTGTGGTCAT-3' (20 bp, Tm 60˚C) and the amplicon size was 156 bp. The PCR reaction was performed in a final volume of 10 μl, comprising 5 μl of 2x SYBR Premix EX Taq buffer, 0.5 μl of each 5'and 3' primer (10 pM/μl), and 2 μl, of sample cDNA. A known concentration of the PCR product was then 10-fold serially diluted from 100 pg/μl to 0.1 pg/μl and used to establish a standard curve. The real-time PCR conditions were as follows: 1 cycle at 96˚C for 20 sec, followed by 40 cycles of 3 sec at 96˚C for denaturation, 15 sec at 60˚C for annealing, and 15 sec at 72˚C for extension. The melting program was performed at 72-95˚C at a heating rate of 1˚C per

Table I. Clinicopathological features of primary BC patient and control tissues (surrounding normal tissues and normal bladder mucosae).

	BC (140)			
Variables	NMIBC	MIBC	Control	P-value
No.	97	43	97	
Mean age ± SD	63.45±13.79	67.60±9.84	61.98±14.32	0.083[a]
Sex (%)				0.975[a]
Male	80 (82.5%)	36 (83.7%)	81 (83.5%)	
Female	17 (17.5%)	7 (16.3%)	16 (16.5%)	
Operation (%)				<0.001[b]
TUR-BT	97 (100.0%)	17 (39.5%)		
Radical cystectomy	0	26 (60.5%)		
Tumor size (%)				0.003[b]
≤1 cm	16 (16.5%)	2 (4.7%)		
2-3 cm	37 (38.1%)	11 (25.6%)		
>3 cm	37 (38.1%)	28 (65.1%)		
Multiplicity (%)				0.108[b]
Single	52 (53.6%)	30 (69.8%)		
2-7	28 (28.9%)	7 (16.3%)		
>7	11 (11.3%)	4 (9.3%)		
Grade, 2004 WHO grading system (%)				<0.001[b]
Low	72 (74.2%)	8 (18.6%)		
High	25 (25.8%)	35 (81.4%)		
Stage (%)				<0.001[b]
TaN0M0	26 (26.8%)			
T1N0M0	71 (73.2%)			
T2N0M0		13 (30.2%)		
T3N0M0		6 (14.0%)		
T≥4 or N≥1 or M1		24 (55.8%)		
Chemotherapy (%)				<0.001[b]
No	97 (100.0%)	23 (53.5%)		
Yes	0	20 (46.5%)		
BCG therapy (%)				<0.001[b]
No	56 (57.7%)	38 (88.4%)		
Yes	40 (41.2%)	5 (11.6%)		
Recurrence, no. of patients (%)				
No	59 (60.8%)	-		
Yes	38 (39.2%)	-		
Progression, no. of patients (%)				0.126[b]
No	79 (81.4%)	30 (69.8%)		
Yes	18 (18.6%)	13 (30.2%)		
Survival, no. of patients (%)				0.009[b]
Alive	64 (66.0%)	21 (48.8%)		
Non-cancer-specific death	18 (18.6%)	3 (7.0%)		
Cancer-specific death	15 (15.5%)	19 (44.2%)		
Mean follow-up, months (range)	72.95 (3.20–172.20)	36.18 (3.00–141.40)		

[a]P-value obtained using Kruskal-Wallis H test (BC compared with control). [b]P-value obtained using the Mann-Whitney U test (NMIBC compared with MIBC). BC, bladder cancer; BCG, Bacillus Calmette-Guerin; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; SD, standard deviation.

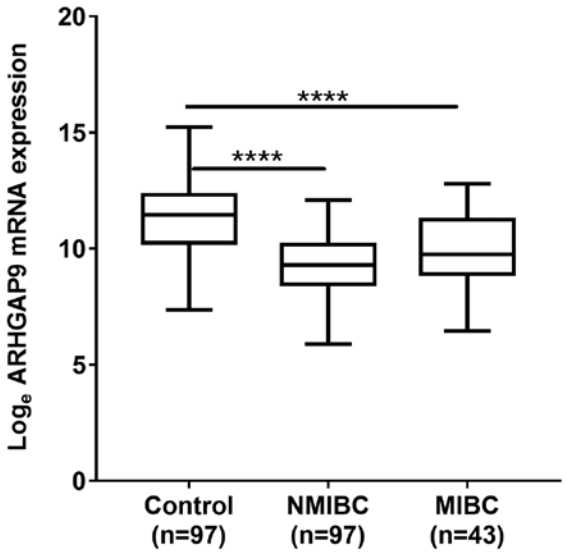

Figure 1. Expression of mRNA encoding ARHGAP9 in BC tissue. Expression of *ARHGAP9* in NMIBC and MIBC tissue was significantly lower compared with normal control tissue samples. BC, bladder cancer; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer. Control samples represent normal bladder mucosae and normal tissues surrounding bladder cancer. The P-value was calculated using the Mann-Whitney U test. ****P<0.0001. ARHGAP9, Rho GTPase-activating protein 9; BC, bladder cancer; MIBC, muscle invasive BC; NMIBC, non-muscle invasive bladder cancer.

45 sec. Rotor-Gene Q software 2.3.1.49 was used for capturing and analyzing spectral data. All samples were run in triplicate. Gene expression was normalized to the expression of *GAPDH*.

Statistical analysis. To reduce variation among microarrays, the intensity values for each microarray were rescaled using a quantile normalization method (19). Gene expression values were loge-transformed and median-centered across samples. The significance of various clinicopathological variables was evaluated using univariate and multivariate Cox proportional hazard regression models. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated to investigate relative risk. Survival curves to determine the prognostic value of the genetic biomarker were plotted using the Kaplan-Meier method and compared using the log-rank test. The *Kruskal-Wallis H* test and *Mann-Whitney U* test were used to examine expression of *ARHGAP9* in BC tissues versus control tissues. Correlations between *ARHGAP9* and genes involved in the MAPK and Hedgehog signaling pathways were examined by calculating non-parametric Spearman's correlation coefficients. Statistical analyses were performed using IBM SPSS Statistics ver. 20.0 (IBM) and GraphPad Prism 7 (GraphPad Software). P-values <0.05 were considered significant.

Results

Expression of ARHGAP9 mRNA in BC tissue. Microarray analysis revealed that expression of mRNA encoding *ARHGAP9* in BC tissues was lower than that in control samples. The validation test showed that the real-time PCR results were identical to those of the microarray, i.e., expression of mRNA encoding *ARHGAP9* was significantly lower in NMIBC and MIBC tissues than in normal control tissues (P<0.001; Fig. 1).

Expression of ARHGAP9 correlates with NMIBC prognosis. Univariate and multivariate Cox regression analyses revealed that expression of *ARHGAP9* in NMIBC patients was an independent predictor of recurrence-free survival (RFS) (HR, 2.436; 95% CI, 1.132-5.243; P=0.023; Table II). Kaplan-Meier analysis demonstrated that NMIBC patients with *ARHGAP9* expression levels in the upper 50th percentile experienced less recurrence than those with expression levels in the lower 50th percentile (log-rank test, P=0.043; Fig. 2A). Particularly, for T1 high grade(HG) BC patients, univariate and multivariate Cox regression analysis identified *ARHGAP9* expression as an independent risk factor for T1HG BC recurrence (HR, 7.264; 95% CI, 1.291-45.091; P=0.025) and progression (HR, 14.987; 95% CI, 1.093-205.567; P=0.043; Table III). The RFS and progression-free survival (PFS) of T1HG BC patients with *ARHGAP9* expression levels in the upper 50th percentile experienced less recurrence and progression than those with expression levels in the lower 50th percentile (log-rank test, P=0.013 and 0.026 respectively; Fig. 2B and C).

Expression of ARHGAP9 correlates with MIBC prognosis. For MIBC patients, univariate and multivariate Cox regression analysis identified *ARHGAP9* expression as an independent risk factor for disease progression (HR, 5.241; 95% CI, 1.456-18.870; P=0.011) and cancer-specific death (HR, 2.923; 95% CI, 1.192-7.163; P=0.019) (Tables IV and V). PFS and cancer specific survival (CSS) of patients with *ARHGAP9* expression in the upper 50th percentile were significantly higher than those of patients in the lower 50th percentile (log-rank test, P=0.020 and 0.031, respectively; Fig. 3A and B).

Relationship between ARHGAP9 and genes regulating the MAPK and Hedgehog signaling pathways in BC. To identify whether expression of *ARHGAP9* correlates with that of genes regulating the MAPK and Hedgehog signaling pathways, we undertook gene network depiction and analysis using the GeneMANIA (http://www.genemania.org) web tool. We selected seven genes (*ARHGAP9*, epidermal growth factor receptor (*EGFR*), mitogen-activated protein kinase 1 (*MAPK1*, also known as *ERK2*), mitogen-activated protein kinase 14 (*MAPK14*, also known as *p38α*), mitogen-activated protein kinase kinase 3 (*MKK3*), mitogen-activated protein kinase kinase 6 (*MKK6*), and glioma-associated oncogene homolog 1 (*Gli1*)) showing potential inter-correlations (Supplementary Fig. S1). Non-parametric Spearman's correlation coefficients (based on microarray data) identified interactions among *ARHGAP9*, *EGFR*, *MAPK1* (*ERK2*), *MAPK14* (*p38α*), *MKK3*, *MKK6*, and *Gli1*. Table VI shows that expression of *ARHGAP9* correlated positively with that of *Gli1*, which regulates the Hedgehog signaling pathway. In addition, *ARHGAP9* interacted with *MKK6* and *MAPK1* (*ERK2*), both of which are essential components of the MAPK signal transduction pathway (P<0.05 for both).

Discussion

ARHGAP9 sits adjacent to *Gli1* on human chromosome 12q13.3; two genes have overlapping 16 bases in their 3'-ends (13), suggesting that *Gli1* and *ARHGAP9* may regu-

Table II. Univariate and multivariate Cox regression analysis to predict NMIBC recurrence.

	Univariate Cox analysis		Multivariate Cox analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age				
≤70 (Ref.) vs. >70	2.994 (1.579-5.680)	0.001[a]	1.727 (0.820-3.637)	0.151
Sex				
Male (Ref.) vs. female	1.314 (0.577-2.993)	0.516		
Tumor size				
≤1 cm	Ref.	0.028[a]	Ref.	0.574
2-3 cm	1.700 (0.474-6.100)	0.416	1.251 (0.341-4.593)	0.736
>3 cm	3.686 (1.093-12.425)	0.035[a]	1.779 (0.484-6.547)	0.386
Multiplicity				
Single	Ref.	0.141		
2-7	1.071 (0.479-2.395)	0.867		
>7	2.383 (0.985-5.767)	0.054		
2004 WHO Grade				
Low (Ref.) vs. high	2.450 (1.275-4.708)	0.007[a]	1.823 (0.809-3.568)	0.147
Stage				
Ta (Ref.) vs. T1	2.938 (1.144-7.540)	0.025[a]	2.347 (0.803-6.857)	0.119
BCG				
No (Ref.) vs. yes	1.918 (1.009-3.647)	0.047[a]	1.744 (0.852-3.568)	0.128
ARHGAP9 expression				
High expression (Ref.) vs. Low expression	1.939 (1.009-3.726)	0.047[a]	2.436 (1.132-5.243)	0.023[a]

[a]P<0.05. NMIBC, non-muscle invasive bladder cancer; BCG, Bacillus Calmette-Guerin; CI, confidence interval; HR, hazard ratio; Ref., reference; ARHGAP9, Rho GTPase-activating protein 9.

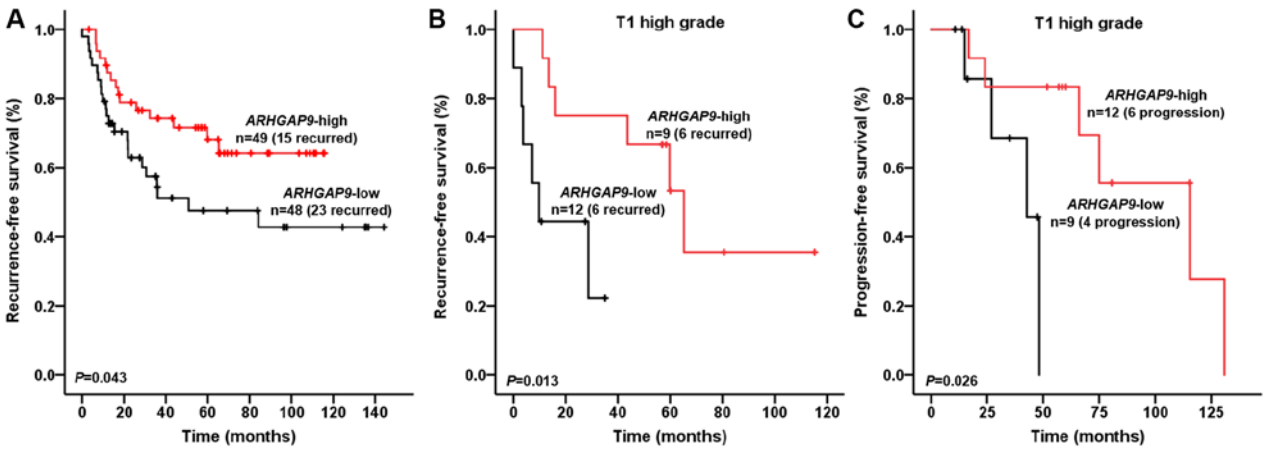

Figure 2. Kaplan-Meier curves showing effect of *ARHGAP9* on the recurrence-free survival and progression-free survival of NMIBC patients. (A) Recurrence-free survival of patients with NMIBC. (B) Recurrence-free survival of patients with T1 high grade BC. (C) Progression-free survival of patients with T1 high grade BC. BC patients were divided into two groups (upper 50th percentile and lower 50th percentile groups) according to the expression of *ARHGAP9*. The recurrence-free survival rate of NMIBC patients, particularly in T1HG BC patients, was significantly higher in the high *ARHGAP9* expression group (log-rank test; P<0.05). The progression-free survival of T1HG BC patients was significantly higher in the high *ARHGAP9* expression group (log-rank test, P<0.05). ARHGAP9, Rho GTPase-activating protein 9; NMIBC, non-muscle invasive bladder cancer; T1HG, T1 high grade; BC, bladder cancer.

late each other. Studies suggest that *Gli1* is down-regulated in BC (25); indeed, *Gli1* is considered to be the most reliable biomarker of Hedgehog pathway activity (25-27). The microarray data presented herein shows that mRNA expression of *Gli1* and *ARHGAP9* were down-regulated in BC tissues, and that there was a positive correlation between the two (Table VI); this indicates that *ARHGAP9*, which lies adjacent to *Gli1*, might be a novel regulator of *Gli1*.

Table III. Univariate and multivariate Cox regression analysis to predict T1 high grade NMIBC recurrence and progression.

	Recurrence				Progression			
	Univariate Cox analysis		Multivariate Cox analysis		Univariate Cox analysis		Multivariate Cox analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age								
≤70 (Ref.) vs.								
>70	2.342 (0.625-8.776)	0.207			1.567 (0.390-6.297)	0.527		
Sex								
Male (Ref.) vs. female	1.327 (0.342-5.154)	0.682			2.748 (0.548-13.781)	0.219		
Tumor size								
≤1 cm	Ref.	0.976			Ref.	0.468		
2-3 cm	29604.104 (0.000-2.839x10^{138})	0.948			9687.884 (0.000-3.269x10^{201})	0.968		
>3 cm	25622.270 (0.000-2.454x10^{138})	0.949			36480.741 (0.000–1.226x10^{202})	0.964		
Multiplicity								
Single	Ref.	0.618			Ref.	0.850		
2-7	1.450 (0.417-5.040)	0.559			1.548 (0.345–6.943)	0.568		
>7	2.933 (0.296-29.074)	0.358			0.000 (0.000-0.000)	0.991		
BCG								
No (Ref.) vs. yes	1.247 (0.336-4.624)	0.741			0.459 (0.119-1.766)	0.257		
ARHGAP9 expression								
High (Ref.) vs. low expression	5.126 (1.247-21.066)	0.023[a]	7.264 (1.291-45.019)	0.025[a]	6.041 (1.026-35.571)	0.047[a]	14.987 (1.093-205.567)	0.043[a]

[a]P<0.05. NMIBC, non-muscle invasive bladder cancer; BCG, Bacillus Calmette-Guerin; CI, confidence interval; HR, hazard ratio; Ref., reference; ARHGAP9, Rho GTPase-activating protein 9.

Table IV. Univariate and multivariate Cox regression analysis to predict MIBC progression.

	Univariate Cox analysis		Multivariate Cox analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age				
≤70 (Ref.) vs. >70	1.302 (0.432-3.926)	0.639		
Sex				
Male (Ref.) vs. female	5.625 (1.766-17.912)	0.003[a]	7.255 (2.062-25.528)	0.002[a]
Operation				
TURBT (Ref.) vs.				
Radical cystectomy	0.948 (0.309-2.905)	0.926		
Tumor size				
≤1 cm	Ref.	0.406		
2-3 cm	12417.036 (0.000–2.033x10^{143})	0.954		
>3 cm	35009.555 (0.000–5.718x10^{143})	0.949		
Multiplicity				
Single	Ref.	0.507		
2-7	0.358 (0.046-2.800)	0.328		
>7	1.483 (0.324-6.787)	0.611		
2004 WHO Grade				
Low (Ref.) vs. high	31.010 (0.132-7298.224)	0.218		
Stage				
T2	Ref.	0.851		
T3	1.630 (0.297-8.958)	0.574		
T4 or N1 or M1	1.229 (0.358-4.222)	0.744		
Chemotherapy				
No (Ref.) vs. yes	3.912 (1.076-14.218)	0.038[a]	2.859 (0.752-10.868)	0.123
ARHGAP9 expression				
High expression (Ref.) vs.				
Low expression	3.818 (1.145-12.733)	0.029[a]	5.241 (1.456-18.870)	0.011[a]

[a]P<0.05. MIBC, muscle invasive bladder cancer; CI, confidence interval; HR, hazard ratio; Ref., reference; ARHGAP9, Rho GTPase-activating protein 9.

As a novel MAP kinase docking protein, ARHGAP9 associates specifically with ERK2 and p38α via complementarily charged residues within the WW domain of ARHGAP9 and the CD domains of ERK2 and p38α. This interaction suppresses MAP kinase activation; but does not affect that of RhoGAP (9). MAPK activation is a common event in tumor progression and metastasis. Inhibition of ERK1/2 and p38 MAP kinase pathways in BC could inhibit proliferation and growth (28). The key target in this signal transduction pathway is EGFR, a receptor tyrosine kinase (29). Binding of EGF to EGFR in BC activates EGFR, which is already overexpressed; furthermore, the Ras-MAPK pathway is activated through the MAPK/ERK pathway. This continuous 'ON' status of MAPK signaling results in overexpression of MEK2 and MKK3, 4, and 6, which lie upstream of MAP kinase (i.e., ERK2 and p38α) and activate ERK2 and p38α, leading to reduced interaction between ARHGAP9 and ERK2 or p38α in BC (this is probably attributable to competitive displacement by overexpressed docking proteins) (Fig. 4). The microarray data revealed a competitive correlation between expression of *ARHGAP9* mRNA and that of *MKK6*, and a positive correlation between *ARHGAP9* and *ERK2* (Table VI). These findings suggest that *ARHGAP9* acts as a tumor suppressor gene in BC. EGFR acts as a receptor molecule in the MAPK signaling pathway, and is a prognostic marker for many cancer types, including BC (30). Our previous study showed that *EGFR* is a progression-related gene in MIBC; increased expression of *EGFR* is associated with a poor prognosis (31). Here, we found that lower expression of *ARHGAP9* was related to poor PFS and CSS (Fig. 3A and B), which is consistent with previous results. However, no definitive evidence has been demonstrated on the recurrence rate of MIBC after radical cystectomy, and the definition of local and distant recurrence is not standardized (32). In our preliminary study, twenty-six MIBC patients received radical cystectomy and only three of them were manifested recurrence, such result should be examined in further study with more samples for the statistically significant validation of the survival analysis.

Furthermore, the *ARHGAP9* mRNA expression could predict the recurrence of NMIBC, that is, lower expression of *ARHGAP9* was related to poor RFS (Fig. 2A). In particular, T1HG BC patients with higher expression of *ARHGAP9* experienced less recurrence and progression (Fig. 2B and C). A

Table V. Univariate and multivariate Cox regression analysis for predicting the cancer-specific survival of patients with MIBC.

	Univariate Cox analysis		Multivariate Cox analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age				
≤70 (Ref.) vs. >70	1.860 (0.791-4.371)	0.155		
Gender				
Male (Ref.) vs. female	3.379 (1.273-8.967)	0.014[a]	4.046 (1.491-10.976)	0.006[a]
Operation				
TURBT (Ref.) vs.				
Radical cystectomy	1.026 (0.435-2.417)	0.954		
Tumor size				
≤1 cm	Ref.	0.386		
2-3 cm	14923.217 (0.000-1.565E+115)	0.941		
>3 cm	32178.497 (0.000-3.369E+115)	0.937		
Multiplicity				
Single	Ref.	0.730		
2-7	0.709 (0.206-2.438)	0.585		
>7	0.611 (0.137-2.725)	0.519		
2004 WHO Grade				
Low (Ref.) vs. high	3.009 (0.699-12.950)	0.139		
Stage				
T2	Ref.	0.480		
T3	0.909 (0.181-4.563)	0.908		
T4 or N1 or M1	1.671 (0.641-4.358)	0.294		
Chemotherapy				
No (Ref.) vs. yes	1.482 (0.633-3.472)	0.365		
ARHGAP9 expression				
High expression (Ref.) vs.				
Low expression	2.554 (1.058-6.163)	0.037[a]	2.923 (1.192-7.163)	0.019[a]

[a]P<0.05. MIBC, muscle invasive bladder cancer; CI, confidence interval; HR, hazard ratio; Ref., reference; ARHGAP9, Rho GTPase-activating protein 9.

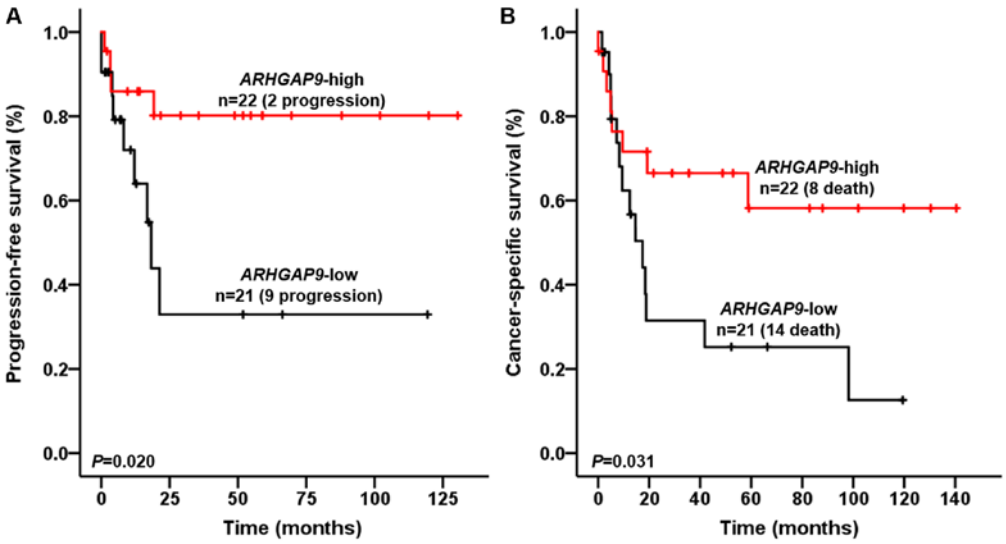

Figure 3. Kaplan-Meier curves demonstrating the effect of *ARHGAP9* on the progression-free survival and cancer-specific survival of MIBC patients. Patient (A) progression-free survival and (B), cancer-specific survival rates are presented. BC patients were divided into two groups (upper 50th percentile and lower 50th percentile groups) according to the expression of *ARHGAP9*. The progression-free survival and cancer-specific survival of MIBC patients were significantly higher in the high *ARHGAP9* expression group (log-rank test, P<0.05). ARHGAP9, Rho GTPase-activating protein 9; MIBC, muscle invasive bladder cancer; BC, bladder cancer.

Table VI. Spearman correlation coefficients of *Gli1*, *ARHGAP9*, *EGFR*, *MKK3*, *MKK6*, *MAPK1* (*ERK2*) and *MAPK14 (p38α)* in BC.

	Gli1	*ARHGAP9*	*EGFR*	*MKK3*	*MKK6*	*MAPK1 (ERK2)*	*MAPK14 (p38α)*
Gli1							
Spearman's Rho	1.000	0.518[b]	-0.009	0.099	-0.042	0.178[a]	-0.202[b]
P-value	.	0.000	0.911	0.205	0.589	0.022	0.009
ARHGAP9							
Spearman's Rho	0.518[b]	1.000	0.084	0.125	-0.168[a]	0.233[b]	-0.138
P-value	0.000	.	0.283	0.109	0.031	0.003	0.076
EGFR							
Spearman's Rho	-0.009	0.084	1.000	0.194[a]	-0.118	0.301[b]	0.192[b]
P-value	0.911	0.283	.	0.012	0.130	0.000	0.013
MKK3							
Spearman's Rho	0.099	0.125	0.194[a]	1.000	0.101	0.327[b]	0.315[b]
P-value	0.205	0.109	0.012	.	0.195	0.000	0.000
MKK6							
Spearman's Rho	-0.042	-0.168[a]	-0.118	0.101	1.000	-0.093	-0.056
P-value	0.589	0.031	0.130	0.195	.	0.233	0.472
MAPK1(ERK2)							
Spearman's Rho	0.178[a]	0.233[b]	0.301[b]	0.327[b]	-0.093	1.000	0.167[a]
P-value	0.022	0.003	0.000	0.000	0.233	.	0.032
MAPK14 (p38α)							
Spearman's Rho	-0.202[b]	-0.138	0.192[a]	0.315[b]	-0.056	0.167[a]	1.000
P-value	0.009	0.076	0.013	0.000	0.472	0.032	.

[a]P<0.05. [b]P<0.01. BC, bladder cancer.

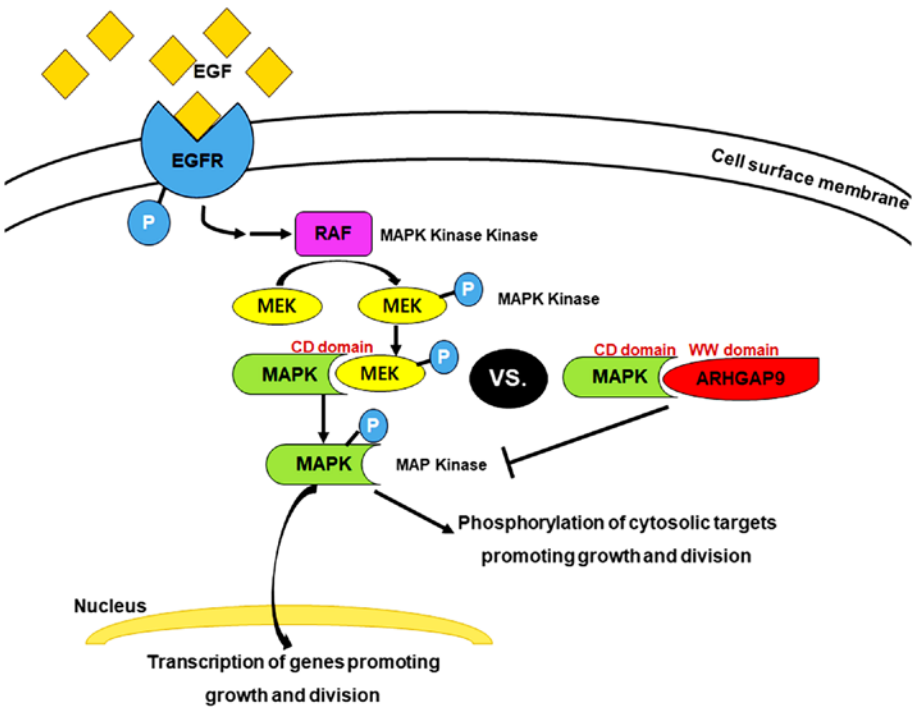

Figure 4. ARHGAP9-mediated regulation of the MAPK signaling pathway in BC. ARHGAP9 associates specifically with ERK2 and p38α via complementarily charged residues within the WW domain of ARHGAP9 and the CD domains of ERK2 and p38α. The binding of EGF to EGFR activates EGFR, which is already overexpressed in BC. Furthermore, the Ras-MAPK pathway is activated through the MAPK/ERK pathway. The increased expression of various upstream kinases (including MEK2 and MKK3, 4 and 6, which interact with ERK2 and p38α, respectively) reduces interaction between ARHGAP9 and ERK2 and p38α in BC. ARHGAP9, Rho GTPase-activating protein 9; BC, bladder cancer.

more careful monitoring and optimal treatment recommendation should be implemented for T1HG BCs because of their highly recurrent nature and risk of progression to MIBC (33), which highlights the strategy for predicting prognosis. This study indicates that *ARHGAP9* gene has a good performance in predicting prognosis of T1HG BC patients.

In addition, TCGA data from the Human Pathology Atlas (https://www.proteinatlas.org/ENSG00000123329-ARHGAP9/pathology/tissue/urothelial+cancer) show that BC patients with higher expression of *ARHGAP9* mRNA tend to survive longer, though it is not statistically significant (P=0.069). On the basis of the results of this study, we can conclude that *ARHGAP9* regulates growth and proliferation of BC by regulating the MAPK signaling pathway. Future studies should use real-time PCR assays to validate the results of microarray tests to confirm reliability of the data. For a better understanding of ARHGAP9, its protein levels in BC should be evaluated and the experimental samples should be increased to reduce the statistical limitations in the future. Moreover, the function of *miR-3620*, which interacted with *ARHGAP9* mRNA, could be clarified by validating the function of *ARHGAP9* in the future.

In conclusion, our findings provide a novel tumor suppressor gene in BC, which could be served as an independent prognostic marker for stratification of NMIBC and MIBC patients into favorable and poor prognosis. Moreover, a new paradigm in BC tumorigenesis and pathogenesis is estimated, since this novel gene seems to involve in the crucial tumorigenesis signaling pathways.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XMP, PJ, SJY and WJK designed the study and all experiments. XMP performed the experiments. YHK, YJB, YX, SPS and SKM collected patient samples. XMP, CY, HWK and WTK assisted with data collection. XMP, JYL, IYK, YHC, EJC and SJY analyzed the data. WJK provided funding. XMP, SJY and WJK wrote the manuscript.

Ethics approval and consent to participate

The collection and analysis of all samples were approved by the Institutional Review Board at Chungbuk National University (approval no. GR2010-12-010). The study methodologies conformed with the standards set by the Declaration of Helsinki. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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