

DDX39 acts as a suppressor of invasion for bladder cancer

Minoru Kato,^{1,2} Min Wei,¹ Shotaro Yamano,¹ Anna Kakehashi,¹ Satoshi Tamada,² Tatsuya Nakatani² and Hideki Wanibuchi^{1,3}

¹Department of Pathology, ²Department of Urology, Osaka City University Graduate School of Medicine, Osaka, Japan

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The object of the present study was to identify markers for predicting urinary bladder cancer progression by comparative proteome analysis of bladder cancers and paired normal mucosas. We found that DDX39 was overexpressed in four of six bladder cancers examined compared with respective control tissues. Immunohistochemical analysis using 303 bladder cancer specimens revealed that DDX39 was inversely correlated to pT stage and histological grade progression. The incidence of DDX39^{high} tumors (positive cells $\geq 50\%$) was 68.6%, 43.5%, 20.0%, and 5.3% in pTa, pT1, pTis, and \geq pT2 tumors, respectively, and 65.2%, 60.7%, and 19.6% in G1, G2, and G3 tumors, respectively. The incidence of DDX39^{high} tumors was significantly lower in pT1 and \geq pT2 compared to pTa tumors, and also significantly lower in G3 compared to G1 and G2 tumors. Follow-up analysis ($n = 105$) revealed that DDX39^{low} tumors (positive cells $< 50\%$) were associated with disease progression (hazard ratio 7.485; $P = 0.0083$). Furthermore, DDX39-knockdown bladder cancer cells increased their invasion ability compared to negative control cells. These results suggest that DDX39 is a suppressor of invasion and loss of its function predicts disease progression in bladder cancers. (*Cancer Sci* 2012; 103: 1363–1369)

Urinary bladder cancers account for approximately 54% of cancers of the urinary system (kidney, renal pelvis, ureters, bladder, and urethra).⁽¹⁾ Approximately 90% of all bladder cancers are urothelial carcinomas.⁽²⁾ At initial presentation, up to 70% of tumors are non-muscle-invasive, whereas the remainder present with muscle-invasive disease.⁽³⁾ The treatment for bladder cancer completely differs depending on stage. Generally, non-muscle-invasive bladder cancer (NMIBC) requires transurethral resection of the bladder tumor (TUR-Bt), whereas most muscle-invasive bladder cancer (MIBC) requires radical cystectomy with or without systemic chemotherapy. However, the prognosis for advanced bladder cancer is poor despite recent therapeutic advances.⁽⁴⁾ To date, pathological data, including grade, stage, and associated carcinoma *in situ* (CIS) at initial presentation, have provided some insight into predicting the likelihood of progression of bladder cancer.^(5,6) Nevertheless, the ability to predict progression remains a challenge as bladder tumors with the same stage and grade have a heterogeneous clinical outcome. This might be due to differences in molecular expression profile. Furthermore, understanding the molecular biology of bladder cancer may provide new therapeutic strategies.

Various molecules have been reported to be associated with the progression of bladder cancer. Tumor suppressor genes, such as *p53*, have been widely studied in bladder cancer, however, its predictive value in assessing the risk of disease progression remains controversial.^(7–9) *Ki-67* has some prognostic value for predicting recurrence, however, further studies are necessary and the marker is not yet clinically applicable.^(10,11)

Recently, proteome analysis has been widely used in the study of urine from bladder cancer patients to identify biomarkers.⁽¹²⁾ Various urinary markers for the early detection of bladder cancer have been reported, but reliable urinary markers capable of predicting cancer progression have not been established. This is partly due to the fact that some of them are not expressed in bladder tissues.⁽¹³⁾ Therefore, investigation of protein expression profiling in bladder cancer tissues will facilitate not only understanding the behavior of cancer cells but also identification of markers of progression of bladder cancer.

The purpose of the present study is to identify markers of bladder cancer progression by comparative proteome analyses of human bladder cancer and paired normal tissues using QSTAR Elite liquid chromatography with tandem mass spectrometry and iTRAQ technology.

Materials and Methods

Patients. Six pairs of snap-frozen bladder urothelial carcinomas and normal mucosa from cystectomy specimens were used for proteome analyses. The clinicopathological characteristics of the bladder carcinomas was as follows: case 1 was pT1, G3; case 2 was pTa, G3; case 3 was pT1, G3; case 4 was pT1, G2; case 5 was pT2a, G3; and case 6 was pT3, G3. Four cases (1–4) were NMIBCs and the remaining cases (5 and 6) were MIBCs. Immunohistochemical analysis was carried out on samples from 303 patients who were treated for bladder cancer by TUR-Bt or cystectomy at Osaka City University Hospital (Osaka, Japan) between 2000 and 2009. There were 248 men (81.8%) and 55 women (18.2%), and the median age was 68 years (range, 33–90 years). Among these patients, those with CIS and those who were incompletely resected and lost to follow-up were excluded from the study. The patients who were treated by total cystectomy were also excluded, because almost all of these patients had already progressed to muscle-invasive disease. One hundred and five TUR-Bt cases (between 2004 and 2007), for which full clinical data were available, were used for follow-up analysis. Pathologic staging was carried out according to the 2002 TNM classification system,⁽¹⁴⁾ and grading was done according to the 1973 World Health Organization criteria for continuity of the study, as many samples were obtained before the 2004 criteria were published. The Institutional Review Board at Osaka City University Graduate School of Medicine approved the use of the specimens and clinical data in accordance with the Declaration of Helsinki and guidelines of Osaka City University Graduate School of Medicine.

Proteome analyses. Pathologic diagnoses of the six urothelial cancers and paired control tissues were confirmed before proteome analyses. Reagents, except for those specifically noted, were obtained from AB Sciex (Foster City, CA, USA). The

³To whom correspondence should be addressed.
E-mail: wani@med.osaka-cu.ac.jp

specimens were homogenized and dissolved in 300 μ L T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) with protease inhibitor. After brief ultrasonication, insoluble material was removed by centrifugation at 13 000g for 15 min at 4°C. Protein concentration of the supernatant was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein reduction, alkylation, and subsequent peptide labeling were carried out using iTRAQ Reagent Multiplex Kit (AB Sciex, Foster City, CA, USA). Samples (100 μ g of each) were resuspended in 20 μ L of dissolution buffer (0.5 M triethylammonium bicarbonate at pH 8.5). One microliter of denaturant (2% SDS) and 2 μ L reducing reagent (50 nM tris-(2-carboxyethyl) phosphine) were added and incubated at 60°C for 60 min. Free sulfhydryl groups of cysteines were blocked with 1 μ L cysteine blocking reagent (20 mM methyl methanethiosulfonate) and incubated at room temperature for 10 min. Trypsin solution (10 μ g) was added to each sample and incubated at 37°C overnight. Tryptic peptides of each sample were labeled with iTRAQ tags by incubation at room temperature for 1 h. Each of the samples was then mixed in one tube and fractionated by six concentrations of KCl solutions (10, 50, 70, 100, 200, and 350 mM) using ICAT cation exchange cartridge. Supernatant was evaporated in a vacuum centrifuge. Peptides of each fraction were resuspended into 2 mL of 2% acetonitrile and desalted using Sep-Pak Light C18 cartridge (Waters, Milford, MA, USA). The supernatant was dissolved in 20 μ L of 0.1% formic acid.

Proteome analysis was carried out with a DiNa-AI nano system (KYA Technologies, Tokyo, Japan) coupled to a QSTAR EliteHybrid mass spectrometer through a NanoSpray ion source. Protein identification was done with ProteinPilot 2.0 software (AB Sciex).

Immunohistochemical analysis of DDX39. Formalin-fixed, paraffin-embedded tissues of 303 patients with bladder cancer were analyzed by immunohistochemical staining. Sections (3 μ m-thick) were cut and deparaffinized in xylene and rehydrated in alcohols and distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 min, followed by washing in PBS three times. Sections were then incubated with 1.5% goat serum in PBS for 15 min to bind non-specific antigens and then with rabbit polyclonal antibody to DDX39 (ab96621, 1:500; Abcam, Cambridge, MA, USA) at 4°C overnight. This was followed by incubation with biotinylated goat anti-rabbit IgG for 30 min and avidin-biotin peroxidase complex for 30 min at room temperature. Antigen was detected with 3,3'-diaminobenzidine and counterstaining with hematoxylin.

Immunohistochemical analysis was carried out by two pathologists who were blinded to the clinical data (S.Y. and H.W.). Immunoreactivity of DDX39 was observed in nuclei of bladder tissues but not in the normal urothelium. Under a microscope at $\times 200$ magnification on six random fields per sample, tissues with $\geq 50\%$ cancer cells immunoreactive for DDX39 were defined as DDX39^{high}, and those with $< 50\%$ cells immunoreactive for DDX39 were defined as DDX39^{low}.

Cell lines. Human bladder cancer cell lines T24, TCCSUP, and UMUC3 were purchased from ATCC (Rockville, MD, USA). All cells were maintained as monolayer cultures at 37°C and 5% CO₂. T24 was grown in McCoy's medium and TCCSUP and UMUC were grown in MEM. All media were supplemented with 10% FBS.

Western blot analyses. Whole cell lysates were collected using a cell scraper and resuspended in CelLytic MT (Sigma, St Louis, MO, USA) with protease inhibitor. The amount of total protein was determined using a BCA protein assay kit (Pierce). Protein (15 μ g of each) was loaded on 10% SDS-polyacrylamide gels. Proteins were transferred to a PVDF membrane and blocked with 5% skimmed milk in TBS buffer containing 0.1% Tween-20. The membrane was probed with primary antibody

for DDX39 (ab50697, 1:100; Abcam) or β -actin (ab49900, 1:100 000; Abcam) for 1 h at room temperature. After washing, the membrane was incubated for 1 h at room temperature linked with HRP-conjugated secondary antibody (#sc-2004, 1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were detected using the ECL Plus Western blotting system (GE Healthcare, Piscataway, NY, USA) and LAS-3000 image analysis system (Fujifilm, Tokyo, Japan).

Real-time PCR. Total RNA was extracted from cell lines using the RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined by Nanodrop (Thermo Scientific). RNA (1 μ g) was used for cDNA synthesis using Advantage RT-for-PCR kit (Takara Bio, Tokyo, Japan). The real time RT-PCR assay was carried out with the Applied Biosystems 7500 Fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR reactions consisted of 10 μ L of 2 \times TaqMan FAST Universal Master Mix (Applied Biosystems), 1 μ L of 20 \times TaqMan Gene Expression Assay (Applied Biosystems), and 1 μ g cDNA solution. The assay IDs used for real-time RT-PCR were as follows: DDX39, Hs00271794_m1; and GAPDH, Hs00266705_g1. The thermal cycle program was: 20 s at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. The data were then quantified using the comparative C_t method for relative gene expression compared with GAPDH as internal control.

Knockdown of DDX39. DDX39 expression was transiently knocked down using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DDX39-specific siRNAs (Silencer Select siRNA; Cat.# s19917 and s19918) were obtained from Life Technologies (Grand Island, NY, USA). Non-targeting control siRNA (PremiR miRNA Precursor Starter Kit, Cat.# AM1540) was obtained from Life Technologies. T24 cells (3×10^5) were transiently transfected with 10 nM s19917, s19918, or control siRNA in a six-well plate. After 24 h, cells were trypsinized and used in cell proliferation and cell invasion assays.

Cell proliferation assay. T24 cells (1×10^4 /well) were seeded in a 96-well plate and transfected with 10 nM DDX39 siRNAs and control siRNA. After 24 h, cell proliferation was measured using a Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. The number of cells was measured with a microplate reader (Bio-Rad, Tokyo, Japan) at 450 nm.

Cell invasion assay. Invasion was assessed in a QCM cell invasion assay (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Briefly, transfectants (2×10^5 cells) were seeded in the upper chamber, whereas the lower chamber was loaded with medium containing 10% FBS. After a 24-h incubation at 37°C, the cells that invaded the reverse side of the insert were dislodged by incubating the insert in 225 μ L cell detachment buffer for 30 min at room temperature. Lysis buffer and CyQuant GR Dye mixture (75 μ L each) were added in detachment buffer and the plate was incubated for 15 min in the dark. Then 200 μ L of the mixture was transferred to a 96-well plate and measured with a fluorescence plate reader at 480/520 nm.

Statistical analysis. Statistical analyses were carried out with SPSS version 19 (IBM, Armonk, NY, USA). Fisher's exact test was used to evaluate the differences in incidence of DDX39 expression patterns among clinical and pathological parameters. The progression-free survival was defined as the time between the date of surgery and the last date of follow-up or date of progression in pT status. The curves were done using the Kaplan-Meier method with the log-rank test to assess statistical significance. Cox proportional hazards analysis was used to determine the relative contribution of various factors to the risk of progression. $P < 0.05$ was considered statistically significant.

Results

Proteome analysis. We identified 493 proteins by proteomic analysis of six sample pairs. Overexpressed proteins were selected according to the criteria that the fold difference had to be >1.2.⁽¹⁵⁾ Fifteen proteins were overexpressed in cancer tissues compared to adjacent normal tissues in four or more of six sample pairs (Table 1). To validate the results of the proteome analysis, immunohistochemical staining of the above proteins in 303 bladder specimens was carried out, except for those that have already been evaluated in bladder cancers (Ezrin, nucleophosmin, prothymosin alpha, S100 calcium binding protein A11, and S100 calcium binding protein P).^(16–19) Actin-related protein 3 homolog B was not evaluated as no commercial antibody was available.

Results of immunohistochemistry analyses of DDX39, B-cell receptor-associated protein 31, chaperonin containing TCP1, FK506 binding protein 4, isocitrate dehydrogenase 1, keratin 19, myosin heavy chain 9 non-muscle, prolyl 4-hydroxylase beta polypeptide, and Y box binding protein 1 revealed that DDX39, but not the other proteins, was expressed in a different manner according to cancer stage and grade as described below, although all of them showed high expression levels in cancer compared to control tissues.

Expression of DDX39 in bladder cancers. The clinicopathological parameters of the patients whose tissues were used for immunohistochemical analysis are summarized in Table 2. As shown in Figure 1, nuclear staining of DDX39 was not observed in normal urothelium (Fig. 1D). Unexpectedly, expression levels of DDX39 were apparently lower in MIBCs when compared with NMIBCs. As summarized in Table 2, the incidence of DDX39^{low} cancers was 31%, 56%, 80%, and 95% in pTa, pT1, pTis, and ≥pT2 tumors, respectively. The incidence of DDX39^{low} cancers was significantly higher in ≥pT2 compared to pT1 and pTa cancers, and also significantly higher in pT1 when compared to pTa. Furthermore, the incidence of DDX39^{low} cancers was 35%, 39%, and 80% in G1, G2, and G3 tumors, respectively, and significantly higher in G3 when compared to G1 and G2 tumors.

Follow-up of patient outcomes and survival analysis. Correlation analysis of DDX39 expression level and recurrence/progression-free survival in 105 bladder cancer patients who were treated by TUR-Bt revealed that DDX39^{low} cancers showed rapid disease progression ($P = 0.0083$; Fig. 2A). Moreover,

Table 2. Clinicopathological characteristics of patients ($n = 303$), stages and grades of bladder cancer, and DDX39 expression

Parameters	DDX39 high expression tumors (%)	P-value
Age (68 ± 10.5 years)		
<65 years ($n = 116$)	52 (44.8)	NS
≥65 years ($n = 187$)	103 (55.1)	
Gender		
Male ($n = 248, 81.8\%$)	127 (51.2)	NS
Female ($n = 55, 18.2\%$)	28 (50.1)	
Stage		
pTa ($n = 169, 55.7\%$)	116 (68.6)	
pT1 ($n = 62, 20.5\%$)	27 (43.5)	0.0005*
≥pT2 ($n = 57, 18.8\%$)	3 (5.3)	<0.0001*
pTis ($n = 15, 5.0\%$)	3 (20.0)	<0.0001†
Grade		
G1 ($n = 66, 21.8\%$)	43 (65.2)	
G2 ($n = 145, 47.9\%$)	88 (60.7)	
G3 ($n = 92, 30.4\%$)	18 (19.6)	<0.0001**††

*Statistically significant from pTa. **Statistically significant from G1.

†Statistically significant from pT1. ††Statistically significant from G2.

NS, not significant.

rapid disease progression was also evident in DDX39^{low} cancers when the 78 pTa cancers were analyzed from the above cases ($P = 0.0027$; Fig. 2B). No association was found with recurrence-free survival in either of the analyses (data not shown).

Univariate and multivariate analyses. Univariate and multivariate analyses of clinicopathological parameters and progression-free survival revealed that low expression of DDX39 was an independent risk factor for progression (Tables 3,4).

Expression levels of DDX39 and invasion ability of bladder cancer cells. The mRNA expression levels of DDX39 were analyzed by real-time PCR in T24, TCCSUP, and UMUC3 cells. T24 cells showed the highest expression level of DDX39 (Fig. 3A) but the lowest invasion ability among the three bladder cancer cell lines (Fig. 3B). As T24 showed an inverse relationship between the expression level of DDX39 and its invasion ability, similar to that observed in the bladder cancer

Table 1. Upregulated proteins in cancer tissues using liquid chromatography with tandem mass spectrometry

Symbol	Case						Full name	Location
	1	2	3	4	5	6		
ACTR3B	4.200	2.359	ND	1.718	1.818	16.933	Actin-related protein 3 homolog B (yeast)	Unknown
BCAP31	2.797	2.186	ND	1.38	2.704	ND	B-cell receptor-associated protein 31	Cytoplasm
CCT4	4.008	1.561	1.650	1.296	ND	7.610	Chaperonin containing TCP1, subunit 4 (delta)	Cytoplasm
DDX39	4.685	1.858	2.116	ND	1.900	ND	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	Nucleus
EZR	4.961	2.315	1.536	1.776	3.113	4.704	Ezrin	Plasma membrane
FKBP4	19.763	3.820	2.308	ND	3.500	ND	FK506 binding protein 4	Nucleus
IDH1	4.618	3.137	1.278	1.580	2.662	ND	Isocitrate dehydrogenase 1 (NADP+), soluble	Cytoplasm
KRT19	3.101	4.917	ND	1.437	5.312	4.614	Keratin 19	Cytoplasm
MYH9	2.918	2.888	1.413	1.473	2.438	6.401	Myosin, heavy chain 9, non-muscle	Cytoplasm
NPM1	1.529	1.240	ND	ND	1.684	11.318	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	Nucleus
P4HB	3.690	1.431	1.378	ND	2.286	10.049	Prolyl 4-hydroxylase, beta polypeptide	Cytoplasm
PTMA	3.477	1.165	1.860	ND	2.102	3.062	Prothymosin, alpha	Nucleus
S100A11	8.414	1.991	1.311	1.512	5.261	9.114	S100 calcium binding protein A11	Cytoplasm
S100P	6.842	5.731	ND	2.128	8.834	3.450	S100 calcium binding protein P	Cytoplasm
YBX1	2.978	1.711	1.644	ND	1.912	ND	Y box binding protein 1	Nucleus

The numbers listed under cases 1–6 indicate the fold change of the protein expression level of cancer tissue compared to normal tissue in each case. ND, not detectable.

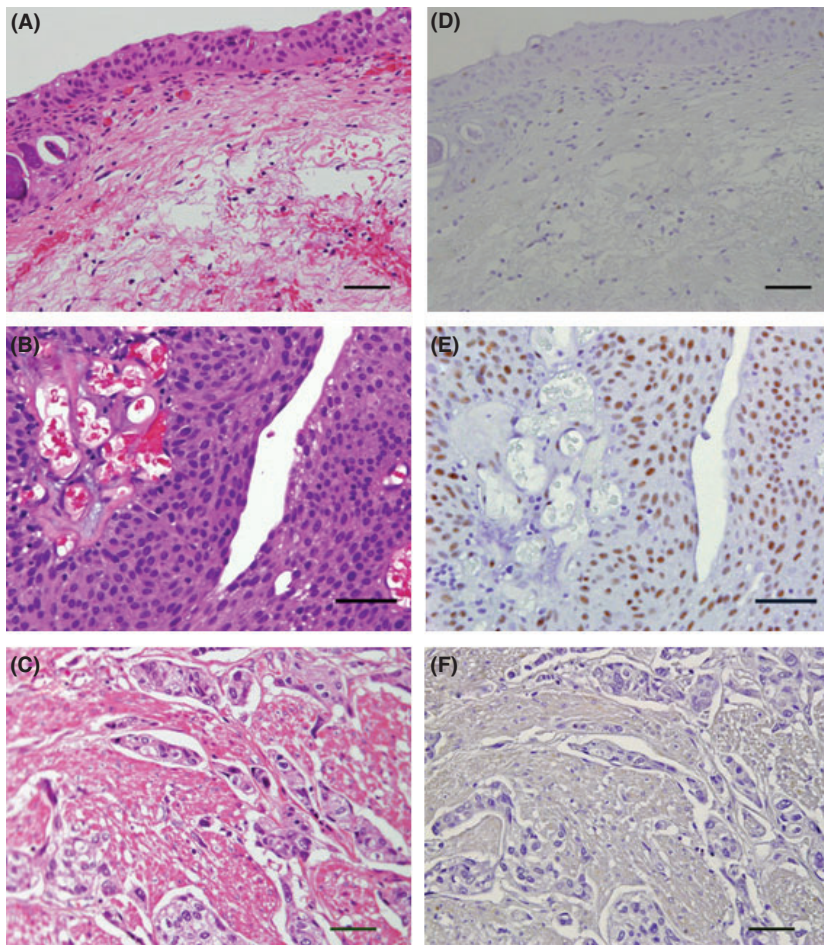


Fig. 1. Hematoxylin-eosin staining (A–C) and immunohistochemical staining (D–F) for DDX39 of normal urothelium and bladder cancers. (A, D) Normal bladder urothelium; (B, E) DDX39^{high} (positive cells \geq 50%) pTa cancer; (C, F) DDX39^{low} (positive cells < 50%) pT2 cancer. Bar = 50 μ m.

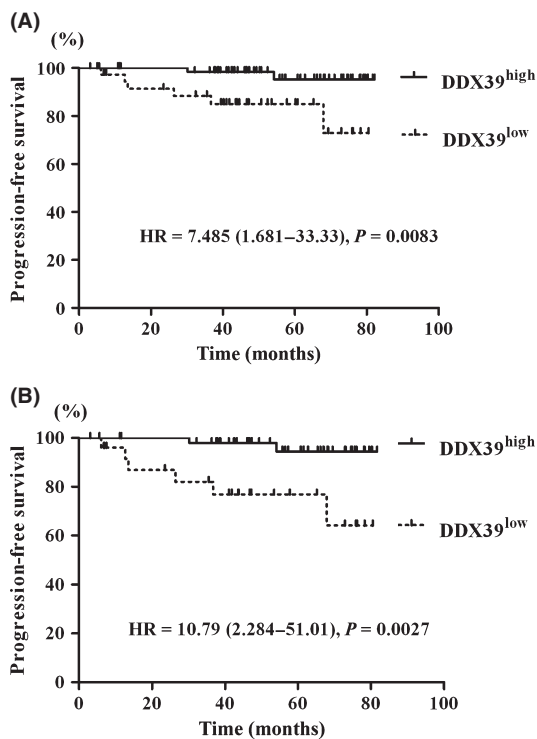


Fig. 2. DDX39^{low} cancers (positive cells < 50%) showed rapid disease progression in pTa and pT1 cancers (A) ($n = 105$) and pTa cancers (B) ($n = 78$). HR, hazard ratio.

specimens analyzed above, we used T24 cells to investigate the effects of DDX39 knockdown on invasion ability.

Effect of DDX39 knockdown on cell proliferation and invasion ability of T24 cells. Western blot analysis showed a remarkable reduction in protein level of DDX39 in T24 cells transfected with si-DDX39 A (s19917) and B (s19918) compared with the negative control (Fig. 4A). Furthermore, real-time PCR analysis showed that si-DDX39 A and B reduced DDX39 mRNA expression levels by 71% and 74%, respectively, compared with the negative control (Fig. 4B). Although knockdown of DDX39 by si-DDX39 A and B had no effect on cell proliferation (Fig. 5A), T24 cells transfected with si-DDX39 A and si-DDX39 B showed 2.36- and 2.65-fold higher invasion activity, respectively, compared to the negative control (Fig. 5B).

Discussion

The results of the present study indicated that the expression level of DDX39 is significantly lower in MIBCs compared to NMIBCs. We also found that the DDX39 expression level was significantly correlated with pT stage and grade, and DDX39^{low} cancers showed rapid disease progression. Furthermore, knockdown of DDX39 increased the invasion ability of bladder cancer cells. These findings indicated that DDX39 is a suppressor of invasion and loss of its function predicts disease progression in bladder cancers. To the best of our knowledge, the present study showed for the first time the relationship between DDX39 expression and cancer cell invasion.

A member of the RNA helicases, DDX39 is involved in pre-mRNA splicing.⁽²⁰⁾ RNA helicase is thought to be required

Table 3. Univariate analyses of various clinicopathological parameters in relation to progression-free survival of patients with bladder cancer

Patients Parameters	Progression-free survival		
	No. cases (%)	No. events	P-value†
Age (years)			
<65	46 (44)	2	0.2971
≥65	59 (56)	6	
Gender			
Male	84 (80)	6	0.6708
Female	21 (20)	2	
Stage			
pTa	78 (74)	8	0.1050
pT1	27 (26)	0	
Grade			
G1 + G2	90 (86)	6	0.3695
G3	15 (14)	2	
Concomitant CIS			
No	98 (93)	7	0.4638
Yes	7 (7)	1	
No. tumors			
Single	76 (72)	4	0.1624
Multiple (2–7)	29 (28)	4	
Tumor size			
<3 cm	101 (96)	8	0.6123
≥3 cm	4 (4)	0	
Tumor status			
Primary	76 (72)	4	0.1830
Recurrent	29 (28)	4	
DDX39 expression			
Low (positive cells <50%)	39 (37)	6	0.0083
High (positive cells ≥50%)	66 (63)	2	

†Log-rank test. CIS, carcinoma *in situ*.

Table 4. Multivariate analyses for progression free survival

Variables	Progression-free survival	
	OR (95% CI)	P-value
Grade		
G1 + G2	1	–
G3	1.084 (0.140–8.420)	0.938
Carcinoma <i>in situ</i>		
–	1	–
+	2.369 (0.178–31.484)	0.514
No. tumors		
Single	1	–
Multiple	2.532 (0.570–11.250)	0.222
Prior recurrence rate		
Primary	1	–
Recurrence	1.035 (0.337–3.176)	0.952
DDX39 expression		
High	1	–
Low	7.171 (1.284–40.063)	0.025

–, normalized.

for the export of mRNA out of the nucleus, transcription, splicing, and transport of mRNA.⁽²¹⁾ Although several other RNA helicases were reported to be dysregulated in cancer, and loss of normal function of RNA helicase could result in abnormal RNA processing,⁽²²⁾ little is known about the exact roles of RNA helicases in carcinogenesis. Sugiura *et al.*⁽²³⁾ reported that DDX39 was upregulated in lung squamous cell

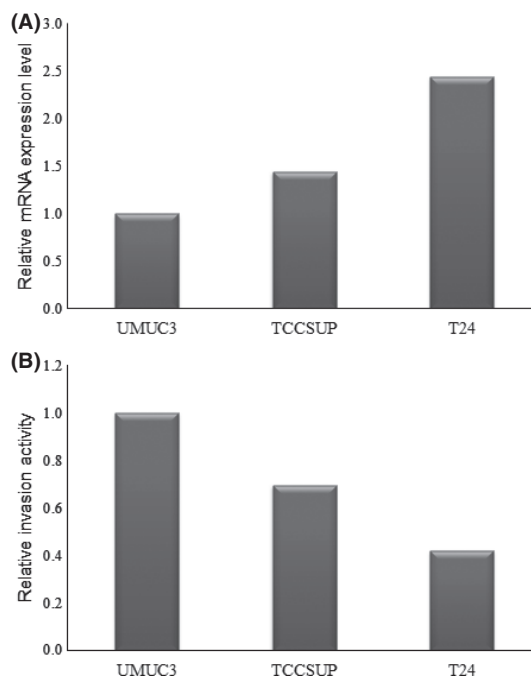


Fig. 3. mRNA expression of DDX39 (A) and cell invasion ability (B) of UMUC3, TCCSUP, and T24 bladder cancer cells. The DDX39 expression level was highest, but the invasion ability was lowest, in T24 cells compared to the other two cell lines.

cancer and promoted cancer cell growth. However, we found that DDX39 inhibits the invasion, but had no effect on proliferation, of bladder cancer cells. Although the exact role of DDX39 in bladder carcinogenesis is not known, the fact that the expression level of DDX39 is significantly higher in NMIBCs compared to MIBCs, and that DDX39 has no effect on cell proliferation and is not expressed in normal bladder mucosa, suggested that DDX39 in NMIBCs may exert a protective role against bladder cancer invasion. Furthermore, based on the finding that DDX39^{low} pTa cancers showed rapid disease progression, DDX39 might serve as a marker for NMIBCs that are likely to progress; those showing low levels of DDX39 may require more intensive therapy and closer follow-up. Further study is needed to evaluate the underlying mechanisms by which DDX39 inhibits the invasion of bladder cancer.

Matrix metalloproteinase 2 and 9 and E-cadherin were reported to be associated with bladder cancer invasion.^(24,25) In the present study, the mRNA expression level of MMP2, MMP9, and E-cadherin did not change significantly by DDX39 knockdown (data not shown). These results suggested that DDX39 inhibited invasion by mechanisms independent of these proteins.

Recurrence and progression are the main problems for NMIBCs, but few reports are available identifying molecules that could predict progression. The European Association of Urology (EAU) guideline on non-muscle-invasive urothelial carcinoma of the bladder has already proven to be useful to predict recurrence and progression,⁽²⁶⁾ but its classification system is quite complicated to apply clinically. In addition, stratification based on the EAU guidelines for recurrence is not fully applicable to Japanese patients with bladder cancers.⁽²⁷⁾ In the present study, cancer stage, histological grade, concomitant CIS, number of tumors, size of tumors, and prior recurrence rate could not predict cancer progression. This might be partially due to the sample size of our patients' dataset. But the results of the present study showed that only

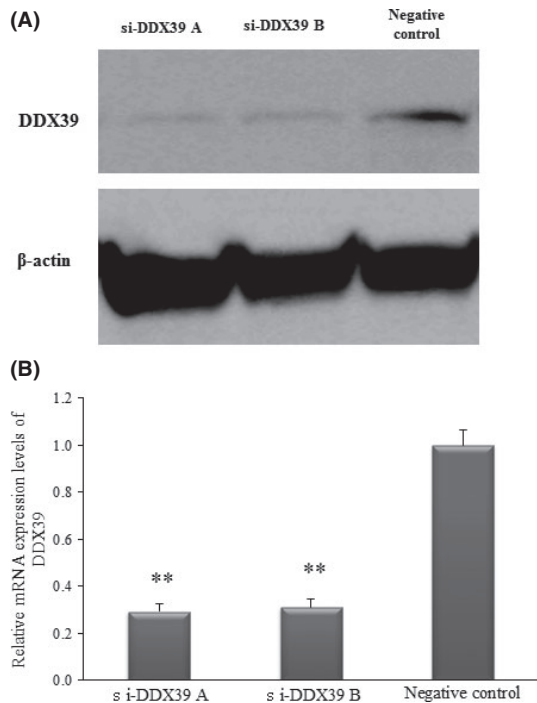


Fig. 4. Two siRNAs for DDX39 (si-DDX39 A and si-DDX39 B) down-regulated the protein (A) and mRNA expression of DDX39 (B). ** $P < 0.01$.

DDX39 had prognostic value for predicting progression of NMIBC.

In conclusion, our results suggest that DDX39 is a suppressor of invasion and could be a useful molecular marker for predicting progression of urothelial carcinoma and a novel target for clinical therapy.

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References

- Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277–300.
- Epstein JI, Amin MB, Reuter VR, Mostofi FK. The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. *Am J Surg Pathol* 1998; **22**: 1435–48.
- Rubben H, Lutzeyer W, Fischer N, Deutz F, Lagrange W, Giani G. Natural history and treatment of low and high risk superficial bladder tumors. *J Urol* 1988; **139**: 283–5.
- Thurman SA, DeWeese TL. Multimodality therapy for the treatment of muscle-invasive bladder cancer. *Semin Urol Oncol* 2000; **18**: 313–22.
- Althausen AF, Prout GR Jr, Daly JJ. Non-invasive papillary carcinoma of the bladder associated with carcinoma in situ. *J Urol* 1976; **116**: 575–80.
- Herr HW. Tumor progression and survival of patients with high grade, non-invasive papillary (TaG3) bladder tumors: 15-year outcome. *J Urol* 2000; **163**: 60–1; discussion 1–2.
- Habuchi T, Marberger M, Droller MJ *et al*. Prognostic markers for bladder cancer: International Consensus Panel on bladder tumor markers. *Urology* 2005; **66**: 64–74.

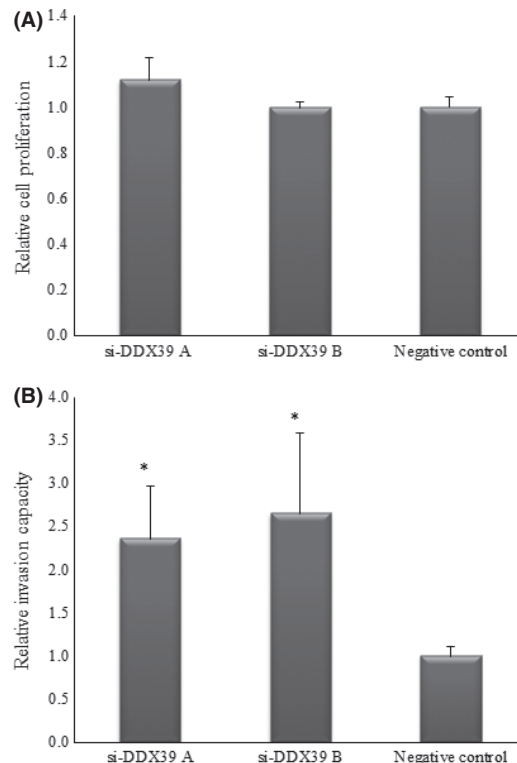


Fig. 5. siRNA knockdown effects on cell proliferation and invasion ability using T24 bladder cancer cells. (A) No significant difference in cell proliferation was found between si-DDX39 transfected cells and control cells. (B) Cells transfected with si-DDX39 A and si-DDX39 B gained significantly higher invasion ability compared to control cells. * $P < 0.05$.

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Disclosure Statement

The authors have no conflict of interest.

- atenolol using iTRAQ-coupled two-dimensional LC-MS/MS. *Mol Cell Proteomics* 2008; **7**: 1007–18.
- 16 Palou J, Algaba F, Vera I, Rodriguez O, Villavicencio H, Sanchez-Carbayo M. Protein expression patterns of ezrin are predictors of progression in T1G3 bladder tumours treated with nonmaintenance bacillus Calmette–Guerin. *Eur Urol* 2009; **56**: 829–36.
 - 17 Tsui KH, Juang HH, Lee TH, Chang PL, Chen CL, Yung BY. Association of nucleophosmin/B23 with bladder cancer recurrence based on immunohistochemical assessment in clinical samples. *Acta Pharmacol Sin* 2008; **29**: 364–70.
 - 18 Tzai TS, Tsai YS, Shiau AL, Wu CL, Shieh GS, Tsai HT. Urine prothymosin-alpha as novel tumor marker for detection and follow-up of bladder cancer. *Urology* 2006; **67**: 294–9.
 - 19 Higgins JP, Kaygusuz G, Wang L *et al*. Placental S100 (S100P) and GATA3: markers for transitional epithelium and urothelial carcinoma discovered by complementary DNA microarray. *Am J Surg Pathol* 2007; **31**: 673–80.
 - 20 Pryor A, Tung L, Yang Z, Kapadia F, Chang TH, Johnson LF. Growth-regulated expression and G0-specific turnover of the mRNA that encodes URH49, a mammalian DEXH/D box protein that is highly related to the mRNA export protein UAP56. *Nucleic Acids Res* 2004; **32**: 1857–65.
 - 21 Sugiura T, Sakurai K, Nagano Y. Intracellular characterization of DDX39, a novel growth-associated RNA helicase. *Exp Cell Res* 2007; **313**: 782–90.
 - 22 Abdelhaleem M. Over-expression of RNA helicases in cancer. *Anticancer Res* 2004; **24**: 3951–3.
 - 23 Sugiura T, Nagano Y, Noguchi Y. DDX39, upregulated in lung squamous cell cancer, displays RNA helicase activities and promotes cancer cell growth. *Cancer Biol Ther* 2007; **6**: 957–64.
 - 24 Lipponen PK, Eskelinen MJ. Reduced expression of E-cadherin is related to invasive disease and frequent recurrence in bladder cancer. *J Cancer Res Clin Oncol* 1995; **121**: 303–8.
 - 25 Kumar B, Koul S, Petersen J *et al*. p38 mitogen-activated protein kinase-driven MAPKAPK2 regulates invasion of bladder cancer by modulation of MMP-2 and MMP-9 activity. *Cancer Res* 2010; **70**: 832–41.
 - 26 Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Bohle A, Palou-Redorta J. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder. *Eur Urol* 2008; **54**: 303–14.
 - 27 Sakano S, Matsuyama H, Takai K *et al*. Risk group stratification to predict recurrence after transurethral resection in Japanese patients with stage Ta and T1 bladder tumours: validation study on the European Association of Urology guidelines. *BJU Int* 2011; **107**: 1598–604.