Clinical significance of CD44 variant 9 expression as a prognostic indicator in bladder cancer

KEITA KOBAYASHI, HIROAKI MATSUMOTO, HIDEYASU MATSUYAMA, NAKANORI FUJII, RYO INOUE, YOSHIAKI YAMAMOTO and KAZUHIRO NAGAO

Department of Urology, Graduate School of Medicine, Yamaguchi University, Ube, Yamaguchi 755-8505, Japan

Received March 8, 2016; Accepted June 30, 2016

DOI: 10.3892/or.2016.5061

Abstract. CD44, a major surface receptor for hyaluronic acid, has multiple isoforms and represents a major cancer stem cell marker for various epithelial tumors. CD44 variant 9 (CD44v9) was correlated with recurrence and metastasis in gastric and colon cancer. We examined its role in invasion and as a biomarker for the basal muscle invasive molecular subtype showing worse prognosis, and for tumor progression in high risk (pT1/high grade) non-muscle invasive bladder cancers (NMIBCs). CD44v9, cytokeratin 5/6 (CK5/6), and cytokeratin 20 (CK20) expression was evaluated by immunohistochemistry in 98 pathologically confirmed specimens (36 muscle and 62 high-risk non-muscle) and correlated to clinical outcome. *In vitro* analysis was performed using two human bladder cancer cell lines (HT1376 and 5637). The CD44v9 high-expressing group exhibited significantly lower progression-free and cancer-specific survival rates in both muscle (P=0.0349 and 0.0382, respectively) and non-muscle (P=0.0002 and 0.0079) invasive patients. CD44v9 expression was significantly correlated with CK5/6 (P<0.001), colocalizing at the muscle invasion front but distinctly separated from CK20 in non-muscle invasion. CD44 and CD44v9 siRNA knockdown demonstrated significantly lower Matrigel invasion ability and significantly shorter migration distance (all P<0.01). CD44 and CD44v9 knockdown increased E-cadherin and decreased N-cadherin, snail, and slug epithelial-mesenchymal transition marker protein expression. Thus, higher CD44v9 expression was associated with worse prognosis, likely impacting invasion and migration via the epithelial-mesenchymal transition. Together, these findings suggest that CD44v9 expression might be a useful predictive biomarker in basal-type muscle and high-risk NMIBC.

Correspondence to: Professor Hideyasu Matsuyama, Department of Urology, Graduate School of Medicine, Yamaguchi University, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: hidde@yamaguchi-u.ac.jp

Key words: bladder cancer, CD44 variant 9, invasion, epithelial-mesenchymal transition, progression-free survival

Introduction

Bladder urothelial carcinoma (UC) ranks as one of the most common urological malignancies worldwide. Approximately 70% of patients with bladder UC present with non-muscle invasive bladder cancer (NMIBC). Of these, ~20% subsequently develop muscle invasion associated with a strong propensity toward lethal metastases (1,2). Muscle-invasive bladder cancer (MIBC) represents a worse prognosis, with a 5-year survival of ~50% even after curative surgery (3). In particular, patients with pathological T1 (invading to the lamina propria of the bladder) and/or a higher tumor grade ('high-risk' NMIBC) are at high risk of developing MIBC.

CD44 is a major surface receptor for hyaluronate that has been shown to be relevant to several physiological processes including leukocyte homing and activation, intercellular adhesion and cell-matrix adhesion, and cell migration, as well as tumor cell invasion and metastasis (4-6). CD44 has multiple isoforms generated through alternative splicing of 10 variant exons, and has been identified as one of the major cancer stem cell markers for various epithelial tumors (7). Recent studies have revealed that the interaction of CD44 variants (CD44v) with the cystine transporter subunit xCT stabilizes cancer cells and provides a defense against reactive oxygen species (8).

In bladder UC, immunohistochemical (IHC) expression of both standard CD44 and the CD44 variant 6 (CD44v6) isoform is significantly reduced in relation to the pathological stage (9). Thus, the expression of CD44 and its variant CD44v6 might have an independent prognostic value in predicting a prolonged survival in invasive bladder cancer; however, these findings are still controversial (10). In addition, CD44 variant 9 (CD44v9) has been reported as a cancer stem cell marker as well as a prognostic marker in colorectal and early gastric cancers (11,12). Furthermore, recent studies have identified two types of molecular classifications of MIBC, termed luminal and basal types, wherein the latter demonstrates the worse prognoses (13).

The aim of this study was to examine whether CD44v9 expression is relevant to the new molecular classification of MIBC, in particular to the basal type, and to explore the clinical significance and mechanism of CD44v9 function in tumor progression. To our knowledge this is the first study to show the histopathological location of basal subtype marker expression and its clinical impact in high-risk NMIBC.

Materials and methods

Patient samples. Written informed consent was obtained from all patients to use their sample and the Institutional Review Board at Yamaguchi University Hospital approved this study (approval no. 17). Tumor specimens were obtained from 36 patients with MIBC treated with radical cystectomy with standard lymphadenectomy and from 62 patients with NMIBC treated with transurethral resection (TUR-BT) and diagnosed as high-grade pT1 (high-risk NMIBC) in Yamaguchi University Hospital between January 2001 and December 2012. All specimens were diagnosed as UC by the pathologist. Details of patient backgrounds are listed in Table I.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue specimens were used for IHC staining. For each sample, 5 μm-thick sections were deparaffinized in xylene, dehydrated in ethanol, and incubated in 0.3% hydrogen peroxide solution in methanol for 10 min. The sections were then microwaved in 0.01 M citrate-buffered solution (pH 6.0) for 15 min and covered in blocking solution (IMMUNO SHOT; Cosmo Bio Co., Ltd., Tokyo, Japan) for 30 min. After addition of a blocking solution, primary antibodies [anti-CD44v9 (1/500 dilution, kindly provided by Professor Saya, Keio University, Tokyo, Japan), anti-cytokeratin 5/6 (CK5/6) (1/200 dilution), or anti-cytokeratin 20 (CK20) (1/100 dilution) (both from Dako, Kyoto, Japan)] were added according to manufacturer's instructions, followed by incubation with the respective secondary antibody (Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature. Double IHC studies were performed on paraffin-embedded sections using double labeling with CD44v9 and CK5/6 or CK20 by diaminobenzidine and fast red. To evaluate the IHC, the H-score was used in this study. Briefly, >500 tumor cells were counted with different three fields of views in each case, and the H-score was subsequently calculated by multiplying the percentage of the positive cells by the intensity (strongly stained, 3x; moderately stained, 2x; and weakly stained, 1x), yielding a possible range of 0-300 (14,15). Paraffin-embedded cell pellets were obtained by freezing aliquots of two human bladder cancer cell lines (HT1376, 5637) at -80˚C followed by fixation in 10% formalin and subsequent processing for use in IHC staining as described (16).

Cell culture. The human bladder cancer cell lines HT1376 and 5637 and the human breast cancer cell line MDA-MB-468 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The MDA-MB-468 cell line was selected based on previous reports of elevated CD44v9 expression. Cells were routinely cultured in DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Biological Industries, Cromwell, CT, USA) and antibiotic antimycotic solution (Sigma-Aldrich, Tokyo, Japan) in a 5% CO_2 atmosphere at 37˚C.

siRNA knockdown of CD44 and CD44v9. CD44, CD44v9, and control siRNAs were obtained from Thermo Fisher Scientific, Inc. siRNA sequences were as follows: CD44

Table I. Clinicopathological characteristics of MIBC patients treated with radical cystectomy and high-risk NMIBC patients treated with TUR-BT.

A, Characteristics of MIBC cases (n=36)

Characteristics	n (%)
Age in years, median (range)	63 (42-79)
Gender	
Male	32 (89)
Female	4 (11)
Clinical T category	
cTis	1 (3)
cT1	3 (8)
cT2	8 (22)
cT3	16 (44)
cT4	8 (22)
Pathological T category	
pTa	2 (6)
pT1	8 (22)
pT2	9 (25)
pT3	10 (28)
pT4	7 (19)
Grade	
G1	1 (3)
G2	9 (25)
G3	23 (64)
Gx	3 (8)
Histological type	
UC	29 (81)
SCC	2 (6)
Other	5 (13)
LVI	
Negative	16 (44)
Positive	16 (44)
Unidentified	4 (12)
Neoadjuvant therapy	
No	21 (58)
Yes	15 (42)
Adjuvant therapy	
No	24 (67)
Yes	12 (33)

B, Characteristics of high-risk NMIBC cases (n=62)

Characteristics	n (%)
Age in years, median (range)	71 (51-88)
Gender	
Male	51 (82)
Female	11 (18)
Primary/recurrence	
Primary	55 (89)
Recurrence	7 (11)

Table I. Continued.

Histological type	
UC	55 (89)
UC + SCC	3 (5)
UC + AC	4 (6)
LVI	
Negative	31 (50)
Positive	5 (8)
Unidentified	26 (42)
CIS component	
No	26 (42)
Yes	36 (58)
2nd TUR-BT	
No	38 (61)
Yes	24 (39)

MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; UC, urothelial carcinoma; SCC, squamous cell carcinoma; LVI, lymphovascular invasion; AC, adenocarcinoma; CIS, carcinoma *in situ*.

siRNA sense, 5'-UAU UCC ACG UGG AGA AAA Att-3' and antisense, 5'-UUU UUC UCC ACG UGG AAU Aca-3'; CD44v9 siRNA sense, 5'-CUA CUU UAC UGG AAG GUU Att-3' and antisense, 5'-UAA CCU UCC AGU AAA GUA Gtt-3'. High GC duplex was used as the negative control siRNA. Each cell line was transiently transfected with siRNA using Lipofectamine RNAi MAX (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection, cells were incubated at 37˚C in a CO_2 incubator for 48 h. Quantitative evaluation of mRNA and protein expression was verified by western blotting and RT-PCR.

RT-PCR. Total cellular RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.). A total of 1 mg RNA was subjected to cDNA synthesis using the Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. PCR reactions were carried out in the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc.) using SYBR-Green Supermix (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The primer sequences were as follows: *CD44* forward, 5'-CCG CTA TGT CCA GAA AGG A-3' and reverse, 5'-CTG TCT GTG CTG TCG GTG AT-3'; *CD44 standard isoform* forward, 5'-AAA GGA GCA GCA CTT CAG GA-3' and reverse, 5'-TGT GTC TTG GTC TCT GGT AGC-3'; *CD44v9* forward, 5'-GGC TTG GAA GAA GAT AAA GAC C-3' and reverse, 5'-TGC TTG ATG TCA GAG TAG AAG TTG-3'; and β-actin forward, 5'-GCA TCC TCA CCC TGA AGT A-3' and reverse, 5'-TGT GGT GCC AGA TTT TCT CC-3'. The differences in gene expression levels between samples were measured using the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry. Aliquots of cells ($5x10^5$) were prepared in assay tubes. After the addition of 2% FBS and 0.02% sodium azide in PBS followed by a rinse and centrifugation, cells were incubated in diluted primary antibody (anti-CD44v9) for 30 min at 4˚C. Cells were then washed twice with 2% FBS and 0.02% sodium azide in PBS, and resuspended cells were incubated in phycoerythrin (PE)-labeled secondary antibody for 30 min at 4˚C in the dark. After being rinsed twice, the conjugated cells were analyzed according to the manufacturer's instructions (Cell Analyzer EC800; Sony, Tokyo, Japan).

Western blotting. Protein lysates from each tested cell sample were prepared in a radio-immunoprecipitation assay buffer with proteinase inhibitors. Each lysate sample (30 μg) was separated by SDS-PAGE, and electro-transferred to a PVDF membrane. Following blocking in 5% non-fat milk or 5% BSA, these membranes were incubated with each primary antibody overnight at 4˚C. After being washed in TBS with 0.05% Tween-20 (TBST), the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After subsequent washing with TBST, membrane signals were detected using an ECL detection system (ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc., Berkeley, CA, USA). Primary antibodies were used at the following dilutions: anti-CD44 (1/1,000 dilution) and anti-β-actin (1/1,000 dilution), both from Abcam (Cambridge, UK); and anti-E-cadherin (1/1,000 dilution); anti-N-cadherin (1/500 dilution); anti-snail (1/500 dilution); and anti-slug (1/500 dilution), from Santa Cruz Biotechnology (Dallas, TX, USA). β-actin was used for protein band normalization.

Cell invasion assay. Equal numbers of transfected and control cells ($1x10^5$) in DMEM without FBS were added to the upper insert invasion chambers with Matrigel (BD BioCoat Matrigel Invasion Chamber, 12 wells, 8 μm; Becton-Dickinson, Bedford, MA, USA) and the control chambers without Matrigel. DMEM supplemented with 10% FBS was added to the lower compartment, and the chambers were incubated at 37˚C for 48 h. After the incubation period, cells from the upper surface of the filter were wiped off with a cotton swab. The lower surface of the filter was stained with Diff-Quik (Dade Behring AG, Düdingen, Switzerland). The number of cells having migrated to the bottom of the chamber was counted from five randomly selected fields using a light microscope. The mean number of cells was calculated per field. We evaluated the invasion capacity using the ratio between the number of cells in the invasion chamber and the number of cells in the control chamber. Two sets of experiments were carried out, each in triplicate.

Wound healing assay. The effect of lithium on cell migratory activity was examined using a scratch-wound assay. Each set of transfected and control cells was seeded in a 12-well chamber in DMEM with 10% FBS and grown until confluency. One linear scar was drawn in the monolayer with a 200-μl tip (width, 500 μm). Wound closure (cell migration) was observed under a phase-contrast microscope (x100 magnification; Olympus, Tokyo, Japan), and photographed when the wound was made and at designated times after wounding. Cells were counted within the initial and the remaining wound area and the wound closure was expressed as the percentage of migrating cells

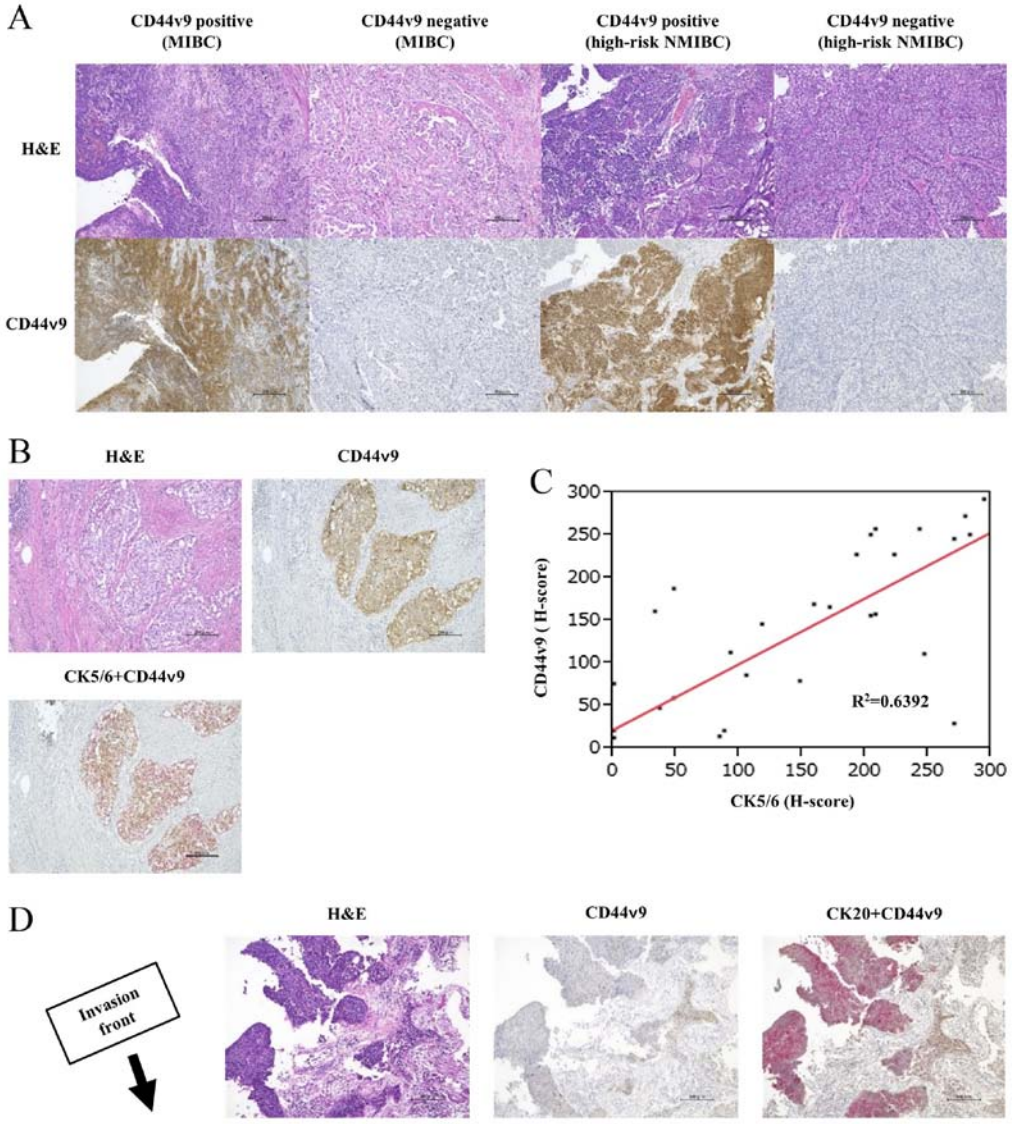

Figure 1. Histological (H&E) and IHC staining with (A) CD44v9, (B and C) CK5/6, CK20, and double staining of CD44v9 (brown) with CK5/6 (red) in MIBC, or (D) with CK20 (red) in high-risk NMIBC. (A) The higher and lower expression staining levels of CD44v9 in MIBC and high-risk NMIBC specimens. Note that CD44v9 is predominantly located either in the cytoplasm or in the cellular membrane. (B) A case with co-localization of positive CD44v9 with CK5/6. Here, the tumor had spread to the inguinal lymph nodes 7 months after radical cystectomy (pT2aN0M0), and the patient died of bladder cancer 18 months following the operation. (C) Significant correlation of the H-score between CD44v9 and CK5/6 (R^2=0.6392, Pearson's product-moment correlation coefficient). (D) A case wherein the positive expression site of CD44v9 is completely separated from that of CK20, and is localized to the invasive front of the tumor in high-risk NMIBC. Here, bladder recurrence occurred within 12 months after transurethral resection (pT1/high grade) and developed to MIBC 25 months following the operation. IHC, immunohistochemical; CD44v9, CD44 variant 9; CK5/6, cytokeratin 5/6; CK20, cytokeratin 20; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer.

compared to the control. Two sets of experiments were carried out, each in duplicate.

Statistical analysis. Statistical analysis was performed using JMP version 10 (SAS, Cary, NC, USA). Differences between the two groups were analyzed using Student's t-test and the Cox proportional hazard model was applied to determine prognostic factors for progression. Progression-free survival and cancer specific-free survival were calculated using the Kaplan-Meier method and compared by a log-rank test. The Pearson's product-moment correlation coefficient was applied to study the correlation of the H-score in IHC. $P<0.05$ was regarded as statistically significant. To investigate the relation of IHC CD44v9 expression status to patient outcome, a receiver operating characteristic (ROC) curve was applied to determine the optimal cut-off value to discriminate cancer-specific death. H-scores of 153 and 170 were set as the cut-offs in MIBC [area under the curve (AUC) =0.6428] and high-risk NMIBC (AUC = 0.6354), respectively.

Results

Correlation of CD44v9 overexpression to patient outcome. The CD44v9 expression level was evaluated by IHC in 36 MIBC and 62 high-risk NMIBC tissues. Fig. 1A shows the representative pictures of higher and lower expression staining in MIBC and high-risk NMIBC specimens, respectively. Table I shows the clinicopathological characteristics of MIBC

Table II. The relationship between the expression of CD44v9 and clinicopathological prognosis factor for progression in MIBC and high-risk NMIBC.

A, MIBC (n=36)

Characteristics	CD44v9 negative (n=25) n (%)	CD44v9 positive (n=11) n (%)
Clinical T category		
<3	9 (36)	3 (27)
≥3	16 (64)	8 (73)
Pathological T category		
<3	14 (56)	5 (45)
≥3	11 (44)	6 (55)
Lymph node metastasis		
Negative	23 (92)	9 (82)
Positive	2 (8)	2 (18)
LVI		
Negative	13 (52)	3 (27)
Positive	9 (36)	7 (64)
Unidentified	3 (12)	1 (9)

B, High-risk NMIBC (n=62)

Characteristics	CD44v9 negative (n=38) n (%)	CD44v9 positive (n=24) n (%)
No. of tumors		
Single	12 (32)	4 (17)
Multiple	26 (68)	20 (83)
Tumor size (cm)		
<3	30 (79)	19 (79)
≥3	8 (21)	5 (21)
Primary/recurrence		
Primary	33 (87)	21 (87)
Recurrence	5 (13)	3 (13)
CIS component		
No	13 (34)	13 (54)
Yes	25 (66)	11 (46)

CD44v9, CD44 variant 9; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; LVI, lymphovascular invasion; CIS, carcinoma *in situ*.

and high-risk NMIBC patients with a median follow-up period of 39 months (range, 3-104 months) and 50 months (range, 18-125 months), respectively. In MIBC, 25 specimens (69.4%) showed lower CD44v9 expression (H-score <153), whereas 11 specimens (30.6%) showed higher expression. In high-risk NMIBC, 38 specimens (61.2%) showed lower CD44v9 expression (H-score <170), whereas 24 specimens (38.8%) showed higher expression. Table II shows the prognosis factors in CD44v9-positive and -negative groups. In MIBC, the positive rate of lymphovascular invasion and lymph node metastasis were higher in CD44v9-positive group than in negative group (64 vs. 36%, 18 vs. 8%) (Table IIA). In univariate and multivariate analysis, the expression of CD44v9 and recurrent tumor were significant predictors for progression-free survival in high-risk NMIBC (Table III; both P<0.01). Patients with higher CD44v9 expression exhibited significantly lower progression-free survival and cancer-specific survival than those with lower CD44v9 expression in MIBC (Fig. 2A and B; both P<0.05) as well as in high-risk NMIBC (Fig. 2C and D; P<0.01, P<0.05).

Colocalization of CD44v9 with CK5/6 expression and specific localization of CD44v9-positive cells in the invasive front of high-risk NMIBC samples. We compared the expression site of CK5/6, a basal-type marker, and CK20, a luminal-type marker, to that of CD44v9 using IHC. Fig. 1B depicts a representative MIBC sample showing the corresponding sites of expression of CD44v9 and CK5/6. In MIBC, the H-score of CD44v9 expression was significantly correlated with that of CK5/6 (Fig. 1C; R2=0.6329, P<0.001). In high-risk NMIBC, the expression sites of CD44v9 and CK5/6 were colocalized in the invasive front of the tumor, which is defined as the deepest invasion site in this cancer. In contrast, the expression sites of CK20 were localized to the superficial tissue, and distinctly separated from the expression site of CD44v9 in high-risk NMIBC (Fig. 1D).

Effect of siRNA-induced knockdown of CD44v9 in 5637 and HT1376 cell lines. CD44v9 expression was evaluated by IHC (Fig. 3A) and flow cytometry (Fig. 3B) and western blotting (Fig. 3C) using the 5637 and HT1376 cell lines. The two cell lines were positive for both CK5/6 and CD44v9/CD44 expressions, while being negative for CK20 in IHC (data not shown). Fig. 3D shows the inhibitory effect of CD44 and CD44v9 siRNA on protein and mRNA expression by western blotting and RT-PCR, respectively.

Effect of siRNA-induced knockdown of CD44v9 on cell migration and invasion ability in the 5637 and HT1376 cell lines. We examined the cell migration and invasion ability associated with the suppression of CD44v9 expression. Initially, we measured the invasion ability of HT1376 and 5637 cells after transient transfection with scrambled or with CD44 and CD44v9 siRNA using a Matrigel invasion assay. The invasion ability of the cells transfected with CD44 and CD44v9 siRNA was significantly lower than that of cells transfected with scrambled siRNA (Fig. 4A and B). Secondly, we measured the migration ability of CD44 and CD44v9 knockdown cells using a wound healing assay. CD44/CD44v9 knockdown cells showed significantly decreased migration ability as compared to scrambled siRNA transfected cells (Fig. 4C and D). No significant difference in the proliferation ability or resistance to cisplatin was observed between the cells transfected with CD44/CD44v9 siRNA and those transfected with control siRNA (data not shown).

Correlation of CD44v9 expression with EMT. We evaluated the correlation of CD44v9 to the EMT markers E-cadherin, N-cadherin, snail, and slug by western blotting (Fig. 5). The

Table III. Univariate and multivariate analysis of prognosis factors associated with progression-free survival in high-risk NMIBC.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Primary or recurrence				
Primary vs. recurrence	0.25 (0.09-0.80)	0.022	0.19 (0.06-0.63)	0.009
No. of tumors				
Single vs. multiple	0.53 (0.12-1.61)	0.287		
Tumor size (cm)				
<3 vs. ≥3	0.85 (0.30-3.03)	0.787		
CIS component				
No vs. Yes	0.83 (0.28-2.20)	0.725		
Expression of CD44v9				
Negative vs. positive	0.18 (0.05-0.49)	0.007	0.15 (0.05-0.43)	0.003

NMIBC, non-muscle invasive bladder cancer; HR, hazard ratio; CI, confidence interval; CIS, carcinoma *in situ*; CD44v9, CD44 variant 9.

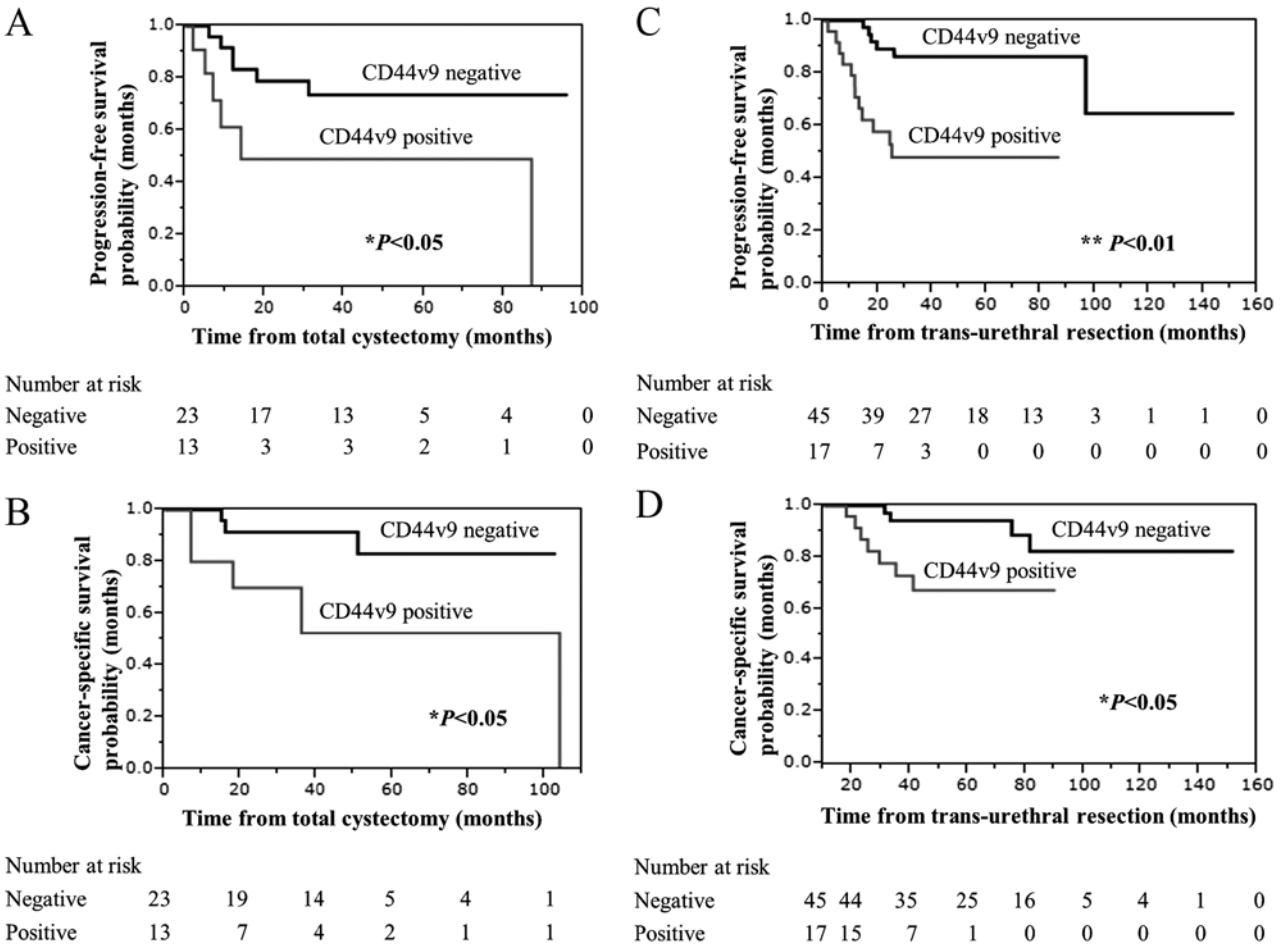

Figure 2. Kaplan-Meier estimates of (A) progression-free survival and (B) cancer-specific survival in MIBC patients, and (C) progression-free survival and (D) cancer-specific survival in high-risk NMIBC patients. Patients with higher expression of CD44v9 (gray line) exhibited significantly poorer prognosis than those with lower expression (black line) in both MIBC and high-risk NMIBC (*P<0.05; **P<0.01). MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; CD44v9, CD44 variant 9.

cells transfected with CD44 and CD44v9 siRNA showed increased expression of E-cadherin and decreased expression of N-cadherin, snail, and slug compared with those transfected with the scrambled siRNA.

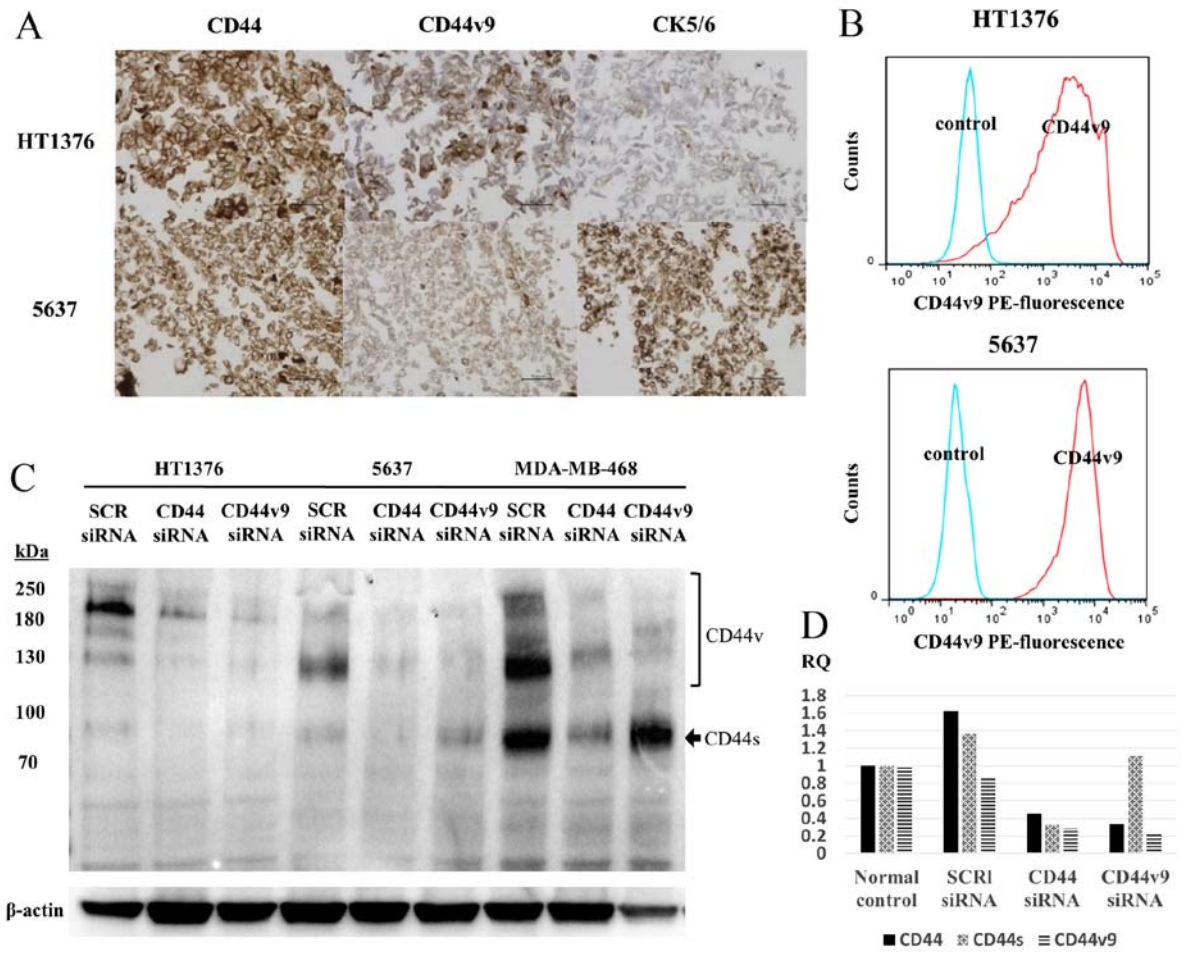

Figure 3. (A) IHC staining of CD44, CD44v9, and CK5/6 and (B) flow cytometry analysis with an anti-CD44v9 antibody and PE-labeled anti-rat IgG antibody in the human bladder cancer cell lines, HT1376 and 5637. (C) Western blotting of CD44v9 expression after CD44 and CD44v9 siRNA transfection in HT1376, 5637, and MDA-MB-468 cells and (D) quantitative comparison of CD44v9 expression between control and CD44v9 siRNA knockdown by RT-PCR. (B) Cells expressing CD44v9 (AUC of the red line) are predominant in both HT1376 and 5637 cell lines in flow cytometry analysis. The MDA-MB-468 cell line, a CD44v9-positive breast cancer cell line, was used as a CD44v9-positive control. (D) The inhibition of CD44v9 was determined using RT-PCR in HT1376. IHC, immunohistochemical; CD44v9, CD44 variant 9; CK5/6, cytokeratin 5/6; PE, phycoerythrin; AUC, area under the curve.

Discussion

As patients with MIBC and high-risk NMIBC show poor prognosis, the identification of the molecular markers for these conditions is pivotal to improve the clinical management of these UC patients. Recently, CD44v9 has been reported as a cancer stem cell marker as well as a prognostic marker in colorectal and early gastric cancers (11,12). In advanced head and neck cancer, high CD44v9 expression was associated with a significantly worse prognosis than was low expression (17). In this study, we demonstrated that the expression of CD44v9 is closely associated with tumor progression and cancer-associated death in MIBC as well as in high-risk NMIBC via the possible mechanism of increasing the properties of tumor invasion and migration and enhancing the EMT in human bladder cancer.

Miyake *et al* reported that an elevated ratio of the CD44v8-10 to CD44s ratio demonstrated a shorter disease-free survival than did a normal ratio in patients with UC (17). In our study, the patients with higher CD44v9 expression exhibited shorter progression-free and cancer-specific survival in MIBC and high-risk NMIBC, respectively. Our results are in good agreement with the previous report that CD44v8-10 overexpression in human bladder cancer cells was associated with the augmentation of malignant progression (18).

Recent studies have provided evidence that MIBC can be classified as two major basal and luminal subtypes that are similar to the intrinsic subtypes of breast cancer (13,19,20). The basal subtype is characterized by EMT, and shows enrichment with squamous features and better chemosensitivity than the luminal subtype. The observed colocalization of CD44v9 with CK5/6 suggests that CD44v9 is a potential biomarker for the basal subtype in UC. Although CK20, a luminal subtype marker, was also expressed in most high-risk NMIBC samples, the expression site was predominantly localized to the surface of the tumor with a distinct separation from the site of CD44v9 expression. Notably, the highest site of CD44v9 expression was located at the invasion front (submucosal layer) of the tumors with shorter progression-free survival and cancer-specific survival within high-risk NMIBC. Our results might be further supported by the report that tumor cells maintain an epithelial phenotype with high CD44v expression at the invasive front (21). The decreased invasion as well as migration ability pursuant to CD44v9 knockdown might explain the functional

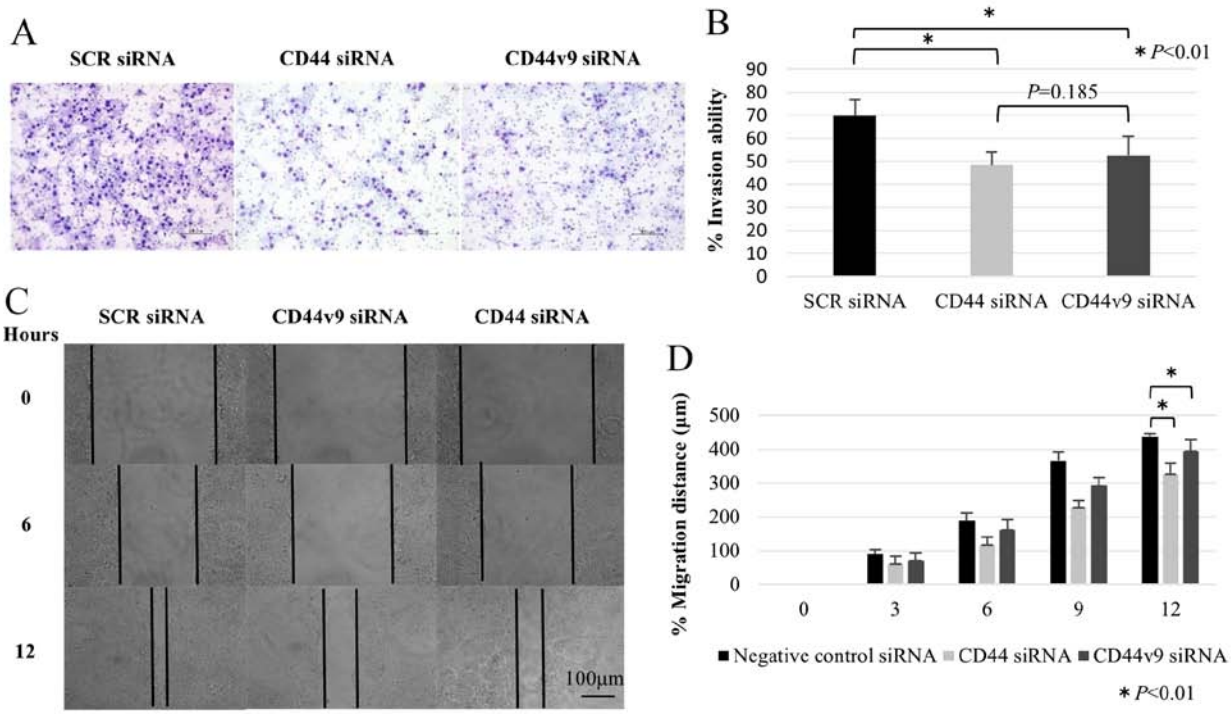

Figure 4. Representative images showing the inhibition of invasion and migration ability by (A and C) CD44 and CD44v9 knockdown and the quantitative comparison of CD44 and CD44v9 siRNA to control siRNA in (B) HT1376 and (D) 5637 cells. Invasion ability was significantly decreased by CD44 and CD44v9 knockdown (P<0.01) in HT1376 cells. Similar results were obtained in 5637 cells following CD44 and CD44v9 knockdown (*P<0.01) (data not shown). Migration ability was significantly decreased by CD44 and CD44v9 knockdown (P<0.01 and P<0.05, respectively) after 12 h following wounding in 5637 cells. Similar results were obtained in HT1376 cells after 48 h (both P<0.01) (data not shown). CD44v9, CD44 variant 9.

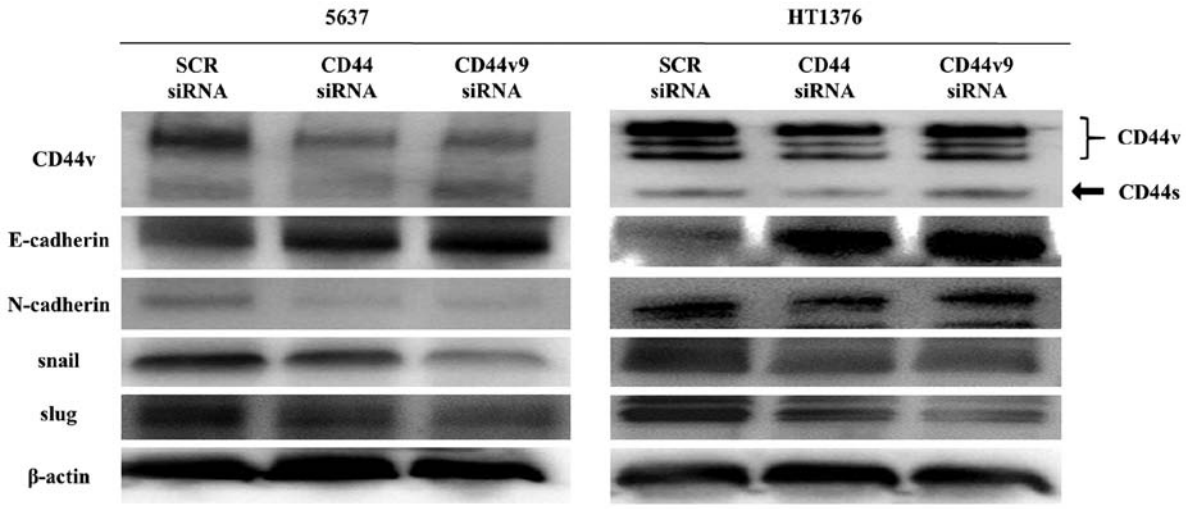

Figure 5. Western blotting of the correlation between CD44v9 and the EMT in 5637 and HT1376 cells. A switch in cadherin expression (E-cadherin, restoration of expression; N-cadherin, decreased expression) was noted, as was the downregulation of representative EMT marker molecules (snail, slug) following CD44 and CD44v9 knockdown. CD44s is the CD44 standard isoform. CD44v9, CD44 variant 9; EMT, epithelial-mesenchymal transition.

role of CD44v9 that confers an increased malignant phenotype on the tumor at the invasion front, leading to the development of tumor progression in high-risk NMIBC. Based on our results and past reports, we propose that CD44v9 expression might therefore serve as a clinically useful prognostic marker for oncological outcome as well as a good marker for defining basal subtype in high-risk NMIBC.

CD44v8-10, just as CD44v9, and CD44v6 have been shown to enhance the metastatic potential of colon cancer and melanoma cells, respectively. CD44v6 interacts with c-Met, a receptor tyrosine kinase that binds hepatocyte growth factor, and thereby increases the potential of melanoma cells to migrate to the brain. CD44v9 expression has been shown to rely on the activity of the cystine transporter subunit xCT for the control of the cell's redox status (22). Therefore, CD44v9-positive tumor cells have an ability to suppress the production of reactive oxygen species, resulting in therapeutic resistance, tumor recurrence and metastasis (8,23,24). CD44v is predominantly

expressed in epithelial cancer cells, whereas CD44s is mainly expressed in mesenchymal cancer cells. CD44 splicing is regulated by ESRP1, redox stress-induced Wnt activation, and TGF-β signaling. EMT-inducing transcriptional factors (e.g., snail and slug) enhance the invasive and migratory phenotype through the cadherin switch and a change in the alternative splicing of CD44 (25). The decreased expression of E-cadherin in association with N-cadherin increase and of decreased expression EMT markers by CD44v9 knockdown in our results are in good agreement with the previous report, and suggest the close association of CD44v9 expression with EMT in high-risk NMIBC.

A limitation of this study is that we could not distinguish the role of CD44v9 from that of CD44. Knockdown of CD44 had a similar effect on invasion and migration abilities as did that of CD44v9. These results imply the possibility that the other CD44v isoforms have similar ability. Nevertheless, the strong association of CD44v9 expression with patient outcome, and its specific localization to the invasion front demonstrate the clinical benefit that the expression status of CD44v9, as determined by IHC, might provide toward the prediction of poor prognosis or of tumor progression in patients with high-risk NMIBC.

In conclusion, this study demonstrated that CD44v9 is a possible prognostic marker for progression and cancer-specific death in MIBC and high-risk NMIBC. In particular, the IHC finding of the specific localization of CD44v9 to the invasion front using TUR-BT specimens might aid in selecting the patients who will likely progress to MIBC, and thereby offering such patients more aggressive treatment options such as radical cystectomy or cisplatin-based chemotherapy in patients in high-risk NMIBC. To confirm these results, more cohorts with larger patient numbers should be utilized.

Acknowledgements

We would like to thank Professor Hideyuki Saya for providing us with CD44v9 antibody and Professor Koji Tamada for help with technical guidance. We would like to thank Editage (www.editage.jp) for English language editing.

References

1. Nargund VH, Tanabalan CK and Kabir MN: Management of non-muscle-invasive (superficial) bladder cancer. Semin Oncol 39: 559-572, 2012.
2. Al Hussain TO and Akhtar M: Molecular basis of urinary bladder cancer. Adv Anat Pathol 20: 53-60, 2013.
3. May M, Helke C, Nitzke T, Vogler H and Hoschke B: Survival rates after radical cystectomy according to tumor stage of bladder carcinoma at first presentation. Urol Int 72: 103-111, 2004.
4. Günthert U, Hofmann M, Rudy W, Reber S, Zöller M, Haussmann I, Matzku S, Wenzel A, Ponta H and Herrlich P: A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65: 13-24, 1991.
5. Nagano O and Saya H: Mechanism and biological significance of CD44 cleavage. Cancer Sci 95: 930-935, 2004.
6. Ponta H, Sherman L and Herrlich PA: CD44: From adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4: 33-45, 2003.
7. Kimura Y, Goi T, Nakazawa T, Hirono Y, Katayama K, Urano T and Yamaguchi A: CD44variant exon 9 plays an important role in colon cancer initiating cells. Oncotarget 4: 785-791, 2013.
8. Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, Oshima M, Ikeda T, Asaba R, Yagi H, *et al*: CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. Cancer Cell 19: 387-400, 2011.
9. Hong RL, Pu YS, Hsieh TS, Chu JS and Lee WJ: Expressions of E-cadherin and exon v6-containing isoforms of CD44 and their prognostic values in human transitional cell carcinoma. J Urol 153: 2025-2028, 1995.
10. Lipponen P, Aaltoma S, Kosma VM, Ala-Opas M and Eskelinen M: Expression of CD44 standard and variant-v6 proteins in transitional cell bladder tumours and their relation to prognosis during a long-term follow-up. J Pathol 186: 157-164, 1998.
11. Hirata K, Suzuki H, Imaeda H, Matsuzaki J, Tsugawa H, Nagano O, Asakura K, Saya H and Hibi T: CD44 variant 9 expression in primary early gastric cancer as a predictive marker for recurrence. Br J Cancer 109: 379-386, 2013.
12. Yamaguchi A, Urano T, Goi T, Saito M, Takeuchi K, Hirose K, Nakagawara G, Shiku H and Furukawa K: Expression of a CD44 variant containing exons 8 to 10 is a useful independent factor for the prediction of prognosis in colorectal cancer patients. J Clin Oncol 14: 1122-1127, 1996.
13. Choi W, Porten S, Kim S, Willis D, Plimack ER, Hoffman-Censits J, Roth B, Cheng T, Tran M, Lee IL, *et al*: Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 25: 152-165, 2014.
14. Ishibashi H, Suzuki T, Suzuki S, Moriya T, Kaneko C, Takizawa T, Sunamori M, Handa M, Kondo T and Sasano H: Sex steroid hormone receptors in human thymoma. J Clin Endocrinol Metab 88: 2309-2317, 2003.
15. Pirker R, Pereira JR, von Pawel J, Krzakowski M, Ramlau R, Park K, de Marinis F, Eberhardt WE, Paz-Ares L, Störkel S, *et al*: EGFR expression as a predictor of survival for first-line chemotherapy plus cetuximab in patients with advanced non-small-cell lung cancer: Analysis of data from the phase 3 FLEX study. Lancet Oncol 13: 33-42, 2012.
16. Subik K, Lee JF, Baxter L, Strzepek T, Costello D, Crowley P, Xing L, Hung MC, Bonfiglio T, Hicks DG, *et al*: The expression patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by immunohistochemical analysis in breast cancer cell lines. Breast Cancer (Auckl) 4: 35-41, 2010.
17. Miyake H, Eto H, Arakawa S, Kamidono S and Hara I: Over expression of CD44V8-10 in urinary exfoliated cells as an independent prognostic predictor in patients with urothelial cancer. J Urol 167: 1282-1287, 2002.
18. Muramaki M, Miyake H, Kamidono S and Hara I: Over expression of CD44V8-10 in human bladder cancer cells decreases their interaction with hyaluronic acid and potentiates their malignant progression. J Urol 171: 426-430, 2004.
19. Damrauer JS, Hoadley KA, Chism DD, Fan C, Tiganelli CJ, Wobker SE, Yeh JJ, Milowsky MI, Iyer G, Parker JS, *et al*: Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc Natl Acad Sci USA 111: 3110-3115, 2014.
20. McConkey DJ, Choi W and Dinney CP: New insights into subtypes of invasive bladder cancer: Considerations of the clinician. Eur Urol 66: 609-610, 2014.
21. Yoshida GJ and Saya H: Inversed relationship between CD44 variant and c-Myc due to oxidative stress-induced canonical Wnt activation. Biochem Biophys Res Commun 443: 622-627, 2014.
22. Yoshikawa M, Tsuchihashi K, Ishimoto T, Yae T, Motohara T, Sugihara E, Onishi N, Masuko T, Yoshizawa K, Kawashiri S, *et al*: xCT inhibition depletes CD44v-expressing tumor cells that are resistant to EGFR-targeted therapy in head and neck squamous cell carcinoma. Cancer Res 73: 1855-1866, 2013.
23. Tsugawa H, Suzuki H, Saya H, Hatakeyama M, Hirayama T, Hirata K, Nagano O, Matsuzaki J and Hibi T: Reactive oxygen species-induced autophagic degradation of *Helicobacter pylori* CagA is specifically suppressed in cancer stem-like cells. Cell Host Microbe 12: 764-777, 2012.
24. Yae T, Tsuchihashi K, Ishimoto T, Motohara T, Yoshikawa M, Yoshida GJ, Wada T, Masuko T, Mogushi K, Tanaka H, *et al*: Alternative splicing of CD44 mRNA by ESRP1 enhances lung colonization of metastatic cancer cell. Nat Commun 3: 883, 2012.
25. Yoshida GJ and Saya H: The novel anti-tumor therapy targeting the 'functional' cancer stem cell markers. Clin Exp Pharmacol 4: 147, 2014.