CD44v6 expression is related to mesenchymal phenotype and poor prognosis in patients with colorectal cancer

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Abstract. CD44 standard isoform (CD44s) is a cancer stem cell marker in many tumors, and is one of the CD44 isoforms. CD44v6 has been reported to correlate with tumor progression and poor prognosis in colorectal cancer. However, the relevance of CD44s and CD44v6 to epithelial-mesenchymal transition (EMT) remains unclear. Immunohistochemistry was performed to investigate the clinical importance of CD44s and CD44v6 and their relevance to EMT in 113 patients with stage II/III colorectal cancer treated by curative resection. The relevance of CD44v6 knockdown to the phenotype of colon cancer cells was examined using small interfering RNA (siRNA) specific for CD44v6 *in vitro*. CD44v6 expression showed a significant inverse correlation with E-cadherin expression (P=0.0007) and a positive correlation with vimentin expression (P=0.0096). A multivariate analysis showed that high CD44v6 expression was an independent poor prognostic factor for disease-free survival (P=0.01, HR=3.05) and overall survival (P=0.025, HR=3.16). The clinical significance and the relevance of CD44s expression to EMT markers was noted to a lesser extent compared to CD44v6 expression. The knockdown of CD44v6 decreased vimentin expression, cell invasion and HGF-induced cell migration, but conferred only a slight effect on E-cadherin expression in colon cancer cells (HCT116 and LoVo). CD44v6 is related to poor outcome of patients with colorectal cancer via upregulation of the mesenchymal phenotype.

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Abbreviations: EMT, epithelial-mesenchymal transition; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction; DFS, disease-free survival; OS, overall survival; HGF, hepatocyte growth factor; TGF-β, transforming growth factor-β; TNFα, tumor necrosis factor α

Key words: CD44v6, colorectal cancer, epithelial-mesenchymal transition

Introduction

Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer-related death in the Western world (1,2). Patients with early stage disease generally have an excellent prognosis after curative resection, while the prognosis for patients with distant metastases remains poor (3). Research has demonstrated that epithelial-mesenchymal transition (EMT) plays a key role in the early process of the metastasis of cancer cells. EMT has been implicated in the development of invasive and metastatic tumor cells during tumor progression (4-6). This process involves the acquisition of the expression of mesenchymal molecules, such as vimentin and fibronectin, together with the loss of epithelial cell adhesion molecules such as E-cadherin (7).

CD44 is a cell adhesion molecule, which belong to a family of hyaluronan binding proteins. The smallest and most abundant isoform of CD44 is the so-called standard form (CD44s). The different isoforms are mainly generated by alternative splicing of 10 variant exons that account for sequences located in the extracellular part of the CD44 protein (8). Recently, CD44s has been considered to be one of the cancer stem cell markers in many solid tumors, including colorectal cancer (9,10). CD44s can contribute to the activation of stem cell regulatory genes and can be a target of these genes (11). However, clinically, the value of CD44v6 rather than CD44s has been reported by many investigators as an immunohistochemical prognostic markers in colorectal cancer. Increased expression of CD44v6 has been reported in lymph node metastases, linked to adverse prognosis independent of Dukes' stage and UICC stage (12-16).

HGF-mediated activation of the c-Met tyrosine kinase pathway induces the proliferation, motility, adhesion and invasion of colon cancer cells (17-21). The HGF/c-Met pathway can contribute to EMT, including malignant tumor progression (22,23). The activation of c-Met and the downstream signaling pathway to extracellular signal-regulated kinase (Erk) in response to HGF requires the presence of the CD44v6-containing isoform (24,25).

A recent study reported that the hyaluronan-CD44 interaction plays a key role in EMT-associated fibrotic disorder (26). With regard to cancer progression and metastasis, the relevance of CD44s and/or CD44v6 to EMT is still poorly understood.

In this study, we investigated whether CD44s and CD44v6 expression is associated with the EMT phenomenon in patients with colorectal cancer using immunohistochemistry and *in vitro* analysis.

Materials and methods

Patients and tissue samples. Among 166 patients with colorectal cancer who underwent curative surgery between 2000 and 2007 at the Department of Gastroenterological Surgery of the Kumamoto University Hospital, Kumamoto, Japan, we selected 113 patients diagnosed with stage II and III disease, according to the 7th edition of the UICC classification (27). None of the patients had undergone preoperative chemotherapy. Tissue specimens were collected from the patients after informed consent had been obtained, in accordance with the institutional guidelines of our hospital.

Cell lines and reagents. The human colon carcinoma cell line, LoVo, was purchased from Riken Bioresource Center (Osaka, Japan) and was cultured in Ham's F12 medium (Wako, Osaka, Japan). The HCT116 cell line was purchased from the ATCC (Manassas, VA, USA) and cultured in RPMI medium (Wako). All media were supplemented with 10% fetal bovine serum (Gibco, Tokyo, Japan), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cells were incubated at 37˚C in a humidified chamber supplemented with 5% CO_2. The anti-CD44s (clone, SFF-304) and CD44v6 (clone, VFF-18) antibodies were purchased from Bender MedSystems (Vienna, Austria). The antibodies against E-cadherin and fibronectin were purchased from BD-Biosciences (San Jose, CA, USA). The anti-vimentin antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-β-actin antibody was purchased from Cell Signaling Technology (Tokyo, Japan).

Immunohistochemical staining. The immunohistochemical procedure was performed as previously described (28). In brief, after deparaffinization and rehydration of 4-μm sections, the endogenous peroxidase activity of the specimens was blocked with a methanol solution containing 3% hydrogen peroxide for 10 min at room temperature. Heat-induced antigen retrieval by microwave pretreatment in citrate buffer solution at pH 6.0 (for E-cadherin) or pH 9.0 (for CD44s, CD44v6 and vimentin) for 5-20 min was performed. Samples were incubated with the primary antibodies overnight at 4˚C at the dilutions noted below. Anti-CD44s was used at a 1:100 dilution; anti-CD44v6 was used at 1:500; anti-E-cadherin was used at 1:1,200 and anti-vimentin was used at 1:50. A subsequent reaction was performed with the EnVision Plus detection system (Dako Co., Tokyo, Japan). A positive reaction was visualized with a diaminobenzidine solution, followed by counterstaining with Mayer's hematoxylin.

Evaluation and scoring. We randomly selected 5 fields within the tumor invasive front under high power magnification (x400) for evaluation. Each molecule expressed on cancer cells was quantified as a percentage of the total number of stained cells. For the expression of CD44s and CD44v6, we applied a three-grade scoring system of: i) strong, ii) moderate, or iii) weak/none. Strong staining was defined as staining in >25% of the tumor cells, moderate staining was indicated when <25% of the cells were stained, and staining in <10% of the tumor cells or an absence of staining was scored as weak or none (13,15). Both CD44s and CD44v6 were considered to be highly expressed when the staining was strong. For the expression of E-cadherin and vimentin, the median value of staining was determined to be the cut-off value. The immunostaining results were independently evaluated by two investigators who were blinded with respect to the clinical and histopathologic features.

Invasion assay. Cell invasion was assessed using the Matrigel invasion chamber (BD Biosciences, San Jose, CA, USA) as previously described (28). Cells ($1x10^5$/well) transfected with either negative-control siRNA (200 nM) or CD44v6 siRNA (200 nM) were plated on Transwell chambers precoated with Matrigel in a 24-well plate. After the cells were incubated for 24 h at 37˚C in a humidified incubator with 5% CO_2, the non-invading cells were removed with cotton swabs. The cells that had invaded through the membrane were fixed in 100% methanol and stained with toluidine blue. In five randomly selected fields, the number of invading cells was counted under a light microscope. Each experiment was performed in triplicate.

Wound healing assay. The migration activity was determined using the wound healing assay. A suspension of LoVo cells ($2.5x10^5$/well) was poured into each well using a 12-well plate. After 24 h of incubation, the cells were transfected with siRNA and grown until subconfluence. The confluent cell layer was scratched with a pipette tip, followed by medium replacement with or without 50 ng/ml recombinant HGF (R&D Systems, Minneapolis, MN, USA). The wound distances were measured and averaged from 5 points per wound area as a baseline width. After 24 h, the width of the mean wound distance was calculated. To evaluate the 'wound closure', five randomly selected points along each wound were marked, and the horizontal distance the migrating cells traveled into the wound was measured. The data are reported as the means ± SD.

RNA extraction, cDNA synthesis and quantitative RT-PCR assay. Total RNA was obtained from the cell lines using the RNeasy Mini kit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. cDNA was synthesized with the SuperScript III Transcriptor First Strand cDNA Synthesis system for RT-PCR (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was performed using a LightCycler 480 II system (Roche Diagnostics, Tokyo, Japan) as previously described (29). To perform qPCR, primers were designed using the Roche Webpage and the Universal Probe Library following the manufacturer's recommendations. The primers used were as follows: CD44v6 forward, 5'-AACAGCTACCCA GAAGGAACAG-3'; CD44v6 reverse, 5'-CTTTGGGTGTTT GGCGATA-3'; and universal probe #145; GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3'; GAPDH reverse, 5'-GC CCAATACGACCAAATCC-3'; and universal probe #60. For amplification, an initial denaturation at 95˚C for 10 min was followed by 15 sec at 95˚C, 15 sec at 60˚C, and 13 sec at 72˚C.

Table I. Expression levels of E-cadherin and vimentin compared to that of CD44v6.

Molecule	Total (n=113)	CD44v6 High (n=38)	CD44v6 Low (n=75)	P-value
E-cadherin				
High	55	10	45	0.0007
Low	58	28	30	
Vimentin				
High	55	25	30	0.0096
Low	58	13	45	

All experiments were performed two times to confirm their reproducibility.

Western blot analysis. For isolating the proteins, cells harvested in 6-well plates were washed once in PBS and lysed in lysis buffer [Tris-HCl (pH 7.4), 25 mmol/l; NaCl, 100 mmol/l; EDTA, 2 mmol/l; Triton X-100, 1%; with 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/l Na_3VO_4, 1 mmol/l phenylmethylsulfonyl fluoride]. Equal amounts of proteins were loaded onto 10% gels and were separated by SDS-PAGE. The resolved proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% low-fat dry milk in TBS-T [25 mM Tris (pH 7.4), 125 mM NaCl, 0.4% Tween 20] for 90 min at room temperature, followed by incubation with the primary antibody at 4˚C overnight. The primary antibodies for CD44v6 (1:1,000), E-cadherin (1:2,000), and vimentin (1:1,000) were the same as used for immunohistochemistry. Primary antibody for fibronectin was used at 1:1,000. The membranes were extensively washed and incubated with a 1:2,000 dilution of HRP-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The membranes were washed and visualized using the chemiluminescence detection reagent kit (ECL Plus; GE Healthcare Corp., Tokyo, Japan).

Small interfering RNA transfection. Two different sequences of small interfering RNA (siRNA) targeting human CD44v6 and negative control siRNA were purchased from Qiagen (Tokyo, Japan). The sequences of CD44v6 were as follows: CD44v6#1 sense, 5'-GGCAACUCCUAGUAGUACATT-3' and antisense, 5'-UGUACUACUAGGAGUUGCCTG-3'; CD44v6#2 sense, 5'-GAAGACUCCCAUUCGACAATT-3' and antisense, 5'-UUGUCGAAUGGGAGUCUUCTT-3'. The cells were transfected with the CD44v6-targeting siRNA or non-silencing siRNA using Lipofectamine 2000 reagent (Qiagen) according to the manufacturer's protocol.

Statistical analyses. Statistical analyses were performed using the StatView 5.0 software program (SAS Institute, Inc., Cary, NC, USA). The progression-free survival (PFS) and overall survival (OS) were calculated using the Kaplan-Meier method and the log-rank test was used to determine the statistical significance. A Cox proportional-hazards model was used to assess the risk ratio with simultaneous contribution from several covariates. Associations among discrete variables were assessed using the χ^2 test. Mean values were compared using the Student's t-test. We analyzed the data by an ANOVA, followed by a Tukey-Kramer post-hoc test to compare multiple samples. P-values <0.05 were considered to indicate a statistically significant result.

Table II. Correlation between the CD44v6 expression pattern and clinicopathological factors of the colorectal cancer patients.

Clinicopathological factors	Total (n=113)	CD44v6 High (n=38)	CD44v6 Low (n=75)	P-value
Age (years)[a]				
≤68	54	14	40	0.10
>68	59	24	35	
Gender				
Male	62	20	42	0.73
Female	51	18	33	
Location of primary tumor				
Colon	78	27	51	0.74
Rectum	35	11	24	
Histological type				
Well	68	18	50	0.048
Other	45	20	25	
Tumor size (mm)[a]				
≤50	54	16	38	0.39
>50	59	22	37	
Lymphatic invasion[b]				
Negative	90	28	62	0.26
Positive	23	10	13	
Venous invasion[b]				
Negative	49	19	30	0.31
Positive	64	19	45	
pT stage				
T1/T2	4	1	3	0.71
T3/T4	109	37	72	
pN stage				
N0	68	21	47	0.45
N1/N2	45	17	28	

[a]The cut-off value was defined as the median value. [b]Pathological vascular invasion. Well, well-differentiated adenocarcinoma.

Results

Relevance of CD44v6 and CD44s to EMT markers in patients with colorectal cancer. The expression of CD44s, CD44v6 and E-cadherin was observed on the membrane of cancer cells, while vimentin expression was observed in the cytoplasm. Although CD44s expression was noted in both

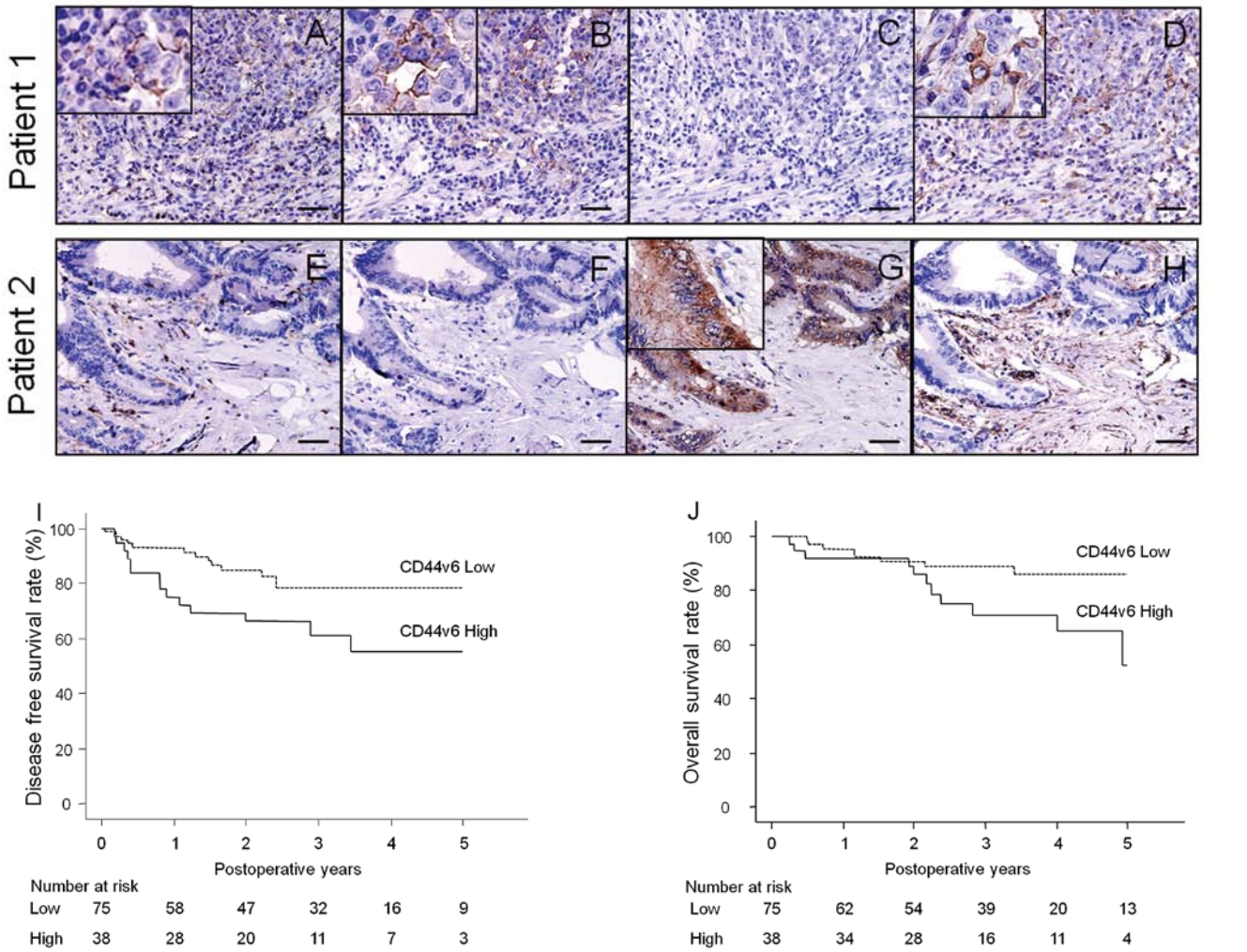

Figure 1. Clinical significance of CD44v6 and its relevance to EMT as evaluated by immunohistochemical analysis. Representative images of immunohistochemical staining using antibodies against CD44s (A and E), CD44v6 (B and F), E-cadherin (C and G), and vimentin (D and H) of specimens obtained from 2 patients with colorectal cancer. Patient 1 showed poorly differentiated cancer without E-cadherin expression. Patient 2 showed well-differentiated cancer with E-cadherin expression. Scale bar, 50 μm. Kaplan-Meier curves of the recurrence-free survival (P=0.010) (I) and overall survival (P=0.025) (J) of the patients according to CD44v6 expression.

cancer and stromal cells, such as fibroblasts and immune cells, CD44v6 expression was localized to the cancer cells. CD44v6 expression showed a significant inverse correlation with E-cadherin expression (P=0.0007) and a positive correlation with vimentin expression (P=0.0096) (Table I), and the results from two representative patients are shown in Fig. 1A-H. In contrast, CD44s expression showed an inverse correlation with vimentin expression (P=0.031), and no correlation with E-cadherin expression (data not shown). High CD44s expression was not associated with a poor prognosis (data not shown). Based on these results, we examined the role of CD44v6 in EMT of colon cancer.

Clinicopathological features of patients and the impact of CD44v6 expression on the prognosis of stage II or III colorectal cancer. Table II shows the association between CD44v6 expression and the clinicopathological features of the 113 patients. A high level of CD44v6 expression was inversely correlated with histological differentiation of the tumor (P=0.048). To examine the prognostic value of CD44v6 expression, univariate and multivariate analyses were carried out (Table III). High CD44v6 expression was found to be an independent poor prognostic factor in disease-free survival (DFS) and overall survival (OS) (data not shown). Kaplan-Meier curves of the DFS and OS determined based on CD44v6 expression are shown in Fig. 1I and J (P=0.03 and P=0.047, respectively).

Knockdown of CD44v6 results in the downregulation of mesenchymal markers. Since we hypothesized that CD44v6 has a role in the EMT phenomenon of colon cancer, we assessed whether CD44v6 knockdown impairs EMT in colon cancer cells. We transfected HCT116 and LoVo cells with two different siRNAs against CD44v6, and both reduced CD44v6 mRNA and protein expression compared with the control siRNA (Fig. 2A and B). We used siCD44v6#1 in the subsequent experiments. Knockdown of CD44v6 downregulated the protein expression of vimentin and fibronectin when compared with the control, while little change was noted in E-cadherin expression (Fig. 2B). Vimentin expression in HCT116 cells and fibronectin expression in LoVo cells were too small to compare.

Table III. Results of the univariate and multivariate analyses for disease-free survival.

		Univariate analysis			Multivariate analysis		
Clinicopathological factors	Total (n=113)	HR	95% CI	P-value	HR	95% CI	P-value
Age (years)[a]							
≤68	54	1.00	0.55-2.52	0.67	1.00	0.74-4.11	0.21
>68	59	1.18			1.74		
Gender							
Male	62	1.00	0.49-2.21	0.92	1.52	0.61-3.78	0.37
Female	51	1.04			1.00		
Location of primary tumor							
Colon	78	1.00	0.90-4.11	0.09	1.00	0.60-3.36	0.43
Rectum	35	1.92			1.41		
Tumor size (mm)[a]							
≤50	54	1.00	0.83-3.98	0.13	1.00	0.68-3.57	0.30
>50	59	1.82			1.56		
Histological type							
Well	68	1.27	0.57-2.82	0.56	3.03	1.09-8.46	0.03
Other	45	1.00			1.00		
Lymphatic invasion[b]							
Negative	23	1.00	0.97-4.81	0.06	1.00	0.98-8.98	0.05
Positive	90	2.15			2.97		
Venous invasion[b]							
Negative	64	1.00	0.63-3.04	0.41	1.00	0.44-2.72	0.85
Positive	49	1.39			1.09		
Invasion of primary tumor							
Negative	98	1.00	0.62-4.32	0.32	1.00	0.78-7.16	0.13
Positive	15	1.63			2.36		
Lymph node metastasis							
Negative	68	1.00	1.19-5.55	0.02	1.00	1.26-7.54	0.01
Positive	45	2.57			3.08		
CD44v6							
Low	75	1.00	1.11-5.04	0.03	1.00	1.31-7.07	0.01
High	38	2.37			3.05		

[a]The cut-off value was defined as the median value. [b]Pathological vascular invasion. HR, hazard ratio; CI, confidence interval. Significance was set at P<0.05. Well, well-differentiated adenocarcinoma.

Downregulation of CD44v6 decreases cancer cell invasion, migration and HGF-induced cell scattering. The Matrigel invasion assay revealed that the knockdown of CD44v6 decreased the invasive activity of HCT116 and LoVo cells (fold-changes were 0.34 and 0.50; P=0.0003 and 0.0001) (Fig. 2C). In addition, knockdown of CD44v6 inhibited the migration of HCT116 and LoVo cells (fold-changes, 0.54 and 0.34; P=0.0085 and 0.0094, respectively) (Fig. 2D). The knockdown of CD44v6 did not cause any marked change in cell proliferation in either cell line (data not shown).

Based on previous reports indicating that CD44v6 induces cell scattering through HGF-cMet signaling in certain cancer cell cultures (24,30), we examined whether CD44v6 mediates this phenomenon. HGF stimulation of the cancer cells for 48 h led to an increase in cell migration (4.4-fold, P<0.0001) and CD44v6 knockdown decreased the HGF-mediated cell migration (0.55-fold, P<0.05) (Fig. 2E) in HCT116 cells. A similar effect was noted in LoVo cells, but the difference was not significant (data not shown). Taken together, these results indicate that CD44v6 supports HGF-induced cell scattering.

Discussion

The present study demonstrated that expression of CD44v6, but not CD44s, was highly correlated with the downregulation of E-cadherin and upregulation of vimentin expression in human samples, and CD44v6 supports the mesenchymal phenotype, such as cellular invasion, migration, HGF-induced

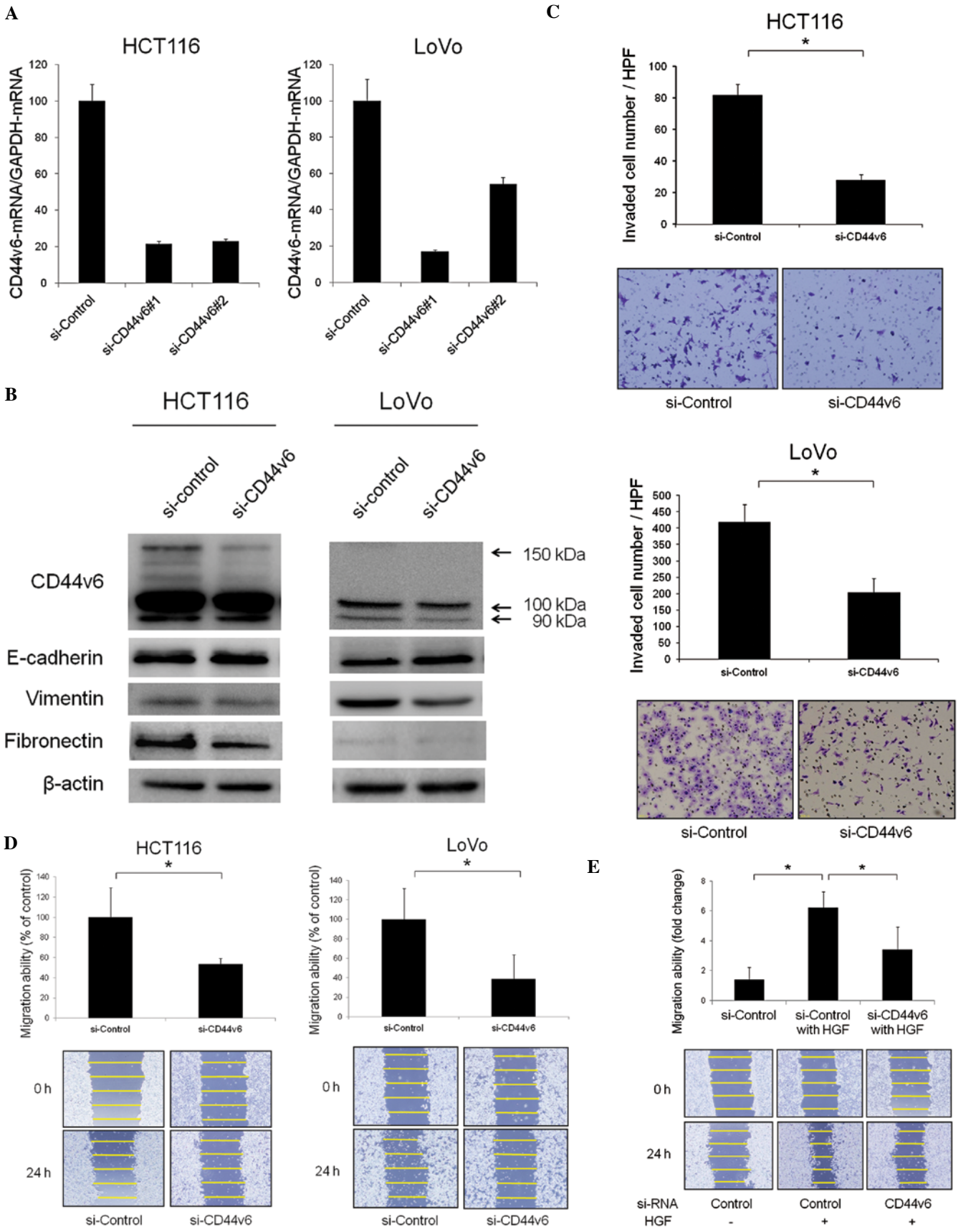

Figure 2. CD44v6 knockdown downregulates the mesenchymal phenotype. (A) Two different siRNAs downregulated the mRNA expression of CD44v6 in 2 different colon cancer cell lines in comparison to cells treated with the control siRNA. Bar, SD. (B) Western blot analysis showed that CD44v6 knockdown by CD44v6#1-siRNA reduced the vimentin expression in LoVo cells and fibronectin expression in HCT116 cells, but only slightly affected the expression of E-cadherin in both cell lines. (C) CD44v6 knockdown reduced the invasive ability of both cell lines. HCT116 and LoVo cells were treated with siRNA for 48 h and were then used for the invasion assay. (D) CD44v6 knockdown reduced the migratory ability of both cell lines. HCT116 and LoVo cells were treated with siRNA for 48 h and then were used for the migration assay. (E) The HGF-induced cellular migration was evaluated by the wound healing assay. LoVo cells were treated with si-Control or si-CD44v6 for 48 h, and were then stimulated with 50 ng/ml of HGF for 24 h ($^{*}P<0.05$).

Table IV. Summary of immunohistochemical-based studies for CD44v6 in colorectal cancer.

Authors (ref.)	Year of study	No. of patients	Stage	Clone no.	Cut-off	Effect of high CD44v6 expression on survival
Mulder *et al* (13)	1994	68	Dukes' A-D	VFF4, 7	(-) <10%, (+) 10-50%, (++) >50%	Adverse effect
Wielenga *et al* (15)	1993	70	Dukes' A-D	VFF4, 7	10%	Dukes' stage
Finke *et al* (12)	1995	102	I-IV	VFF7	(-) 0, (+) ≤20%, (+) 20-70%, (++) ≥70%	UICC stage
Koretz *et al* (43)	1995	180	Dukes' A-D	VFF7	10%	No effect
Gotley *et al* (42)	1996	109	Dukes' A-D	2F10, 2G9		Not done
Nanashima *et al* (39)	1999	113	Dukes' B-D, metastatic tumor	VFF7	(-), (+)	Favorable effect
Neumayer *et al* (44)	1999	81	Adenoma, carcinoma-in-adenoma, T1	VFF7	10%	Not done
Nanashima *et al* (38)	2001	62	Liver metastases	VFF7	10%	Favorable effect
Günther *et al* (41)	2002	116	I-III	2F10	(-) <10%, (+) 10-50%, (++) >50%	No effect
Köbel *et al* (16)	2004	145	I-IV	VFF7	10%	Adverse effect
Kuhn *et al* (45)	2007	170	I-IV	VFF18		No effect
Peng *et al* (14)	2008	179	II/III	VFF7	10%	Adverse effect
Zlobec *et al* (40)	2009	1,279	I-IV, metastatic tumor	VFF18	30%	Favorable effect

cell scattering, and expression of mesenchymal markers, in colon cancer cells *in vitro*. Clinical research using immunohistochemistry also showed that a high level of CD44v6 expression was an independent prognostic factor for DFS and OS of stage II/III colorectal cancer patients following curative resection.

The levels of CD44s and CD44v6 expression in patients with colorectal cancer using immunohistochemical analysis remain controversial. Concerning CD44s, several studies have reported that CD44s expression is associated with an advanced stage of disease and a poor prognosis (31-34), whereas other studies found no significant correlation between CD44s expression and the progression of colorectal cancer patients (35,36). Furthermore, the absence of CD44 expression in the stromal matrix was reported to be associated with a poor prognosis (37). Concerning CD44v6, its increased expression has been associated with poor prognosis, linked to adverse prognosis independent of Dukes' and UICC stages (12-16). However, others have reported that CD44v6 expression is associated with a favorable prognosis (38-40). Various clones of antibody, CD44s and CD44v6, appeared to affect the outcome of their clinical significance in patients with colorectal cancer. The clinical outcome and information regarding the antibodies used in previous reports are listed in Table IV.

Although many previous studies concerning CD44v6 in colon cancer have reported the significance of CD44v6 expression using immunohistochemistry-based prognostic studies (12-16,38-45), the effect of CD44v6 expression on EMT is unclear. This study suggests a molecular mechanism for how CD44v6 expression is linked to the malignant phenotype. The relationship of CD44v6 and EMT markers was particularly clear in the invasive front of colorectal cancer. In addition, we observed that a high level of CD44v6 expression was inversely correlated with cell differentiation. This result is consistent with previous studies (46,47), and CD44v6 may be related to the phenotype of poorly differentiated cancer cells that is defined by a lack of cellular polarity and regularity.

To confirm these clinical observations, we examined the role of CD44v6 in colon cancer cells using RNA interference. The knockdown of CD44v6 decreased cell invasive and migratory capabilities, and also decreased vimentin expression, but had no obvious effect on E-cadherin expression in colon cancer cells. HGF/c-Met signaling and cell scattering were previously reported to be regulated by CD44v6 expression (24,48), and we herein showed that CD44v6 expression was related to HGF-induced cell scattering. When this phenotypic change occurred, the E-cadherin expression level did not decrease after HGF treatment (data not shown).

This study was limited with regard to how CD44v6-expressing cells functionally differ from CD44s-expressing cells. A recent study indicated that the functional role of CD44s expression on breast cancer cells differed from that of a variant isoform in EMT regulation (49). Variant-specific functional roles have been identified not only for CD44v6, but also for CD44v3 in colorectal cancer (21,50,51). Although our clinical outcome showed the relevance of CD44v6 to tumor progression and EMT phenomenon, further investigation is required to determine how the cellular phenotype is regulated under various microenvironmental conditions.

In conclusion, the clinical outcome suggests that CD44v6 but not CD44s is associated with E-cadherin downregulation and vimentin upregulation and thereby relates to tumor progression. *In vitro* analysis supported that CD44v6 may affect the mesenchymal phenotype of colon cancer cells.

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