The role of CD44 in the TGF-β-mediated mesenchymal phenotype

*Tumor and Stem Cell Biology*

CD44s regulates the TGF-β-mediated mesenchymal phenotype and is associated with poor prognosis in patients with hepatocellular carcinoma

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Abstract

The prognosis for individuals diagnosed with hepatocellular carcinoma (HCC) remains poor due to the high frequency of invasive tumor growth, intrahepatic spread, and extrahepatic metastasis. Here, we investigated the role of the standard isoform of CD44 (CD44s), a major adhesion molecule of the extracellular matrix and a cancer stem cell marker, in the TGF-β-mediated mesenchymal phenotype of HCC. We found that CD44s was the dominant form of CD44 mRNA expressed in HCC cells. Overexpression of CD44s promoted tumor invasiveness and increased the expression of vimentin, a mesenchymal marker, in HCC cells. Loss of CD44s abrogated these changes. Also in the setting of CD44s overexpression, treatment with TGF-β1 induced the mesenchymal phenotype of HCC cells, which was characterized by low E-cadherin and high vimentin expression. Loss of CD44s inhibited TGF-β-mediated vimentin expression, mesenchymal spindle-like morphology, and tumor invasiveness. Clinically, overexpression of CD44s was associated with low expression of E-cadherin, high expression of vimentin, a high percentage of phospho-Smad2-positive nuclei, and poor prognosis in HCC patients, including reduced disease-free and overall survival. Together, our findings suggest that CD44s plays a critical role in the TGF-β-mediated mesenchymal phenotype and therefore represents a potential therapeutic target for HCC.
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Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent and the third most deadly type of cancer worldwide. In fact, it is diagnosed in more than half a million people worldwide each year (1). Surgical resection and liver transplantation are available options for the treatment of early-stage HCC, however, the prognosis of HCC remains poor due to a high level of tumor invasiveness, frequent intrahepatic spread, extrahepatic metastasis, and resistance to chemotherapy (2).

Epithelial-mesenchymal transition (EMT) has been shown to be a pivotal mechanism contributing to cancer invasion and metastasis including HCC (3-7), because epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells in the developmental process. In recent metastasis researches, EMT also shown to an important role in stem-like properties (8).

CD44, a major adhesion molecule of the extracellular matrix, has been implicated in a wide variety of physiological processes, including leukocyte homing and activation, wound healing and cell migration (9, 10). Cells produce CD44 protein isoforms through the process of alternative mRNA splicing. The CD44 standard isoform (CD44s) is expressed predominantly in hematopoietic cells and normal epithelial cell subsets, while the variant isoform (CD44v) is expressed by some epithelial cells during embryonic development, during lymphocyte maturation and activation, and by several types of carcinoma cells. Recently, cancer stem cells (CSCs) in many tumors have been identified by positive expression of CD44, either individually or in combination with other markers and these cells have been shown to be involved in tumor progression and metastasis (10-16). Although TGF-β signaling is a major regulator of EMT and it maintains the mesenchymal phenotype and stem cell states in an autocrine fashion in cancer (17), the underlying molecular mechanisms that integrate the
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mesenchymal phenotype with the EMT process and with CSC properties still remain unknown. Therefore, we hypothesize that CD44, a CSC marker, plays an important role in inducing EMT or in maintaining the mesenchymal phenotype in HCC.

**Materials and Methods**

**Cell lines, culture conditions, and reagents**

The human HCC lines PLC/PRF/5, HuH1, HLF, and HLE were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). SK HEP-1 cells were purchased from American Type Culture Collection (VA, USA). The cells were routinely maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). The cells were incubated at 37°C in a 5% CO₂ air-humidified atmosphere. Purified recombinant human TGF-β1 (R&D Systems) was reconstituted in sterile 4 mM HCl containing 1 mg/ml bovine serum albumin (Sigma). TGF-β1 was used at the indicated concentrations in serum-free medium.

**Plasmids and small interfering RNA (siRNA) transfection**

The cDNA corresponding to human CD44s was introduced into the pcDNA3.1 expression plasmid (Invitrogen) (18). PLC/PRF/5 cells were transfected with the resulting plasmids using Lipofectamine 2000 (Invitrogen). CD44 expression was transiently downregulated using a predesigned siRNA duplex directed against CD44, and a nontargeting siRNA was used as a negative control. The sequences of the siRNA (chimeric RNA-DNA) duplexes (Japan Bioservice) were as follows (18): CD44 siRNA, 5’-AAAUGGUCGUACAGCAUUCTT-3’ and 5’-GAUGCUGUAGCGACCAUUUTT-3’, and control siRNA, 5’-CGUACGCGGAAUACUUGCATT-3’ and
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5'-UCGAAGUAUUCCGCGUACGTT-3'. HCC cells were transfected with the annealed siRNA for 24 to 72 hours using Lipofectamine 2000.

Protein extraction and Western blot analysis

Protein extraction from cultivated cells and Western blot analyses were performed as previously described (19, 20). Briefly, the cells were lysed in cell lysis buffer containing 25 mM Tris (pH 7.4), 100 mM NaCl, and 1% Tween 20. Equal amount of proteins were loaded onto 10% gels and separated using SDS-PAGE. The resolved proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Inc.). 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 1 hour at room temperature, followed by incubation with a primary antibody at 4°C overnight. The blots were extensively washed with TBS-T and incubated with an HRP-conjugated secondary antibody diluted 1:2,000 in TBS-T for 1 hour at room temperature. The membranes were washed and visualized using a Chemiluminescent Detection Reagent Kit (ECL; GE Healthcare Corp.). Primary antibodies for E-cadherin (1:1,000 dilution; BD Transduction Laboratories), vimentin (1:1,000 dilution; Santa Cruz), CD44s (1:1,000 dilution; Bender Medsystems), phospho-Smad2 (1:500 dilution; Cell Signaling), Smad2/3 (1:1,000 dilution; Cell Signaling), and β-actin (1:1,000 dilution; Cell Signaling) were used for this study.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described (19, 20). Total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN), and cDNA was synthesized with the SuperScript III Transcriptor First Strand cDNA...
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Synthesis System for RT-PCR (Invitrogen) according to the manufacturers’ instructions. qRT-PCR was performed using a LightCycler 480 II instrument (Roche). To determine the differences in the gene expression levels between specimens, the $2^{-\Delta\Delta Ct}$ method was used to measure the fold changes among the samples (21). To perform qRT-PCR, primers were designed using the Universal Probe Library (Roche) following the manufacturer’s recommendations. The primer sequences and probes used for real-time PCR were as follows: E-cadherin, 5'-TTGACGCGAGCTACAC-3', 5'-GTCCAGCTGGTCAAATCTT-3', and universal probe #80; vimentin, 5'-TACAGGAAGCTGCTGAAGG-3', 5'-ACCAGAGGAGGTAAATCC-3', and universal probe #13; CD44, 5'-GCAGTCAACAGTCGAGAAG-3', 5'-TGTCCTCCACAGCTCCATT-3', and universal probe #29; GAPDH, 5'-AGCCACATCGCTCAAGAC-3', 5'-GCCATACCCCAATCC-3', and universal probe #60; EpCAM, 5'-AGTTGGTGCAACAAAATACTGTCAT-3', 5'-TCCCAAGTTTTGAGGCAATCC-3', and universal probe #8; CD133, 5'-TCCACAGAAATTACCTACATTGG-3', 5'-CAGCAGAGGCAGATGACCA-3', and universal probe #83; Bmi1, 5'-TTCTTTGACCAGACAGGATGG-3', 5'-GCATCACAGTCATTGCTGCTT-3', and universal probe #63; CD13, 5'-TTGCTACAGTCCATGCTGCTGCT-3', 5'-CAGAACCCTACATGCTGCTGCT-3', and universal probe #75; and CD90, 5'-CAGAACCCTACATGCTGCTGCT-3', and universal probe #66. To analyze the CD44 splice variants in human HCC cell lines, the following human primer sets (forward and reverse, respectively) were used: CD44, 5'-TCCAGACGAAGCAGATGG-3' and 5'-GCATCACAGTCATTGGCTGCT-3'; GAPDH, 5'-AGCCACATCGCTCAAGAC-3' and 5'-GCCCAATACGACAAATCC-3'.
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**Invasion assay**

An *in vitro* cell invasion assay was performed as previously described (20). Briefly, the invasion rate of tumor cells that migrated through transwell inserts (8-μm pore size) with a uniform layer of BD Matrigel basement membrane matrix (BD Biosciences) was assessed according to the manufacturer’s recommended protocol. The cells were seeded (5 × 10⁴) in 500 μl of serum-free medium into the upper chamber of the insert, and medium containing 10% FBS was added to the lower chamber. After 22 hours, the non-invading cells were removed with a cotton swab, and the invading cells were stained with 1% toluidine blue and counted under a microscope.

**Patients and treatment**

Among the 235 consecutive patients who had undergone curative hepatic resection between 2004 and 2007 in the Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 150 primary HCC samples were analyzed in this study. None of the patients received any preoperative anticancer treatment. The pathologic diagnoses and the clinicopathological factors were established based on the general guideline for primary liver cancer as defined by the Liver Cancer Study Group of Japan (22, 23) and the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) staging system (24). The median follow-up duration after surgery was 44 months. This study was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Kumamoto University (Kumamoto, Japan).

**Immunohistochemistry and scoring**
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The sample processing and immunohistochemistry (IHC) procedures were performed as described in a previous report (20). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide, and the sections were incubated with diluted antibodies. A subsequent reaction was performed with a biotin-free horseradish peroxidase enzyme-labeled polymer from the Envision Plus detection system (Dako Co.). Phospho-Smad2 antibody binding was detected using the Vectastain ABC ‘Elite’ avidin/biotin/peroxidase kit (Vector Laboratories Inc.). A positive reaction was visualized with the addition of diaminobenzidine solution, which was followed by counterstaining with Mayer’s hematoxylin. Primary antibodies for E-cadherin (1:100 dilution; BD), vimentin (1:50 dilution; Santa Cruz), CD44s (1:300 dilution; Bender MedSystems), and phospho-Smad2 (1:100 dilution; Cell Signaling) were used for this study. All of the IHC staining results were independently scored by two pathologists. The membranous E-cadherin, cytoplasmic vimentin, and membranous CD44s expression was interpreted according to the guidelines published in previous studies (5, 25, 26). For membranous E-cadherin, the 2+ and 3+ samples were defined as positive IHC results. For cytoplasmic vimentin, membranous CD44s, and phospho-Smad2-positive nuclei, the 3+ specimens were defined as positive IHC results.

Statistical analyses

All of the experiments were performed in triplicate, and the data shown are representative of the results. The data are presented as the means ± standard deviation (SD). Independent Student’s t-tests were used to compare the continuous variables between the two
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groups, and categorical variables were compared using the \( \chi^2 \) test. Overall survival and
disease-free survival were calculated using the Kaplan-Meier method and compared using the
log-rank test. Statistical analyses were performed as indicated with a statistical analysis
software program (Excel Statistics, Social Survey Research Information Co.). Differences
were considered to be significant if \( P<0.05 \).

## Results

**CD44 standard isoform expression is associated with a mesenchymal phenotype in hepatocellular carcinoma cells**

We examined the expression of CD44 and its association with EMT markers (E-cadherin and vimentin) in five HCC cell lines (PLC/PRF/5, HuH1, HLF, HLE, and SK HEP-1). At the mRNA level, the high CD44 expressing cell lines HLF, HLE, and SK HEP-1 showed high expression levels of vimentin, whereas the low CD44 expressing cell lines PLC/PRF/5 and HuH1 showed high expression levels of E-cadherin, as determined by real-time PCR (Fig. 1A). Recently, we demonstrated that the CD44 isoform expressed in the tumor cells of Gan mice, as well as in human gastrointestinal cell lines, consists mostly of variant isoforms (CD44v8-10) containing amino acids derived from exons 8 to 10 (18, 27). To determine the predominant isoform of CD44 expressed in the HCC cell lines, we examined the expression levels of the different isoforms using RT-PCR according to the same method. Unlike in human colon cancer cell lines, our RT-PCR analysis revealed that CD44s mRNA, rather than CD44v8-10mRNA, was the dominant form present in the human HCC cell lines HLF, HLE, and SK HEP-1 (Fig. 1B). We confirmed the associations of CD44s with E-cadherin and vimentin expression at the protein level (Fig 1C). These results suggest that high levels of CD44s expression are related to a mesenchymal phenotype, which includes
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downregulation of E-cadherin and upregulation of vimentin, in HCC cells. We also examined
the expression of other CSC markers that were previously reported in HCC (12, 28-31) and
compared them with the expression of E-cadherin and vimentin. However, no correlations
were observed that were similar to that of CD44 and vimentin. In addition, the expression
levels of EpCAM, CD133, and CD13 appeared to be similar to the expression level of
E-cadherin (Supplementary Fig. S1).

Overexpression of CD44s promotes the expression of vimentin and tumor invasiveness of
hepatocellular carcinoma cells while the knockdown of CD44s attenuates these changes
To investigate whether CD44s regulates the mesenchymal phenotype of HCC cells,
we transiently transfected PLC/PRF/5 cells with a human CD44s expression vector. The
overexpression of CD44s increased the expression of vimentin but did not decrease the
expression of E-cadherin (Fig. 2A, B). Furthermore, the overexpression of CD44s increased
the in vitro invasion of the transfected cells by 7-fold (Fig. 2C). Next, we examined the effect
of the siRNA knockdown of CD44 in HLF cells, which normally have high CD44 expression,
to determine whether CD44s is essential for the expression of vimentin in HCC cells. CD44
siRNA attenuated the expression of vimentin at the mRNA and protein levels (Fig. 2D, E).
Furthermore, in HLF cells pretreated with CD44 siRNA, the addition of a CD44s expression
vector reversibly increased the expression of vimentin (Fig. 2E). The results from an in vitro
invasion assay revealed that HLF cells transfected with CD44 siRNA exhibited a decrease in
invasion compared with cells transfected with a control siRNA (Fig. 2F). We observed
similar results in high CD44s expressing HLE and SK HEP-1 cells (Supplementary Fig. S2).
Overexpression of CD44s in PLC/PRF/5 cells did not decrease the expression of E-cadherin.
Moreover, we could not detect the upregulation of E-cadherin in the CD44 knockdown cells.
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These results suggest that CD44 regulates the expression of vimentin and tumor cell invasion in HCC cells.

**CD44s is induced by TGF-β and regulates the TGF-β-mediated mesenchymal phenotype in hepatocellular carcinoma cells**

TGF-β signaling is central to tumorigenesis and tumor progression because it regulates many critical cellular processes, including cell proliferation, EMT, and stem cell maintenance (32). HLF and HLE cells express detectable levels of phosphorylated Smad2, and the expression of E-cadherin was increased after incubation with a TGFβ type I receptor kinase inhibitor in HLF and HLE cells, suggesting that TGF-β signaling plays a crucial role in EMT in HCC cells (33). TGF-β1 is overexpressed in tumor cells, and this overexpression correlates with a poor prognosis in patients with HCC (34, 35). Thus, we investigated the role of CD44 in TGF-β signaling. We screened the activation status of TGF-β signaling by measuring phosphorylated Smad2 (phospho-Smad2) expression in the five HCC cell lines. As reported previously, HLF and HLE cells expressed detectable levels of phosphorylated Smad2 whereas SK HEP-1 cells did not (Fig. 3A). The precise mechanism of CD44 expression in SK HEP-1 cells with low phospho-Smad2 expression is unclear from our study. SK HEP-1 cells are originally derived from endothelial cells, which express CD44 (36, 37). In human endothelial cells, other growth factors, such as basic fibroblast growth factor, vascular endothelial growth factor, and hepatocyte growth factor/scatter factor (HGF/SF), can induce the expression of CD44 (37, 38). Therefore, an alternative mechanism may be associated with the expression of CD44 in SK HEP-1 cells. We next examined the role of CD44 in the TGF-β-mediated mesenchymal phenotype of HCC cells. Treatment with TGF-β1 induced a mesenchymal spindle-like morphology in PLC/PRF/5 cells (Supplementary Fig. S3A).
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Following treatment with TGF-β1 (5 ng/ml), phospho-Smad2 expression in PLC/PRF/5 cells progressively increased in a time-dependent manner and peaked after 60 minutes (Fig. 3B). The downregulation of E-cadherin, the upregulation of vimentin and the expression of CD44s were induced after 24 hours of treatment with TGF-β1 in PLC/PRF/5 cells (Fig. 3B). The expression of E-cadherin mRNA was decreased after 6 hours and restored after 24 hours (Supplementary Fig. S3B). The expression of vimentin and CD44 mRNA were elevated after 24 hours. Several transcription factors, including Snail, Slug, and Twist1 promote EMT in epithelial cells (4). The expression of Snail mRNA was increased after treatment with TGF-β1 and peaked after 3 hours (Supplementary Fig. S3C). The expression of Slug was not affected, and no expression of Twist1 in response to TGF-β1 was detected in PLC/PRF/5 cells. These results suggest that TGF-β signaling triggered the mesenchymal phenotype and induced the expression of CD44s in HCC cells. Next, we examined the effects of CD44s on the TGF-β-mediated mesenchymal phenotype. HLF cells also displayed progressively increased phospho-Smad2 levels in a time-dependent manner following incubation with TGF-β1 (5 ng/ml), and the levels peaked after 60 minutes (Supplementary Fig. S3D). Additionally upregulation of vimentin was induced by TGF-β1 after 24 hours of treatment in HLF cells (Fig. 3C). These results indicate that TGF-β signaling enhances the expression of vimentin in HLF cells. CD44 siRNA inhibited the TGF-β-mediated vimentin expression, mesenchymal spindle-like morphology, and tumor invasiveness in HLF cells (Fig. 3D, E, F). These results suggest that CD44s is essential for the TGF-β-mediated mesenchymal phenotype in HCC cells.

CD44s expression is associated with E-cadherin and vimentin expression and poor prognosis in patients with hepatocellular carcinoma
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To confirm the in vitro finding that the TGF-β-mediated mesenchymal phenotype and induction of CD44s are correlated in HCC cells, we analyzed the expression levels of CD44s, E-cadherin, vimentin, and phospho-Smad2 by IHC in 150 HCC patient samples. High expression of CD44s (e.g., Case 2 shown in Fig. 4A) was detected in 25% (37/150) of the samples, and low E-cadherin expression was significantly associated with high vimentin expression (P=0.004). High CD44s expression was significantly associated with low E-cadherin expression (P=0.039), high vimentin expression (P<0.001), and high phospho-Smad2 nuclear positivity (P<0.001) (Table 1). These data suggest that the activation of TGF-β signaling induces CD44s expression and a mesenchymal phenotype in patients with HCC. With regard to clinicopathological factors, a significant correlation was demonstrated between high CD44s expression and large tumor size (P=0.003), multiple tumors (P=0.032), and poor tumor differentiation (P=0.020) (Table 2). We further investigated the association between the expression levels of CD44s, vimentin, and E-cadherin and the clinicopathological factors in HCC patients. In a subgroup of patients with a CD44s_{high}/vimentin_{high}/E-cadherin_{low} expression profile, the incidence of vascular invasion was significantly higher than that in a subgroup of patients with a CD44s_{low}/vimentin_{low}/E-cadherin_{high} expression profile (25% versus 0%, respectively, P<0.001) (Supplementary Table S1). In all 150 HCC patients studied, high CD44s expression was more associated with frequent vascular invasion than low CD44s expression was, but this correlation did not reach a statistically significant difference (19% versus 10%, P=0.136). These results suggest that not only the mesenchymal phenotype via CD44s but also the loss of E-cadherin expression plays an important role of the vascular invasion in HCC patients. Notably, high CD44s expression was significantly associated with shorter disease-free survival (P=0.023) (Fig. 4B) and shorter overall survival (P=0.013) (Fig. 4C).
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**Discussion**

In the present study, CD44s, but not the variant isoforms, regulates the mesenchymal phenotype in HCC cells. In patients with HCC, tumoral CD44s overexpression was associated with the mesenchymal phenotype, as characterized by low E-cadherin expression and high vimentin expression. Interestingly, CD44s mRNA was the dominant form of CD44 mRNA present in human HCC cell lines, whereas the presence of high levels of the CD44v have been proposed as an important metastatic tumor marker in a number of cancers such as colon and lung cancer (39, 40). In breast cancer, CD44s was essential for the response to TGF-β during EMT, and the gain of CD44s expression was accompanied by a loss of expression of the variant isoforms (41). These findings suggest that the dominant form of CD44 isoforms in different tumors varies according to the location of the cancer cells.

TGF-β primes cancer cells for pulmonary metastasis and metastatic colonization of cancer cells in bones (42, 43). A recent study demonstrated that TGF-β in the blood activates TGF-β signaling in cancer cells, resulting in their transition to an invasive mesenchymal-like phenotype with enhanced metastatic potential (44). Furthermore, a link between TGF-β and the CD44\textsuperscript{high} population has been described in breast cancer and glioblastoma. TGF-β increases the CD44\textsuperscript{high}/CD24\textsuperscript{low} population, which is enriched in CSCs, through the induction of EMT in breast cancer (8). Additionally, glioma-initiating cells (GICs) expressed high levels of CD44, and the inhibition of TGF-β signaling decreased the GIC population in glioblastomas (45). These results agree with our studies demonstrating a novel role for CD44s in the TGF-β-mediated mesenchymal phenotype in HCC cells.

Although TGF-β signaling can induce the mesenchymal phenotype as characterized by low E-cadherin expression and high vimentin expression in epithelial cells, the mediators
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between TGF-β signaling and the mesenchymal phenotype are still unclear. The present study suggests that CD44 plays a downstream role in TGF-β signaling by regulating the expression of vimentin and tumor cell invasiveness and that CD44 does not affect the expression of E-cadherin, which may be suppressed by other mechanisms involved in the TGF-β pathway (Fig. 4D). The expression of Snail was elevated after 3 hours, and the expression of E-cadherin was suppressed after 6 hours. The induction of Snail expression in response to TGF-β is mediated by Smad2/3. Smad3 binds to the Snail promoter and activates its transcription (46). Smad3/4 and Snail interact to form a transcriptional repressor complex, which targets the promoter of E-cadherin (47). The time course of the activation of TGF-β signaling, and the expression of Snail and E-cadherin in the present study suggested that TGF-β signaling may suppress the expression of E-cadherin via Snail in HCC cells.

We demonstrated that high CD44s expression was a poor prognostic factor following curative hepatic resection of primary HCC. In addition to CD44s, several other variant isoforms (CD44v5, CD44v6, CD44v7-8, CD44v10) correlate with a worse prognosis in HCC patients, as determined by IHC analysis (26). Our investigations regarding clinicopathological factors demonstrated a significant correlation between CD44s expression and large tumor size, multiple tumors, and poor tumor differentiation in patients with HCC. Consistent with our in vitro analysis, high CD44s expression correlated with high vimentin expression in human HCC cells. Furthermore, in patients with HCC, we showed that phospho-Smad2 nuclear positivity was associated with high CD44s expression and that a CD44s_high/vimentin_high/E-cadherin_low expression profile was associated with vascular invasion. Together with our in vitro studies, these results suggested that the TGF-β-CD44s axis plays an important role in the vascular invasion of HCC. TGF-β signaling was recently determined to maintain the stem cell-like properties of tumor-initiating cells (32). Chaffer and Weinberg...
The role of CD44 in the TGF-β-mediated mesenchymal phenotype suggested a new concept of cancer cell metastasis in which cancer cells traveling through the circulation system are considered to be CSCs due to their anchorage-independent survival and the fact that these CSCs extravasate and invade into the parenchyma at distant organs (48). These observations support our findings that CD44s, a CSC marker, plays a downstream role in TGF-β signaling in HCC cells. We are continuing our studies to examine whether CD44s has an important role in the modulation of CSC properties in HCC cells.

In conclusion, our study suggests that the standard isoform of CD44, a CSC marker, regulates the TGF-β-mediated mesenchymal phenotype and that its expression indicates poor prognosis in patients with HCC. This information will be valuable for a better understanding of the relationship between CSCs and the mesenchymal phenotype induced by EMT; in addition, our results establish CD44s as a novel therapeutic target for HCC.
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**Disclosure of Potential Conflicts of Interest**

All of the authors declare that they do not have any potential conflicts of interest.
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Transforming growth factor betas and their signaling receptors in human


al. CD44 is involved in tumor angiogenesis; an activation antigen on human
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Table 1. Correlation between CD44s expression and E-cadherin/vimentin/phospho-Smad2 expression in 150 HCC patients.

<table>
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<th>E-cadherin</th>
<th>Vimentin</th>
<th>phospho-Smad2</th>
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</thead>
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<td></td>
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<td>High (n=58)</td>
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<td>Low (n=126)</td>
<td>High (n=24)</td>
<td>P-value</td>
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<tr>
<td></td>
<td>Low (n=123)</td>
<td>High (n=27)</td>
<td>P-value</td>
</tr>
<tr>
<td>CD44s Low</td>
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<td>49</td>
<td>0.039</td>
</tr>
<tr>
<td>CD44s High</td>
<td>28</td>
<td>9</td>
<td>18</td>
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</table>

*estimated by χ² test.

Abbreviations: CD44s, CD44 standard isoform; phospho-Smad2, phosphorylated Smad2.
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Table 2. Correlation between CD44s expression and the clinicopathological factors of 150 HCC patients.

<table>
<thead>
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<th>CD44s high expression (n=37)</th>
<th>CD44s low expression (n=113)</th>
<th>P-value</th>
</tr>
</thead>
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<td>35 / 78</td>
<td>0.441</td>
</tr>
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<td><strong>Sex</strong> Male / Female</td>
<td>30 / 7</td>
<td>93 / 20</td>
<td>0.867</td>
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<td><strong>HBs-Ag</strong> Negative / Positive</td>
<td>28 / 9</td>
<td>79 / 34</td>
<td>0.501</td>
</tr>
<tr>
<td><strong>HCV-Ab</strong> Negative / Positive</td>
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<td>61 / 52</td>
<td>0.994</td>
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<td><strong>Child-Pugh classification</strong> A / B</td>
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<td>103 / 10</td>
<td>0.412</td>
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<td>60 / 53</td>
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<td>18 / 19</td>
<td>57 / 56</td>
<td>0.850</td>
</tr>
<tr>
<td><strong>Tumor size</strong> ≤3 / &gt;3 (cm)</td>
<td>5 / 32</td>
<td>45 / 68</td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td><strong>Tumor number</strong> 1 / 2≤</td>
<td>21 / 16</td>
<td>85 / 28</td>
<td><strong>0.032</strong></td>
</tr>
<tr>
<td><strong>Tumor encapsulation</strong> absent / present</td>
<td>2 / 35</td>
<td>15 / 98</td>
<td>0.190</td>
</tr>
<tr>
<td><strong>Tumor differentiation</strong> moderate, well / poor</td>
<td>25 / 12</td>
<td>96 / 17</td>
<td><strong>0.020</strong></td>
</tr>
<tr>
<td><strong>LCSGJ TNM Stage</strong> 1,2 / 3, 4</td>
<td>18 / 19</td>
<td>71 / 42</td>
<td>0.127</td>
</tr>
<tr>
<td><strong>AJCC/UICC TNM Stage</strong> 1,2 / 3, 4</td>
<td>28 / 9</td>
<td>94 / 19</td>
<td>0.309</td>
</tr>
<tr>
<td><strong>Vascular invasion</strong> absent / present</td>
<td>30 / 7</td>
<td>102 / 11</td>
<td>0.136</td>
</tr>
</tbody>
</table>

Vascular invasion, portal vein (3rd branch, 2nd branch, 1st branch, or trunk) or hepatic vein (trunk of hepatic vein or IVC) invasion were defined via macroscopic examination of the resected specimens. Abbreviations: CD44s, CD44 standard isoform; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C antibody; AFP, α-fetoprotein; PIVKA II, protein-induced vitamin K absence-II; LCSGJ, Liver Cancer Study Group of Japan; AJCC/UICC, American Joint Committee on Cancer/International Union Against Cancer.
The role of CD44 in the TGF-β-mediated mesenchymal phenotype

**Figure Legends**

**Figure 1.** CD44s expression is associated with a mesenchymal phenotype in hepatocellular carcinoma cells.

(A) The relative expression levels of CD44, E-cadherin and vimentin mRNA in five HCC cell lines are shown. The data represent the means ± SD (n=3).

(B) RT-PCR analysis of CD44 mRNA in five HCC cell lines. Total RNA isolated from the five HCC cell lines was subjected to RT-PCR analysis with primers targeted to exons 5 and 16 of human CD44 cDNA and to human GAPDH cDNA. The positions of PCR products derived from CD44v8-10 (479 bp) or CD44s (83 bp) cDNAs are indicated. HT29 and HCT116 cell lines (colon cancer cell lines) were used as positive controls.

(C) The expression levels of CD44, E-cadherin, and vimentin protein in the five HCC cell lines, as determined by Western blot analysis.

**Figure 2.** Overexpression of CD44s promotes the expression of vimentin and tumor invasiveness of hepatocellular carcinoma cells while the knockdown of CD44s attenuates these changes.

(A) The relative expression levels of E-cadherin and vimentin mRNA in PLC/PRF/5 cells overexpressing CD44s compared with control cells. The data represent the means ± SD (n=3) (***P<0.001).

(B) The expression levels of CD44s, E-cadherin, and vimentin proteins in PLC/PRF/5 cells overexpressing CD44s compared with control cells.

(C) The invasive capacity of PLC/PRF/5 cells overexpressing CD44s compared with control cells. The data represent the means ± SD (n=3) (*P<0.05).

(D) The relative expression levels of vimentin mRNA in HLF cells transfected with siRNA
The role of CD44 in the TGF-β-mediated mesenchymal phenotype targeted against CD44 compared with control cells. The data represent the means ± SD (n=3) (***(P<0.001).

(E) The expression levels of CD44s and vimentin protein in HLF cells transfected with siRNA targeted against CD44 or transfected with both siRNA targeted against CD44 and an hCD44s expression vector compared with control cells.

(F) The invasive capacity of HLF cells transfected with siRNA targeted against CD44 compared with control cells. The data represent the means ± SD (n=3) (*P<0.05).

Figure 3. CD44s is induced by TGF-β1 and regulates the TGF-β-mediated mesenchymal phenotype in hepatocellular carcinoma cells.

(A) The expression of phosphorylated Smad2 (phospho-Smad2) in the five HCC cell lines, as determined by Western blot analysis.

(B) Immunoblot analysis of phospho-Smad2 and the expression levels of CD44s, E-cadherin, and vimentin following TGF-β1 (5 ng/ml) treatment in PLC/PRF/5 cells.

(C) Immunoblot analysis of phospho-Smad2 and the expression level of vimentin following TGF-β1 (5 ng/ml) treatment in HLF cells.

(D) Immunoblot analysis of the expression levels of CD44s and vimentin with or without TGF-β1 (5 ng/ml) treatment for 24 hours in HLF cells transfected with siRNA targeted against CD44 compared with control cells.

(E) Phase-contrast images with or without TGF-β1 (5 ng/ml) treatment for 24 hours in the HLF cells transfected with siRNA targeted against CD44 compared with control cells. Scale bars, 200 μm.

(F) The invasive capacity of HLF cells with or without TGF-β1 (5 ng/ml) treatment for 24 hours after transfection with siRNA targeted against CD44 compared with untreated control
The role of CD44 in the TGF-β-mediated mesenchymal phenotype cells. The data represent the means ± SD (n=3) (\(*P<0.01\)).

**Figure 4.** CD44s expression is associated with E-cadherin and vimentin expression and poor prognosis in patients with hepatocellular carcinoma.

(A) Immunohistochemical staining of E-cadherin, vimentin, CD44s and phospho-Smad2 in representative cases with low CD44s expression (Case 1) and high CD44s expression (Case 2). Scale bars, 50 μm.

(B) Kaplan-Meier survival analysis of disease-free survival in 150 HCC patients comparing the high CD44s expression group and the low CD44s expression group using the log-rank test.

(C) Kaplan-Meier survival analysis of overall survival in 150 HCC patients comparing the high CD44s expression group and the low CD44s expression group using the log-rank test.

(D) Model of the regulation of the mesenchymal phenotype by CD44s in hepatocellular carcinoma cells following the induction of CD44s expression by TGF-β signaling.

Subsequently, CD44s promotes vimentin expression and tumor cell invasion. The expression of E-cadherin may be suppressed by other mechanisms in the TGF-β signaling pathway.
Figure 1.

A

CD44

E-cadherin

Vimentin

B

C

CD44s

E-cadherin

Vimentin

β-actin
Figure 2.

A

PLC/PRF/5

<table>
<thead>
<tr>
<th></th>
<th>E-cadherin</th>
<th>Vimentin</th>
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</thead>
<tbody>
<tr>
<td>pcDNA3.1-Mock</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pcDNA3.1-hCD44s</td>
<td><strong>1.5</strong></td>
<td><strong>1.5</strong></td>
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B

PLC/PRF/5

<table>
<thead>
<tr>
<th></th>
<th>CD44s</th>
<th>E-cadherin</th>
<th>Vimentin</th>
<th>β-actin</th>
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<td>pcDNA3.1-Mock</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pcDNA3.1-hCD44s</td>
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<td><strong>1.5</strong></td>
<td><strong>1.5</strong></td>
<td><strong>1</strong></td>
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</table>

C

PLC/PRF/5

<table>
<thead>
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<td>pcDNA3.1-hCD44s</td>
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D

HLF

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<tr>
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<tbody>
<tr>
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<tr>
<td>CD44 siRNA</td>
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E

HLF

<table>
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<tr>
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<th>CD44siRNA + hCD44s</th>
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<tr>
<td>CD44 siRNA</td>
<td>CD44siRNA + hCD44s</td>
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F

HLF

<table>
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<tr>
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</tr>
<tr>
<td>CD44 siRNA</td>
<td><strong>1.2</strong></td>
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Figure 3.

A. 

<table>
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<tr>
<th>PLC/PRF/5</th>
<th>Huh7</th>
<th>HLF</th>
<th>HLE</th>
<th>SK HEP-1</th>
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<tbody>
<tr>
<td></td>
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B. 

**PLC/PRF/5**

<table>
<thead>
<tr>
<th>Time</th>
<th>TGF-β1 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
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</tr>
<tr>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
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</tr>
<tr>
<td>90 min</td>
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<tr>
<td>120 min</td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
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</table>

**phospho-Smad2**

**Smad2/3**

**β-actin**

C. 

**HLF**

<table>
<thead>
<tr>
<th>TGF-β1 5 ng/ml</th>
</tr>
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<tbody>
<tr>
<td>-</td>
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<td>+</td>
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<table>
<thead>
<tr>
<th>24 hr</th>
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**phospho-Smad2**

**Smad2/3**

**β-actin**

D. 

**HLF**

<table>
<thead>
<tr>
<th>Control siRNA</th>
<th>CD44 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
</tr>
</tbody>
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**TGF-β1 5 ng/ml**

<table>
<thead>
<tr>
<th>-</th>
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**CD44s**

**Vimentin**

**β-actin**

E. 

**HLF**

<table>
<thead>
<tr>
<th>TGF-β1 5 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
</tr>
</tbody>
</table>

**Control siRNA**

**CD44 siRNA**

F. 

**Invasion**

<table>
<thead>
<tr>
<th>TGF-β1 5 ng/ml</th>
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</thead>
<tbody>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
</tr>
</tbody>
</table>

**Control siRNA**

**CD44 siRNA**

**Fold change**

<table>
<thead>
<tr>
<th>TGF-β1 5 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
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</tbody>
</table>

**Control siRNA**

**CD44 siRNA**
Figure 4.

A

<table>
<thead>
<tr>
<th></th>
<th>E-cadherin</th>
<th>Vimentin</th>
<th>CD44s</th>
<th>phospho-Smad2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
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<tr>
<td>Case 2</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

B

Disease-free survival rates (%)

- CD44s low expression (n=113)
- CD44s high expression (n=37)

Time after hepatic resection (years)

\[ P = 0.023 \]

C

Overall survival rates (%)

- CD44s low expression (n=113)
- CD44s high expression (n=37)

Time after hepatic resection (years)

\[ P = 0.013 \]

D

Hepatocellular carcinoma cell

- TGF-β
- phospho-Smad2/3

CD44s

E-cadherin

Vimentin

Tumor cell invasion
CD44s regulates the TGF-β-mediated mesenchymal phenotype and is associated with poor prognosis in patients with hepatocellular carcinoma

Kosuke Mima, Hirohisa Okabe, Takatsugu Ishimoto, et al.

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