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Predictive value of gene methylation for second recurrence following surgical treatment of first bladder recurrence of a primary upper-tract urothelial carcinoma

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Abstract. The clinical relevance of aberrant DNA promoter methylation is being increasingly recognized in urothelial carcinoma. The present study was conducted to explore the methylation status of patients with upper-tract urothelial carcinoma (UTUC) who experienced bladder recurrence, and to evaluate the predictive value of gene methylation for second bladder recurrence and tumor progression. A total of 85 patients with primary UTUC, who experienced bladder recurrence after radical nephroureterectomy, were enrolled between January 2001 and December 2013. Using methylation-sensitive polymerase chain reaction, the promoter methylation statuses of 10 genes were analyzed in the bladder tumor specimens. Among the patient group, 32 patients experienced second bladder recurrence, and bladder progression was detected in 16. With the exception of *BRCA1*, the methylation rate of the majority of genes tended to gradually increase to varying extents with the number of recurrences; a smaller proportion of primary tumors exhibited gene methylation when compared with the first recurrent tumors and second recurrent tumors. Univariate and multivariate Cox regression analyses revealed that unmethylated *GDF15* [hazard ratio (HR)=0.36; 95% confidence interval (CI), 0.14-0.92] and methylated *VIM* (HR=2.91; 95% CI, 1.11-7.61) in the first recurrent bladder tumor, as well as male gender (HR=2.28; 95% CI, 1.06-4.87), first recurrence interval <8 months (HR=2.34; 95% CI, 1.15-4.78) and primary UTUC tumor size ≥5 cm (HR=3.48; 95% CI, 1.43-8.45) were independent risk factors for a second bladder recurrence after surgery for the first bladder recurrence; the Harrell's concordance index (c-index) for the related nomogram was 0.71 (95% CI: 0.61-0.81). Furthermore, methylated *CDH1* (HR=2.91; 95% CI, 1.08-7.77) and *VIM* (HR=4.91; 95% CI, 1.11-21.7) in the first recurrent bladder tumor, male gender (HR=3.6; 95% CI, 1.1-11.73), and primary tumor stage T2-T4 (HR=4.57; 95% CI, 1.22-17.13), multifocality (HR=3.64; 95% CI, 1.19-11.16) and size ≥5 cm (HR=3.1; 95% CI, 1.91-10.54) for the primary UTUC were considered to be predictors of tumor progression; the c-index for the nomogram was 0.88 (95% CI, 0.69-0.92). The present findings demonstrated that promoter methylation of cancer-related genes was frequently observed in patients with urothelial carcinoma, and that the gene methylation rate of certain genes tended to gradually increase with the number of bladder recurrences. This may be used as a predictive factor for a second bladder recurrence and tumor progression after the surgical treatment of the first bladder recurrence.

Introduction

Upper-tract urothelial carcinoma (UTUC), including ureteral and renal pelvic carcinoma, is a relatively uncommon disease that accounts for 5-10% of cases of urothelial carcinoma (1,2). Radical nephroureterectomy (RNU) with excision of the bladder cuff is the gold-standard treatment for UTUC; however, due to the frequent multifocal nature of urothelial carcinomas, 22-47% of all primary UTUC patients experience bladder recurrence after RNU (3,4). Moreover, the patients who experience bladder tumor recurrence often require more than one transurethral resection of the bladder tumor (TURBT), which leads to increased suffering of the patient. Multiple bladder recurrences treated with repeated TURBT may significantly reduce a patient's quality of life, and some patients must undergo radical cystectomy for bladder tumor progression during the repeated recurrences. A number of previous studies have reported that the risk factors for bladder recurrence include tumor multifocality, tumor site and patient gender (5-7). However, a lack of effective markers remains a challenge with regard to the prediction of bladder recurrence. It is necessary to

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increase the amount of available data addressing second recurrence after surgery for a first bladder recurrence, in order to establish novel prognostic factors and predictive models.

As a major epigenetic mechanism in humans, gene methylation plays an important role in the development, progression and prognosis of various types of carcinoma (8-10). Our previous study evaluated the methylation statuses of 10 selected genes with regard to their prognostic value for bladder recurrence of a primary UTUC treated with RNU, and found that gene methylation was a common status and could predict bladder recurrence in UTUC patients (11). In the present study, to continue our previous research, we collected data from patients who experienced bladder recurrence from a primary UTUC database, and evaluated the predictive value of gene methylation and clinical factors for subsequent outcomes after the surgical treatment of a first bladder recurrence following primary UTUC treated with RNU.

Patients and methods

Patient selection. This was a retrospective study. All patients with primary UTUC in our database had been diagnosed with UTUC and had undergone RNU at Peking University First Hospital (Beijing, China) between January 2001 and December 2013. None of the patients had received neoadjuvant chemotherapy prior to RNU. Following the exclusion of patients with a previous history of bladder cancer, 318 patients remained in the database. Among these 318 primary UTUC patients, 110 experienced bladder recurrence, of which 25 patients were excluded: 9 in whom the first recurrent bladder tumor was stage T0 or Tis, 6 who were lost to follow-up, and 10 for whom paraffin specimens could not be obtained. A total of 85 patients were included in the final analysis. All patients provided written informed consent.

Diagnosis and treatment. The diagnosis, treatment and pathological examination of primary UTUC samples were performed as described in our previous study (11). Bladder recurrence of UTUC was diagnosed by cystoscopy with biopsy, and TURBT was performed according to the standard procedure. Radical cystectomy was performed for recurrent bladder tumors where indicated due to tumor progression; otherwise, repeated TURBT was performed. All patients received one immediate instillation of Mitomycin C or epirubicin within 24 h after TURBT.

All resected specimens were reviewed by two senior pathologists who were blinded to the personal data of the patients. Tumor stage was evaluated according to the 2002 UICC TNM classification of malignant tumors, and tumor grade was assessed according to the 1973 WHO classification (12). The time between primary UTUC and the first bladder recurrence was defined as the first recurrence interval.

Methylation analysis of gene promoters. The methylation statuses of 10 selected genes (*ABCC6*, *BRCA1*, *CDH1*, *GDF15*, *HSPA2*, *RASSF1A*, *SALL3*, *THBS1*, *TMEFF2*, *VIM*) were evaluated in 117 bladder tumors (85 from a first recurrence, and 32 from a second recurrence). DNA extraction, bisulfite transformation and gene methylation status were evaluated according to the procedures described in our previous study (11). The methylation statuses of the genes between urothelial tumors and normal tissues were not compared as all genes investigated in this study have been validated to have a low methylation rate in normal tissues (13-16).

Postoperative follow-up. The patients were followed-up every 3 months for the first 2 years after surgery, and annually thereafter at our institution. The follow-up consisted of physical examination, urinalysis, cytology, chest X-ray, ultrasound or CT/MRI, and cystoscopy. Second bladder recurrence and tumor progression were used as the endpoints in this study. Tumor progression was defined as the presence of a pathologically confirmed, muscle-invasive tumor (above stage T2) in the bladder during follow-up. Patients who were still alive without a second recurrence or tumor progression were censored at the last follow-up, and the survival time was censored at death during follow-up.

Statistical analysis. χ^2 tests were used to compare categorical variables. Binary logistic regression was used to evaluate methylation status with respect to tumor stage and grade. The second bladder recurrence-free survival (BRFS) rate and progression-free survival (PFS) rate after the surgical treatment of the first bladder recurrence were evaluated by the Kaplan-Meier method. Variables influencing BRFS and PFS were compared using Cox proportional hazards regression models. Variables with $P<0.05$ on univariate analysis were also assessed by multivariate analysis. Multivariate Cox regression coefficients were then used to generate nomograms to predict the 6-, 12-, 24- and 36-month BRFS and PFS rates, and Harrell's concordance index (c-index) was used to quantify the discrimination ability of these nomograms, and calibration plots were generated to explore the performance of the nomograms. All statistical analyses were performed using IBM SPSS version 20.0 and R version 3.2.0. Two-sided P-values <0.05 were considered to indicate statistical significance.

Results

Overall results of clinical follow-up. The characteristics of the 85 patients with primary UTUC and first bladder recurrence are presented in Table I. Of the 85 patients, 42 (49.4%) were female and 43 (50.6%) were male, and the median age was 67 years (range, 46-82 years). The median follow-up time was 51 months (range, 5-161 months). During follow-up, there were 31 mortalities (36.5%), of which 29 were due to cancer, and a total of 32 patients (37.6%) developed second bladder recurrence. The median interval between RNU and the first bladder recurrence was 15 months (range, 2-98 months) and the median interval between the first and second bladder recurrences was 31 months (range, 2-126 months). A total of 16 patients experienced tumor progression during follow-up. The median interval between the first bladder recurrence and tumor progression was 41 months (range, 4-126 months) Of the 16 patients with tumor progression, 8 received radical cystectomy and 8 received TURBT.

Gene methylation status and oncological outcomes. The methylation rates of the 10 selected genes in primary UTUC, and first and second recurrent bladder tumors are summarized in Table II and Fig. 1. *GDF15* (primary, 50.6%;

Table I. Characteristics of primary UTUC and first bladder recurrence tumor of all the 85 patients.

Clinicopathologic characteristics	Median (range) or no. (%)
Age	67 (46-82)
Gender	
Female	42 (49.4)
Male	43 (50.6)
No. of subsequent recurrence	
1	85 (100)
2	15 (17.6)
3	10 (11.8)
≥4	7 (8.2)
Bladder tumor progression	
Absent	69 (81.2)
Present	16 (18.8)
Death	
Cancer-specific death	29 (34.1)
Other death	2 (2.4)
Characteristics of primary UTUC	
Tumor stage	
Ta, T1	31 (36.5)
T2	37 (43.5)
T3	17 (20.0)
Tumor grade	
G1	4 (4.7)
G2	55 (64.7)
G3	26 (30.6)
Tumor size	
Small (<5 cm)	76 (89.4)
Large (≥5 cm)	9 (10.6)
Tumor architecture	
Papillary tumor	72 (84.7)
Sessile tumor	13 (15.3)
Tumor location	
Renal pelvis	50 (58.8)
Ureter	35 (41.2)
Tumor multifocality	
Absent	60 (70.6)
Present	25 (29.4)
Characteristics of first bladder recurrence tumor	
Tumor stage	
Ta	45 (53.6)
T1	35 (41.7)
T2	5 (4.7)
Tumor grade	
G1	9 (10.7)
G2	59 (70.2)
G3	16 (19.0)
First recurrence interval	
Long (≥8 months)	59 (69.4)
Short (<8 months)	26 (30.6)
Renal function	
eGFR ≥30 ml/min	76 (89.4)
eGFR <30 ml/min	9 (10.6)

UTUC, upper-tract urothelial carcinoma; eGFR, estimated glomerular filtration rate.

first recurrence, 65.9%; second recurrence, 75%) and *VIM* (primary, 58.8%; first recurrence, 60%; second recurrence, 75%) had the highest methylation rates. With the exception of *BRCA1*, all genes showed a higher methylation rate in the second recurrent tumors compared with the first recurrent tumors, and in the first recurrent tumors compared with the primary UTUC. The associations between gene promoter methylation statuses and pathological tumor characteristics are shown in Table III. Univariate analysis showed that methylated statuses in the *ABCC6*, *BRCA1*, *CDH1*, *GDF15*, *HSPA2* and *RASSF1A* promoters were significantly associated with pT1/T2 stage in the first bladder recurrence. However, on binary logistic regression analysis, after adjusting for clinical and pathological factors, promoter methylation status in any of the 10 genes in the first recurrent bladder tumor was not associated with T1/T2 stage or grade 3 malignancy.

The univariate and multivariate analyses of prognostic significance are shown in Table IV. The 12-, 24-, 36- and 60-month BRFS rates were 75.8, 66.6, 63.6 and 58.2%, respectively. On multivariate analysis, unmethylated *GDF15* [hazard ratio (HR)=0.36; 95% confidence interval (CI), 0.14-0.92] and methylated *VIM* (HR=2.91; 95% CI, 1.11-7.61) in the first recurrent bladder tumor, as well as male gender (HR=2.28; 95% CI, 1.06-4.87), first recurrence interval <8 months (HR=2.34; 95% CI, 1.15-4.78) and primary tumor size ≥5 cm (HR=3.48; 95% CI, 1.43-8.45) were independently associated with second bladder recurrence. The 12-, 24-, 36- and 60-month PFS rates were 93.9, 84.6, 81.5 and 79.7%, respectively. Methylated *CDH1* (HR=2.91; 95% CI, 1.08-7.77) and *VIM* (HR=4.91; 95% CI, 1.11-21.7) in the first recurrent bladder tumor, male gender (HR=3.6; 95% CI, 1.1-11.73), and primary tumor stage T2-T4 (HR=4.57; 95% CI, 1.22-17.13), multifocality (HR=3.64; 95% CI, 1.19-11.16) and size ≥5 cm (HR=3.1; 95% CI, 1.91-10.54) were significantly associated with tumor progression on multivariate analysis.

Predictive model for BRFS and PFS. The nomogram for predicting the probability of BRFS following surgery for first bladder recurrence is illustrated in Fig. 2A, and the c-index of this multivariate model was 0.71 (95% CI: 0.61-0.81). The calibration plots at 1-year and 3-year follow-up for the nomogram are shown in Fig. 2B and C, respectively. The nomogram for predicting the probability of PFS after surgery for first

Table II. The gene methylation rate of primary UTUC, the first bladder recurrence tumor and second bladder recurrence tumor.

Gene promoter	Primary UTUC (%)	First bladder recurrence (%)	Second bladder recurrence (%)
Total no.	85	85	32
ABCC6			
Unmethylated	75 (88.2)	61 (71.8)	22 (68.8)
Methylated	10 (11.8)	24 (28.2)	10 (31.3)
BRCA1			
Unmethylated	70 (82.4)	55 (64.7)	26 (81.3)
Methylated	15 (17.6)	30 (35.3)	6 (18.8)
CDH1			
Unmethylated	74 (87.1)	64 (75.3)	24 (75.0)
Methylated	11 (12.9)	21 (24.7)	8 (25.0)
GDF15			
Unmethylated	42 (49.4)	29 (34.1)	8 (25.0)
Methylated	43 (50.6)	56 (65.9)	24 (75.0)
HSPA2			
Unmethylated	62 (72.9)	38 (44.7)	12 (37.5)
Methylated	23 (27.1)	47 (55.3)	20 (62.5)
RASSF1A			
Unmethylated	70 (82.4)	69 (81.2)	26 (81.2)
Methylated	15 (17.6)	16 (18.8)	6 (18.8)
SALL3			
Unmethylated	62 (72.9)	48 (56.5)	16 (50.0)
Methylated	23 (27.1)	37 (43.5)	16 (50.0)
THBS1			
Unmethylated	63 (74.1)	60 (70.6)	16 (50.0)
Methylated	22 (25.9)	25 (29.4)	16 (50.0)
TMEFF2			
Unmethylated	57 (67.1)	49 (57.6)	18 (56.3)
Methylated	28 (32.9)	36 (42.4)	14 (43.8)
VIM			
Unmethylated	35 (41.2)	34 (40.0)	8 (25.0)
Methylated	50 (58.8)	51 (60.0)	24 (75.0)

UTUC, upper-tract urothelial carcinoma.

bladder recurrence is illustrated in Fig. 3A, and c-index of this multivariate model was 0.88 (95% CI: 0.69-0.92). The calibration plots at 1-year and 3-year follow-up for the nomogram are shown in Fig. 3B and C, respectively.

Discussion

UTUC is a relatively rare cancer. Approximately 60% of UTUCs are invasive at diagnosis, compared with 15-25% of

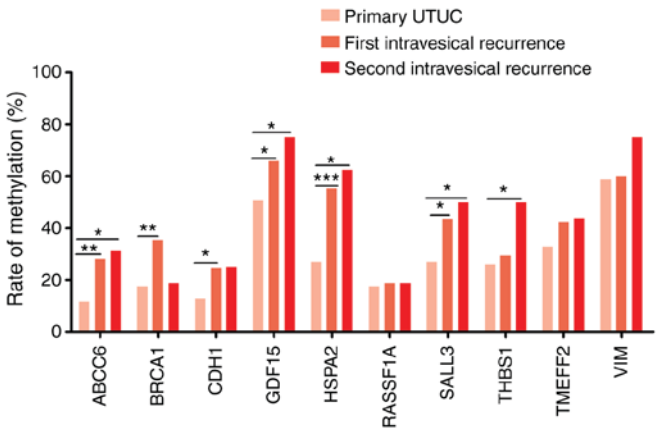

Figure 1. Gene methylation rates in primary UTUC, first recurrent bladder tumor and second recurrent bladder tumor. *P<0.05, **P<0.01, ***P<0.001. UTUC, upper-tract urothelial carcinoma.

all bladder tumors, and thus this disease has a comparatively poor prognosis (6). The European Association of Urology Guidelines reviewed several published studies and reported that four different nomograms are available for predicting survival rates postoperatively, based on standard pathological features (17-20). Recently, several papers reported a number of clinicopathological features and gene promoter methylation statuses of UTUC patients that might affect the probability of bladder recurrence, including patient gender, smoking status, tumor multifocality, surgical management, and *GDF15* and *RASSF1A* promoter methylation (11,21-23). However, although a few papers have addressed the clinical course after the first bladder tumor relapse, none of the studies has provided any significant recommendation concerning bladder surveillance. In the present study, we collected data from UTUC patients who experienced bladder recurrence following a primary UTUC treated with RNU, and evaluated the prognostic value of gene promoter methylation and clinical factors for the subsequent outcomes, including repeated recurrence and progression after TURBT for the first bladder recurrence.

DNA methylation is an important biochemical process that is involved in the normal development of higher organisms. A family of DNA methyltransferases transfer a methyl group from *S*-adenosyl methionine to the fifth carbon of a cytosine residue to form 5-methylcytosine, thereby catalyzing DNA methylation. This modification can be inherited through cell division (24). Beside its involvement in normal development in human beings, DNA methylation is frequently implicated in the onset or course of cancer due to its roles in many other regulatory processes. Several studies have reported that aberrant promoter methylation at several gene loci was associated with bladder urothelial carcinoma (13,25-27). As bladder urothelial carcinoma and UTUC display genomic and clinical similarities, we selected 10 genes (*ABCC6, BRCA1, CDH1, GDF15, HSPA2, RASSF1A, SALL3, THBS1, TMEFF2* and *VIM*) with a high frequency of methylation in bladder urothelial carcinoma and evaluated their methylation statuses in UTUC and their associations with clinical outcomes.

According to our results, gene methylation is common in recurrent bladder tumors. Compared with our previous study that focused on primary UTUC, we found that gene methylation rate exhibits a significant increasing trend with

Table III. Predictive effect of epigenetic biomarkers in first bladder recurrence of UTUC for high tumor stage (T1 and T2) and grade 3 using univariable and multivariable logistic regression.

Promoter methylation status	T1 and T2						Grade 3					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
ABCC6	4.626	1.657-12.912	0.003	1.909	0.503-7.241	0.342	1.70	0.54-5.347	0.364	1.853	0.398-8.631	0.432
BRCA1	3.273	1.294-8.274	0.012	2.051	0.674-6.236	0.206	1.125	0.365-3.472	0.838	0.839	0.210-3.355	0.804
CDH1	4.457	1.520-13.066	0.006	3.663	0.925-14.49	0.064	1.020	0.290-3.584	0.976	1.378	0.269-7.046	0.700
GDF15	3.029	1.149-7.982	0.025	1.106	0.305-4.009	0.878	0.833	0.269-2.577	0.752	0.257	0.049-1.361	0.110
HSPA2	3.314	1.335-8.222	0.01	2.006	0.534-7.531	0.303	1.441	0.472-4.406	0.521	2.510	0.504-12.506	0.261
RASSF1A	3.422	1.07-10.943	0.038	2.913	0.636-13.33	0.168	1.583	0.435-5.761	0.486	1.696	0.327-8.795	0.529
SALL3	2.393	0.994-5.765	0.052	1.016	0.286-3.608	0.98	1.011	0.338-3.027	0.984	0.728	0.157-3.374	0.685
THBS1	2.413	0.929-6.268	0.07	1.005	0.295-3.422	0.994	0.762	0.22-2.639	0.668	0.394	0.073-2.119	0.278
TMEFF2	1.765	0.739-4.216	0.201	0.424	0.103-1.741	0.234	1.073	0.358-3.214	0.900	0.628	0.123-3.202	0.576
VIM	2.352	0.952-5.813	0.064	1.375	0.384-4.928	0.625	2.308	0.676-7.876	0.182	4.668	0.921-23.65	0.063

UTUC, upper-tract urothelial carcinoma; HR, hazard ratio; CI, confidence interval.

the development and recurrence of UTUC (primary UTUC to first bladder recurrence to second recurrence); this partly confirmed the 'intraluminal seeding and implantation' hypothesis, which is the classic mechanism for bladder recurrence of primary UTUC (28,29). Our results indicated that promoter methylation in 10 genes in the first recurrent bladder tumor was not associated with pT1/T2 and grade 3 malignancy. However, a former study (11) showed that certain epigenetic biomarkers of primary UTUC were significantly associated with tumor malignancy (pT3/T4, tumor grade 3 and positive lymph node metastasis). Therefore, we suggested that the tumor stage and grade of the first bladder recurrence could not be predicted by the gene methylation status of the recurrent bladder tumor, and it was difficult to explain this interesting phenomenon with existing theories.

After adjusting for clinical and pathological factors, multivariate analysis showed that male gender, a short interval between initial RNU and first bladder recurrence, unmethylated *GDF15* and methylated *VIM* in the first recurrent bladder tumor, and a primary UTUC tumor size >5 cm were independently associated with repeated bladder relapse after surgery for the first bladder recurrence. *GDF15* encodes a divergent member of the transforming growth factor (TGF)-β superfamily, whose members are required for normal development, differentiation, and tissue homeostasis. The anti-tumorigenic activity of *GDF15* has been suggested to be due to the association between *GDF15* overexpression and tumor growth arrest and increased apoptosis (25). Thus, we hypothesized that this anti-tumorigenic activity reduced bladder recurrence. Male gender, methylated *CDH1* and *VIM* in the first recurrent bladder tumor, and high tumor stage, large tumor size and multifocality of primary UTUC were independently associated with bladder tumor progression. *VIM*, *CDH1* and *GDF15* have been confirmed to be upregulated by promoter demethylation. *VIM* methylation has been found to be more frequent in bladder urothelial carcinoma and UTUC, but rare in normal tissue, and may therefore be useful as a novel diagnosis and detection method in urothelial cancer. Downregulation of *VIM* has also been associated with increased tumor invasion, progression, epithelial to mesenchymal transition (EMT) and poor prognosis in various types of tumor (30-34). Monteiro-Reis *et al* (31) suggested that during early upper urinary tract carcinogenesis, the *VIM* promoter is progressively methylated and the gene is kept silenced, as in normal urothelium; by contrast, in a subset of UTUCs, methylation is decreased, allowing for aberrant vimentin expression, due to stimuli leading to EMT. As a consequence, these tumors may be more prone to local invasion and systemic dissemination, thus fostering disease progression and increasing recurrence. In previous studies, *CDH1* methylation was found to be more frequent in colorectal cancer than in adjacent non-neoplastic margins, and loss of *CDH1* expression in colorectal cancer was associated with an infiltrative tumor growth pattern and lymph node metastasis (35,36). A meta-analysis also identified *CDH1* as a tumor suppressor gene that contributes to the progression of breast cancer, and suggested that *CDH1* hypermethylation could be used as a novel drug target for developing personalized therapy (37). Consistently, the

Table IV. Univariable and multivariable Cox regression analyses predicting bladder recurrence-free survival for UTUC patients and tumor progression of subsequent bladder recurrence.

Gene promoter methylation status and clinicopathological parameter	Second bladder recurrence						Tumor progression					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Parameters of first bladder recurrence tumor												
ABCC6 (M vs. U)	1.20	0.41-3.52	0.739				2.26	0.84-6.09	0.109			
BRCA1 (M vs. U)	0.57	0.22-1.46	0.241				1.06	0.39-2.92	0.909			
CDH1 (M vs. U)	2.85	1.11-7.36	0.030				3.12	1.17-8.33	0.023	2.91	1.08-7.77	0.033
GDF15 (M vs. U)	0.35	0.13-0.96	0.042	0.36	0.14-0.92	0.033	1.06	0.37-3.09	0.906			
HSPA2 (M vs. U)	0.86	0.33-2.26	0.766				0.77	0.29-2.08	0.608			
RASSF1A (M vs. U)	1.70	0.56-5.17	0.354				1.50	0.48-4.65	0.486			
SALL3 (M vs. U)	0.80	0.32-2.02	0.638				1.04	0.39-2.80	0.935			
THBS1 (M vs. U)	0.57	0.19-1.66	0.304				1.71	0.62-4.72	0.301			
TMEFF2 (M vs. U)	0.61	0.22-1.69	0.346				1.09	0.41-2.93	0.865			
VIM (M vs. U)	3.61	1.32-9.87	0.012	2.91	1.11-7.61	0.029	5.22	1.18-23.03	0.029	4.91	1.11-21.70	0.036
Gender (male vs. female)	2.40	1.15-5.01	0.019	2.28	1.06-4.87	0.034	2.77	1.94-8.17	0.045	3.60	1.10-11.73	0.034
Age	0.98	0.95-1.01	0.224				0.99	0.94-1.04	0.598			
First recurrence interval (<8 vs. ≥8 months)	1.90	1.04-3.82	0.044	2.34	1.15-4.78	0.019	0.90	0.31-2.60	0.848			
Tumor stage (T2-T4 vs. T0-T1)	1.48	0.73-2.99	0.276				2.20	0.79-6.18	0.133			
Tumor grade (G3 vs. G1-G2)	1.57	0.71-3.51	0.268				1.48	0.48-4.60	0.497			
Renal function (eGFR<30 vs. eGFR≥30 ml/min)	0.52	0.13-2.19	0.377				0.04	0.00-25.92	0.329			
Parameters of primary UTUC												
Tumor stage (T2-T4 vs. T0-T1)	1.57	0.73-3.40	0.250				3.33	1.94-11.72	0.041	4.57	1.22-17.13	0.024
Tumor grade (G3 vs. G1-G2)	1.34	0.65-2.74	0.426				2.53	1.94-6.78	0.045			
Tumor size (≥5 cm vs. <5 cm)	3.94	1.67-9.32	0.002	3.48	1.43-8.45	0.006	4.22	1.31-13.57	0.016	3.10	1.91-10.54	0.041
Tumor architecture (sessile vs. papillary)	1.31	0.57-3.03	0.530				1.85	0.59-5.74	0.287			
Tumor location (ureter vs. renal pelvis)	0.93	0.46-1.89	0.843				1.19	0.44-3.20	0.730			
Tumor multifocality (yes vs. no)	1.05	0.49-2.28	0.898				2.27	1.82-6.25	0.044	3.64	1.19-11.16	0.023

UTUC, upper-tract urothelial carcinoma; HR, hazard ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate.

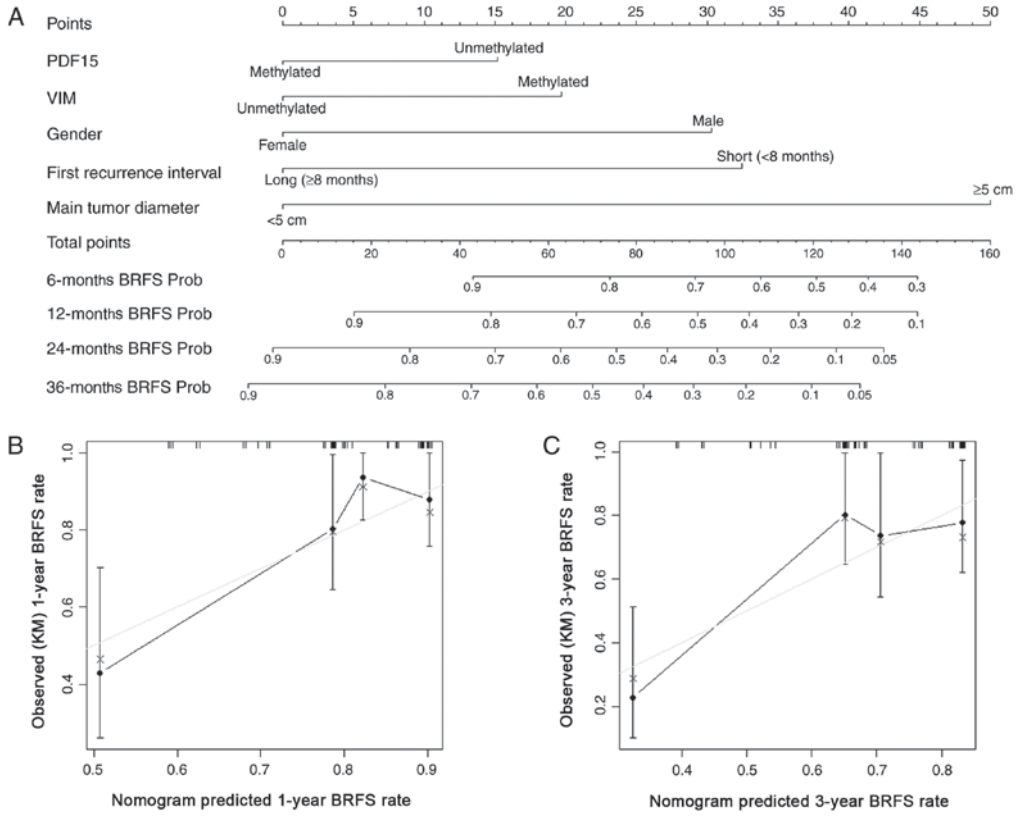

Figure 2. Nomogram for predicting the probability of BRFS after surgery for a first bladder recurrence is illustrated in (A); the c-index of this multivariate model was 0.71 (95% CI, 0.61-0.81). The calibration plots at 1-year and 3-year follow-up for the nomogram are shown in (B) and (C), respectively. BRFS, bladder recurrence-free survival; CI, confidence interval.

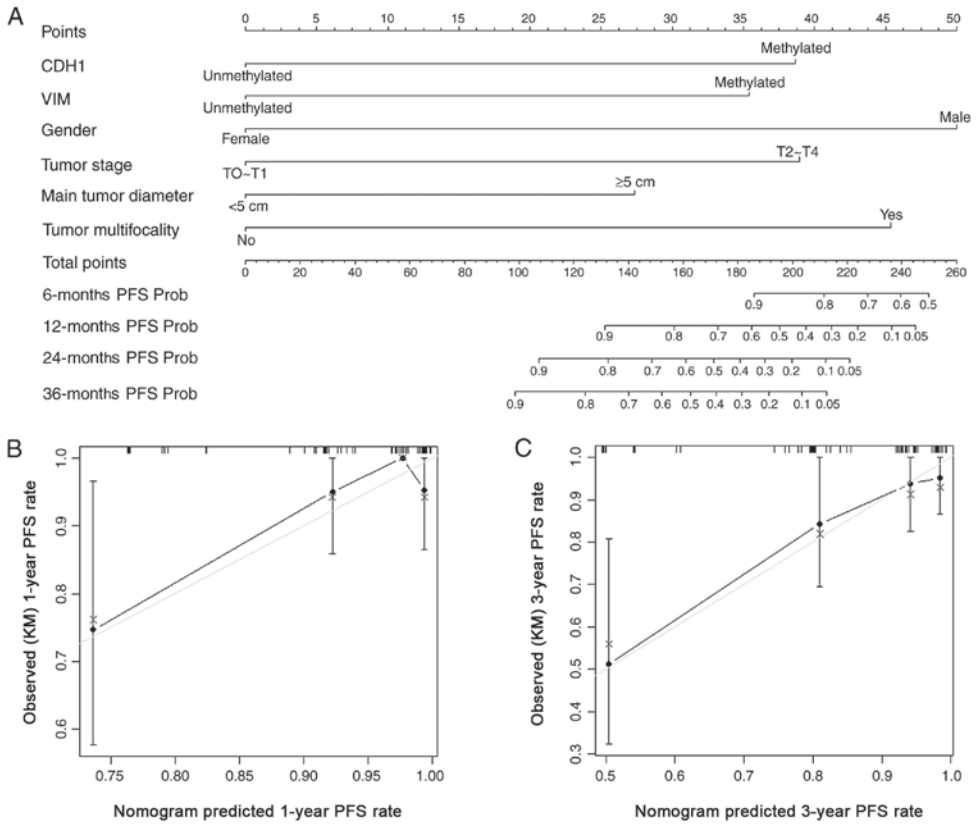

Figure 3. Nomogram for predicting the probability of PFS after surgery for a first bladder recurrence is illustrated in (A); the c- index of this multivariate model was 0.88 (95% CI, 0.69-0.92). The calibration plots at 1-year and 3-year follow-up for the nomogram are shown in (B) and (C), respectively. PFS, progression-free survival; CI, confidence interval.

methylation of *VIM* or *CDH1* predicted poor outcomes in the bladder after surgery for a first bladder recurrence.

Previous studies (11) have indicated that the *GDF15* gene has a diverse range of cancer-specific presentations. For bladder urothelial carcinoma, *GDF15* acts as a tumor suppressor gene, and methylation of *GDF15* is associated with tumor invasion and progression. In the present study, we speculated that patients with *GDF15* promoter methylation in the first recurrent bladder tumor may die due to the aggressive nature of the tumor before a second recurrence, and therefore unmethylated *GDF15* in the first recurrent bladder tumor was a risk factor for subsequent recurrence. Compared with the predictive factors for the first recurrent bladder tumor, the predictive factors for the primary UTUC may be more easily applied in clinical decision-making. Therefore, we also analyzed the gene methylation statuses in primary UTUC; however, none showed any association with subsequent bladder outcomes after first bladder recurrence (data not shown). Nevertheless, certain clinicopathological parameters (T stage, tumor size and tumor multifocality) relating to the primary UTUC had good predictive value for subsequent bladder outcomes. Prior to this study, two other studies had analyzed the bladder outcomes subsequent to surgery for the first bladder recurrence following primary UTUC. Abe *et al* (38) reported that 40% of primary UTUC patients experienced bladder recurrence, of whom 80% developed repeated bladder recurrence (in contrast to 37.6% in the present study), and 20% eventually showed tumor progression (compared with 18.8% in the present study). Tanaka *et al* (39) identified the independent prognostic factors for subsequent bladder outcomes, and the c-indexes of their multivariate models to predict second bladder recurrence and progression were 0.61 and 0.87, respectively. By comparison, regarding the prognostic values of gene methylation, the c-indexes of our predictive models based on gene methylation status and clinical parameters for the prediction of subsequent bladder recurrence and progression were 0.71 and 0.88, respectively.

There were several limitations of the present study, partly due to the intrinsic biases of retrospective analyses, and partly due to the small scale of our study cohort. However, while the study cohort of 85 patients was less than that of the study by Tanaka *et al* (39) (n=241), our data were more comprehensive; in addition to routine clinical parameters, we also evaluated the methylation statuses of 10 genes in primary UTUC and in first recurrent bladder tumors. As a result, we were able to construct more accurate prognostic models to predict second bladder recurrence and tumor progression. Our predictive models will need to be validated by further research. In summary, to the best of our knowledge, this was the first study to evaluate the predictive value of clinical parameters and gene methylation status for the subsequent bladder outcomes after surgery for a first bladder recurrence following a primary UTUC treated by RNU. We speculate that our results may aid to achieve more reasonable and accurate clinical decision-making, and improve the comprehension of bladder recurrence after primary UTUC treated by RNU.

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