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Basic and Translational Research

Decreased *DBC1* Expression Is Associated With Poor Prognosis in Patients With Non-Muscle-Invasive Bladder Cancer

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Purpose: The deleted in bladder cancer 1 (*DBC1*) gene is located within chromosome 9 (9q32-33), a chromosomal region that frequently shows loss of heterozygosity in bladder cancer (BC). It is suspected that it acts as a tumor suppressor gene, but its prognostic value remains unclear. The aim of the present study was to investigate the value of *DBC1* as a prognostic marker in BC.

Materials and Methods: The expression of *DBC1* was determined by real-time polymerase chain reaction analysis in 344 patients with BC (220 non-muscle-invasive BC [NMIBC] and 124 muscle-invasive BC [MIBC]) and in 34 patients with normal bladder mucosa. The results were compared with clinicopathologic parameters, and the prognostic value of *DBC1* was evaluated by Kaplan-Meier analysis and a multivariate Cox regression model.

Results: *DBC1* expression was significantly decreased in patients with MIBC compared with those diagnosed with NMIBC (p=0.010). Patients with aggressive tumor characteristics had lower *DBC1* expression levels in NMIBC (each, $p < 0.05$). By multivariate Cox regression analysis, low *DBC1* expression was a predictor of progression to MIBC (hazard ratio, 7.104; p=0.013). Kaplan-Meier estimates revealed a significant difference in tumor recurrence, progression to MIBC, and cancer-specific survival depending on the level of *DBC1* expression in NMIBC (log-rank test, each, $p < 0.05$).

Conclusions: The expression of *DBC1* was associated with tumor aggressiveness, progression to MIBC, and survival in NMIBC. Our results suggest that *DBC1* expression can be a useful prognostic marker for patients with NMIBC.

Keywords: *Bladder cancer; Human DBC1 protein; Prognosis*

INTRODUCTION

Bladder cancer is the second most common genitourinary tumor in human populations, and it was estimated in 2013 that 72,570 new cases of cancer of the urinary bladder were diagnosed in the United States and 15,210 deaths were attributable to bladder cancer [1]. At the initial diagnosis, 75% of patients present with non-muscle-invasive bladder cancer (NMIBC), which can be managed with a combination of transurethral resection (TUR) and intravesical therapy [2,3]. However, after TUR about three-quarters of patients experience tumor recurrence within 2 years, and 20% to 30% of them experience progression to muscle-invasive bladder cancer (MIBC) [4,5]. Only 20% of bladder

cancer patients are diagnosed with MIBC, which causes most cancer-specific deaths. Although radical cystectomy is considered the gold standard for the treatment of patients with localized MIBC, about 50% of these patients develop metastases within 2 years after cystectomy and subsequently die of the disease [6].

Bladder cancer has diverse biological and functional characteristics. Therefore, it is very difficult for urologists to estimate the success rate of treatment and to counsel patients about prognosis [7]. Prognostic information obtained from conventional histopathological parameters such as tumor stage or grade and lymph node status is insufficient to predict outcome. One limitation of the current use of staging is the fact that tumors of a similar stage and grade can have a significantly different biology [8]. Nomograms and prognostic modeling approaches that simultaneously incorporate many factors have been developed for assessing the risk of individual patients [3,9,10]. The limited value of the established prognostic markers is that these require analysis of new molecular parameters of interest for predicting the prognosis of bladder cancer patients in a clinical setting [11]. The specific alterations in gene expression that occur as a result of the cross-talk between various cellular pathways determine the biological behavior of a tumor, including its growth, recurrence, progression, and metastasis, all of which can influence patient survival [7,11-14]. To detect and monitor cancer and determine a likely prognosis, it is necessary to identify molecular markers of the disease that can be used in a clinic.

The deleted in bladder cancer 1 (*DBC1*) gene, originally named *DBCCR1*, which represents a candidate tumor suppressor gene within a precisely mapped region of loss of heterozygosity on 9q33, shows the loss of one allele in 60% of all bladder tumors or transcriptional silencing in approximately 50% of bladder cancer cell lines by promoter hypermethylation of the 5' region of *DBC1* containing a CpG island [15-18]. Abnormal methylation or deletion of *DBC1* has also been described in other tumors including oral squamous cell carcinoma, non-small-cell lung cancer, and acute lymphoblastic leukemia [19,20]. *DBC1* contains a membrane attack complex/perforin domain, which is a membrane-disrupting protein that is involved in pore formation during complement-mediated cell lysis [16]. *DBC1* is also known to play a role in cell cycle control. Exogenous expression of *DBC1* protein in human bladder tumor cell lines results in suppression of proliferation, and this *DBC1*-mediated growth inhibition is due to an increase in the number of cells in the G1 phase of the cell cycle [16,21]. Thus far, there has been no evidence for an equivalent function of *DBC1*, and clinical evidence is insufficient as to whether the inactivation of the *DBC1* tumor suppressor gene influences genetic susceptibility and the disease course of bladder cancer.

In the current study, we compared the expression levels of *DBC1* between normal and cancer tissue to assess the contribution of this gene in bladder carcinogenesis. More importantly, we assessed the value of *DBC1* as a prognostic indicator for bladder cancer.

MATERIALS AND METHODS

1. Study population

We collected bladder tissue from 344 patients with primary bladder cancer (220 NMIBC and 124 MIBC) and 34 patients with nonmalignant, noninflammatory disease. Cases were recruited from patients with bladder cancer who had been histologically verified with urothelial carcinoma at our institution. To reduce confounding factors affecting the analysis and to delineate a more homogeneous study population, any patients diagnosed with a concomitant carcinoma *in situ*, or for whom data collection was incomplete, as well as patients with short-term follow-up periods (less than 6 months) were excluded. The Ethics Committee of Chungbuk National University approved this protocol, and written informed consent was obtained from each subject. The collection and analyses of all samples was approved by the Institutional Review Board of Chungbuk National University (IRB approval number 2006-01-001), and informed consent was obtained from each subject.

Tumors were staged according to the 2002 TNM classification and the 1973 World Health Organization grading system [22]. All diagnoses were confirmed by pathological analysis of frozen sections from cystectomy and TUR specimens. In the case of NMIBC, a TUR of the tumor was performed. A second TUR was performed 2 to 4 weeks after the initial resection when a bladder cancer specimen did not include proper muscle or when a high-grade tumor was detected. Patients who had multiple tumors, large tumors (≥ 3 cm in diameter), or high-grade NMIBC received one cycle of intravesical treatment (bacille Calmette-Guerin [BCG] or mitomycin-C). Response to treatment was assessed by cystoscopy and urinary cytology. Patients who were free of disease within 3 months of the commencement of treatment were assessed every 3 months for the first 2 years and every 6 months thereafter [5]. We defined recurrence as a relapse of primary NMIBC with a lower or equivalent pathologic stage and progression to invasive bladder cancer.

2. RNA extraction and construction of cDNA

One milliliter of TRIzol (Invitrogen, Carlsbad, CA, USA) was added to control and bladder cancer tissue and the samples were homogenized in a 5-mL glass tube. The homogenate was transferred to a 1.5-mL tube and was mixed with 200 µL chloroform. After incubation for 5 minutes at 4°C, the homogenate was centrifuged for 13 minutes at 13,000× g at 4°C. The upper aqueous phase was transferred to a clean tube and 500 µL isopropanol was added, followed by incubation for 60 minutes at 4°C. The tube was then centrifuged for 8 minutes at 13,000×g and 4°C. Then, the upper aqueous phase was removed, mixed with 500 µL of 75% ethanol, and centrifuged for 5 minutes at 13,000×g and 4°C. After the upper aqueous layer was discarded, the pellet was

dried at room temperature, dissolved with diethylpyrocarbonate-treated water, and stored at –80°C. The quality and integrity of RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under ultraviolet light. The cDNA was then prepared from 1 μg of random priming by using a First-Strand cDNA Synthesis Kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) according to the manufacturer's protocol.

3. Real-time polymerase chain reaction
To quantify the expression levels of *DBC1*, real-time polymerase chain reaction (PCR) amplification was performed by using a Rotor Gene 6000 instrument (Corbett Research, Mortlake, Australia). Real-time PCR assays using SYBR Premix EX Taq (Takara Bio Inc., Otsu, Japan) were carried out in microreaction tubes (Corbett Research). For amplification we used *DBC1* (201 bp) sense (5'-CCC TCG CCC GCC TAC TAT-3') and antisense (5'-GCT GGG CGG GGT TGT AGA-3') primers. The PCR reaction was performed in a final volume of 10 μL, consisting of 5 μL of 2× SYBR premix EX Taq buffer, 0.5 μL of each 5'- and 3'- primer (10 pmol/μL), and 1 μL of the sample cDNA. The product was purified with a QIAquick Extraction kit (QIAGEN, Hilden, Germany), quantified with a spectrometer (MBA2000, Perkin Elmer, Fremont, CA, USA), and sequenced with an automated laser fluorescence sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). The known concentration of the product was 10-fold serially diluted from 100 pg/μL to 0.1 pg/μL. The dilution series of PCR products was used to establish a standard curve of real-time PCR. The real-time PCR conditions were 1 cycle at 96°C for 20 seconds, followed by 40 cycles of 3 seconds at 96°C for denaturation, 15 seconds at 60°C for annealing, and 15 seconds at 72°C for extension. The melting program was performed at 72°C–95°C with a heating rate of 1°C per 45 seconds. Spectral data were captured and analyzed by using Rotor-Gene Real-Time Analysis Software 6.0 Build 14 (Corbett Research). All samples were run in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was applied as an endogenous RNA reference gene. Gene expression was normalized to the expression of *GAPDH*.

4. Statistical analysis
Because the expression levels of *DBC1* were highly skewed to the left, even after log transformation, we performed either a Mann-Whitney U test or a Kruskal-Wallis test. Patients were classified as having a high expression of *DBC1* or a low expression of *DBC1*, with the median of the mRNA expression as the cutoff value. The Kaplan-Meier method was used to estimate the time to recurrence and progression, and differences were assessed by using log-rank statistics. The prognostic value of *DBC1* expression was analyzed by using a multivariate Cox proportional hazard regression model. Statistical analysis was performed by using IBM SPSS ver. 20.0 (IBM Co., Armonk, NY, USA), and a p-value < 0.05 was considered statistically significant.

RESULTS

1. Baseline characteristics
Table 1 lists the baseline characteristics of the 34 control and 344 bladder cancer (220 NMIBC and 124 MIBC) patients included in the study. The mean age of the bladder cancer patients was 66.9 years (range, 24 to 87 years) and that of the controls was 53.9 years (range, 19 to 80 years).

Of the 220 NMIBC patients, 73 (33.2%) experienced re-

TABLE 1. Baseline characteristics

Characteristic	Controls (n=34)	NMIBC (n=220)	MIBC (n=124)
Age (yr), mean±SD (range)	53.85±14.5 (19–80)	66.1±13.8 (24–87)	67.34±9.4 (38–87)
Follow-up period (mo), median (range)	-	62.0 (6.0–220.0)	35.0 (6.0–181)
DBC1 mRNA expression level ($\times 10^4$ copies per μg), median (IQR)[a]	17.49 (9.84–36.12)	36.55 (6.38–109.02)	17.32 (3.87–63.23)
Gender, n (%)			
Male	18 (52.9)	182 (82.7)	103 (83.1)
Female	16 (47.1)	38 (17.2)	21 (16.9)
Grade, n (%)			
G1		72 (32.7)	37 (29.8)
G2	-	118 (53.6)	85 (68.5)
G3	-	30 (13.6)	
TNM stage, n (%)			
TaN0M0	-	84 (38.2)	
T1N0M0	-	136 (61.8)	
T2N0M0	-		60 (48.4)
T3N0M0	-		34 (27.4)
T4 or higher than N0M0	-		30 (24.2)

NMIBC, nonmuscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; SD, standard deviation; IQR, interquartile range.
[a]:p < 0.05 between NMIBC and MIBC, p-values was based on the Mann-Whitney U test.

currence and 22 (10.0%) experienced progression. Four patients with Ta progressed into T1, and the other 18 patients progressed into muscle-invasive disease. Intravesical therapy was performed in 111 patients (50.5%) after TUR; 74 patients were treated with BCG and 37 with mitomycin C. The mean intervals for recurrence and progression were 20.7 months (range, 6.4 to 133.6 months) and 43.0 months (range, 6.6 to 115.4 months), respectively. During follow-up, 14 of 220 patients (6.4%) with NMIBC died of bladder tumors, and the mean interval for cancer-specific survival was 49.5 months (median, 40.5 months; range, 7.4 to 115.4 months).

Of the 124 MIBC patients, 70 cases (56.5%) underwent radical cystectomy and 57 patients (46.0%) received adjuvant chemotherapy. During follow-up, 60 of 124 patients (48.4%) with MIBC experienced progression and 50 (40.7%) died of bladder cancer.

2. mRNA expression levels of *DBC1* in normal and cancer tissue

To identify whether *DBC1* is involved in bladder carcino-

genesis, the mRNA expression level of *DBC1* was analyzed. *DBC1* levels did not differ between normal mucosa and bladder cancer tissue, between normal mucosa and NMIBC, or between normal mucosa and MIBC. However, *DBC1* levels were significantly lower in patients with MIBC than in those with NMIBC (Table 1) (p=0.010).

3. Relationship between *DBC1* mRNA expression levels and clinicopathologic features

The expression of *DBC1* mRNA was significantly lower in cancer specimens from patients with a higher grade, T1 stage, large size, and multiple tumors than in those with a lower grade, Ta stage, small size, and a single tumor in NMIBC (p < 0.001, p=0.002, p=0.013, and p=0.009, respectively) (Table 2). However, *DBC1* expression showed no significant differences according to clinicopathological parameters such as tumor stage, grade, progression, and cancer-specific death in MIBC (data not shown).

4. Prognostic value of *DBC1* mRNA expression levels for recurrence, progression, and survival in NMIBC

In the multivariate analysis, intravesical therapy (hazard ratio [HR], 0.220; p < 0.001) was the independent predictor of tumor recurrence. Tumor size (HR, 4.402; p=0.018), tumor grade 3 (HR, 18.693; p=0.020), intravesical therapy (HR, 0.258; p=0.032), and *DBC1* expression (HR, 7.104; p=0.032) were the independent predictors of progression to MIBC (Table 3).

Analysis using Kaplan-Meier curves revealed prolonged recurrence, progression-free survival, and cancer-specific survival of high *DBC1* expressors compared with low expressors in NMIBC (p=0.023, p=0.024, and p=0.036, respectively) (Fig. 1A–C).

DISCUSSION

In the present study, we investigated the contribution of *DBC1* to an increased risk of bladder cancer and the useful-

TABLE 2. mRNA expression levels of *DBC1* in the control and bladder cancer patients

Group	mRNA expression level ($\times10^4$ copies per μg), median (IQR)	p-value[a]
Control	17.49 (9.84–36.12)	0.414
Bladder cancer	24.42 (6.38–92.61)	
Control	17.49 (9.84–36.12)	0.128
NMIBC	36.55 (6.38–109.02)	
Control	17.49 (9.84–36.12)	0.642
MIBC	17.32 (3.87–63.23)	
NMIBC	36.55 (6.38–109.02)	0.010
MIBC	17.32 (3.87–63.23)	

IQR, interquartile range; NMIBC, nonmuscle invasive bladder cancer; MIBC, muscle invasive bladder cancer.
[a]:p-values were based on the Mann-Whitney U test.

TABLE 3. Multivariate Cox regression analysis for prediction of tumor recurrence, progression to MIBC

Variable	Tumor recurrence		Progression to MIBC	
	HR (95% CI)[a]	p-value	HR (95% CI)[a]	p-value
Age (< 67 yr vs. ≥67 yr)	0.949 (0.585–1.540)	0.834	1.325 (0.496–3.545)	0.575
Gender (male vs. female)	1.381 (0.767–2.488)	0.282	0.394 (0.088–1.767)	0.224
Tumor size (< 3 cm vs. ≥3 cm)	1.271 (0.770–2.100)	0.348	4.402 (1.286–15.074)	0.018
Tumor number (single vs. multiple)	1.153 (0.706–1.883)	0.569	0.818 (0.286–2.341)	0.708
DBC1 expression (high vs. low)	1.115 (0.674–1.842)	0.672	7.104 (1.516–33.293)	0.013
Grade				
G1	1[b]	-	1[b]	-
G2	1.244 (0.610–2.539)	0.548	3.261 (0.309–34.455)	0.326
G3	1.969 (0.815–4.754)	0.132	18.693 (1.594–219.266)	0.020
T stage (Ta vs. T1)	1.535 (0.805–2.926)	0.193	3.872 (0.701–21.381)	0.120
Intravesical therapy (No vs. Yes)	0.220 (0.118–0.411)	< 0.001	0.258 (0.075–0.893)	0.032

MIBC, muscle invasive bladder cancer; HR, hazard ratio; CI, confidence interval.
[a]:Multivariate Cox regression analysis was used to estimate HR with the corresponding 95% CI. [b]:Reference.
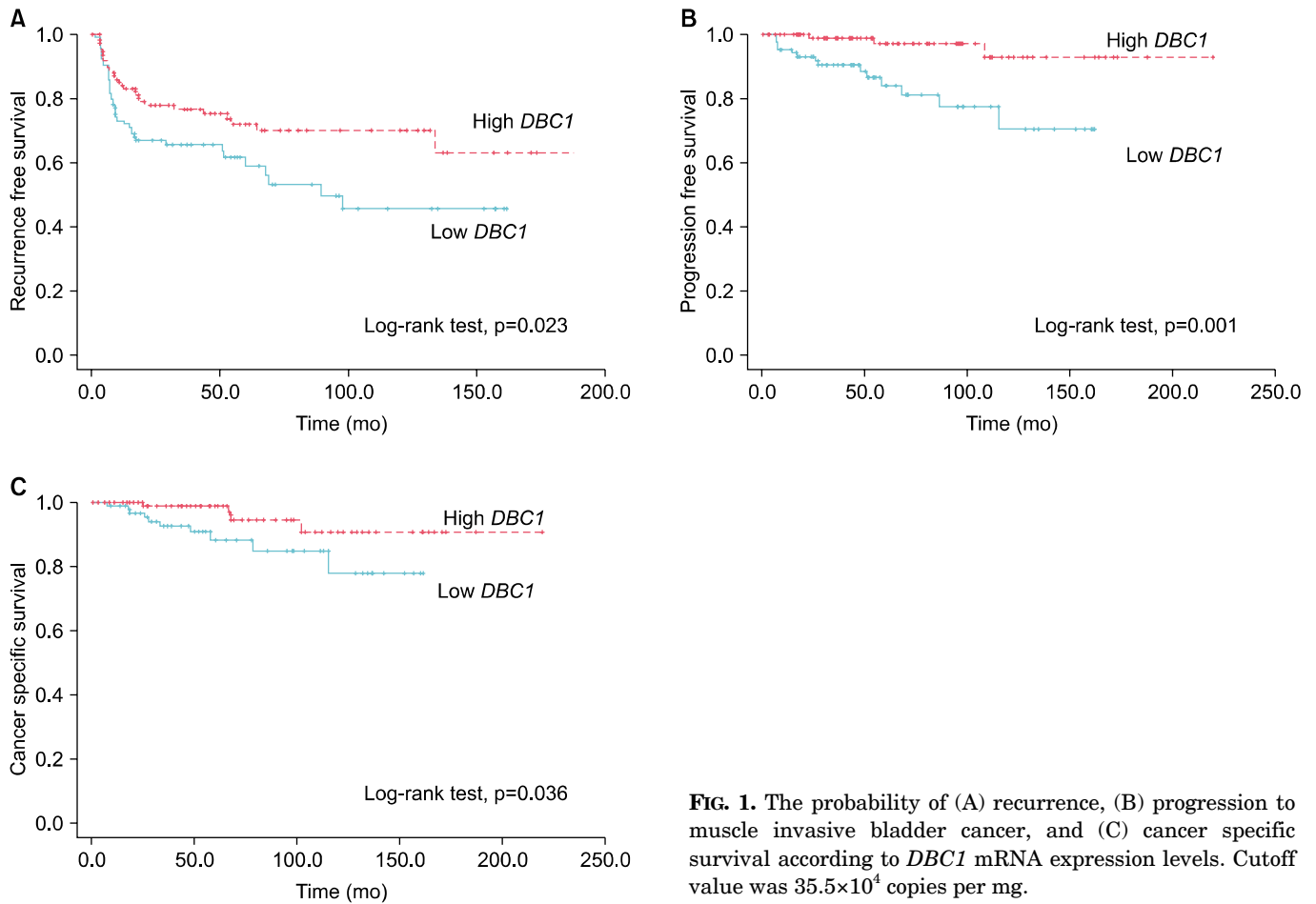
FIG. 1. The probability of (A) recurrence, (B) progression to muscle invasive bladder cancer, and (C) cancer specific survival according to *DBC1* mRNA expression levels. Cutoff value was 35.5×10^4 copies per mg.

ness of *DBC1* expression as a prognostic marker in bladder cancer. *DBC1* was found to be closely associated with clinicopathologic features and cancer-related survival in patients with NMIBC. Moreover, the expression of *DBC1* was an independent predictor of progression to MIBC in patients with NMIBC. Our results suggest that *DBC1* is a useful prognostic marker for NMIBC and might be an important tool for individualized prognosis of patients with NMIBC.

Loss of heterozygosity on chromosome 9 is the most frequently reported genetic event in urothelial carcinoma of the urinary bladder. Approximately 60% to 70% of bladder tumors have loss of heterozygosity at one or more loci on either arm of chromosome 9 and approximately 30% display loss of the entire chromosome. Deletion mapping with microsatellite markers suggests the existence of several putative tumor suppressor loci on chromosome 9, and deletions at chromosome 9 have been found in tumors of various grades and stages [23]. *DBC1* is located within chromosome 9 (9q32–33) and has been identified as a potential tumor suppressor gene that is commonly hypermethylated or deleted in bladder cancer. The 5' region of *DBC1* contains a CpG island that is aberrantly hypermethylated in approximately 50% of bladder cancer cell lines and tumors, 40% of oral squamous cell carcinomas, 80% of non-small-

cell lung cancer cell lines, and a proportion of primary non-small-cell lung cancer [18-20]. In addition, the exogenous expression of *DBC1* protein in human bladder tumor cell lines is known to suppress proliferation, and *DBC1*-mediated growth inhibition is reportedly due to an increase in the number of cells in the G1 phase of the cell cycle, which suggests a role for *DBC1* in cell cycle control [21,24]. At present, however, the exact mechanism of action and the pathways in bladder carcinogenesis are unknown; therefore, *DBC1* has not yet been placed in a functional pathway.

Our data have shown some similarities and differences compared with previous studies. The most striking result was that *DBC1* expression was closely associated with clinicopathologic features such as tumor stage, grade, size, and multiplicity in patients with NMIBC. Moreover, individuals with low *DBC1* expression appeared to have an increased predisposition toward progression to muscle-invasive cancer as well as a reduced rate of survival from NMIBC. Intriguingly, *DBC1* expression was significantly decreased in patients with MIBC compared with those with NMIBC. Decreased *DBC1* expression in MIBC supports the finding that individuals with low *DBC1* expression appear to have an increased predisposition for progression to muscle-invasive cancer. This result was contrary to previous *in vitro* evidence. Habuchi et al. [18,25] reported that

mRNA expression was absent in 5 of 10 (50%) bladder cancer cell lines, and 52% of bladder tumors demonstrated patterns of abnormal hypermethylation without correlation to tumor grade or stage. The results of that study suggested that hypermethylation of the *DBC1* region is one of the earliest alterations in the development of urothelial carcinoma in the bladder and that there may be an age-related hypermethylation-based field defect in normal urothelium. Although *DBC1* mRNA expression appears to be suppressed by hypermethylation of the 5'-region *in vivo*, there was no clear relationship between the methylation level of *DBC1* and its mRNA expression. Unfortunately, there is no clinical evidence as to whether inactivation of the *DBC1* tumor suppressor gene (loss of mRNA expression) influences genetic susceptibility and the prognosis of bladder cancer. We also found highly left-skewed expression levels of *DBC1* in normal urothelium. Normal human urothelial cells showed a decrease in *DBC1* mRNA expression when cultured, with increasing *DBC1* expression as cells passed to senescence, which suggests that *DBC1* may have a role in maintaining cells in a quiescent state [26]. This mechanism might explain the highly left-skewed expression levels of *DBC1* in normal urothelium.

A possible limitation of the present study is that we did not evaluate the protein levels of *DBC1*, such as by Western blot or immunohistochemical staining. We think that further protein study of *DBC1* is needed to confirm its function as a more reliable prognostic marker. Second, the present study had a relatively small sample size, particularly for control cases, which lowered the statistical power. Thus, further research is needed to confirm the prognostic value of *DBC1* as it relates to disease progression and recurrence in NMIBC patients.

CONCLUSIONS

The expression of *DBC1* is associated with tumor aggressiveness in NMIBC. The results of the present study suggest that the *DBC1* gene may be a useful prognostic marker for NMIBC. This result could be an important tool for individualized prognosis for patients with NMIBC. If indeed *DBC1* has a critical role in the disease progression of NMIBC, it could be a potential target for use in the prevention of disease progression, such as with BCG immunotherapy or intravesical chemotherapy, because transcriptional silencing can be reversibly controlled by inhibitors of methylation.

CONFLICTS OF INTEREST
The authors have nothing to disclose.

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