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CD44s signals the acquisition of the mesenchymal phenotype required for anchorage-independent cell survival in hepatocellular carcinoma

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Background: Circulating tumour cells (CTCs) have an important role in metastatic processes, but details of their basic characteristics remain elusive. We hypothesised that CD44-expressing CTCs show a mesenchymal phenotype and high potential for survival in hepatocellular carcinoma (HCC).

Methods: Circulating CD44$^+$CD90$^+$ cells, previously shown to be tumour-initiating cells, were sorted from human blood and their genetic characteristics were compared with those of tumour cells from primary tissues. The mechanism underlying the high survival potential of CD44-expressing cells in the circulatory system was investigated *in vitro*.

Results: CD44$^+$CD90$^+$ cells in the blood acquired epithelial–mesenchymal transition, and CD44 expression remarkably increased from the tissue to the blood. In Li7 and HLE cells, the CD44^{high} population showed higher anoikis resistance and sphere-forming ability than did the CD44^{low} population. This difference was found to be attributed to the upregulation of Twist1 and Akt signal in the CD44^{high} population. Twist1 knockdown showed remarkable reduction in anoikis resistance, sphere formation, and Akt signal in HLE cells. In addition, mesenchymal markers and CD44s expression were downregulated in the Twist1 knockdown.

Conclusions: CD44s symbolises the acquisition of a mesenchymal phenotype regulating anchorage-independent capacity. CD44s-expressing tumour cells in peripheral blood are clinically important therapeutic targets in HCC.

The epithelial–mesenchymal transition (EMT) is considered to be an essential step for malignant cells to progress into the metastatic cascade, invasion, migration, extravasation, translocation into blood circulation, and distant metastases (Polyak and Weinberg, 2009; Hanahan and Weinberg, 2011). So far, many transcription factors have been identified as capable of inducing the EMT process, such as Snail, Slug, Twist1, the forkhead box protein C2, zinc finger E-box-binding homeobox 1 (ZEB1), and ZEB2 (Yang

et al, 2004; Mani *et al*, 2007; Gregory *et al*, 2008; Hoot *et al*, 2008; Vincent *et al*, 2009; Wellner *et al*, 2009; Yang *et al*, 2010). The main difficulty in understanding EMT is due to the variety of roles in which EMT inducers have in intersecting major pathways originating from transforming growth factor (TGF)-β, hepatocyte growth factor, Wnt–β-catenin, and tumour necrosis factor-α, in various solid cancers within each organ-specific microenvironment. In addition, the acquisition of EMT is also linked with

tumour-initiating capacity (Mani *et al*, 2008). This fact has attracted substantial attention by investigators in the last decade.

CD44 was first identified as the lymphocyte homing receptor in 1983 (Gallatin *et al*, 1983). Its function is variable depending on the expression of a variant isoform (CD44v) and posttranslational modification (Ponta *et al*, 2003; Teriete *et al*, 2004). CD44v expression has been observed in epithelial cells as well as in some gastroenterological cancers (Ishimoto *et al*, 2011; Yae *et al*, 2012). On the other hand, the CD44 standard isoform (CD44s), which is known as the haematopoietic isoform, is the most frequently observed tumour-initiating cell (TIC) marker that always occurs with other frequent markers, such as CD34, CD133, ALDH, CD24, CD90, CD24, and EpCAM (Zoller, 2011). However, the role of CD44s in maintaining the stem cell phenotype of malignant cells has not yet been clarified, and TICs cannot be defined by CD44s alone. The role of CD44s in TICs in many malignancies has therefore been a subject of great concern.

Primary liver cancer, which is predominantly of HCC, is the fifth/sixth most common cancer worldwide and the third most frequent cause of cancer mortality (Ferlay *et al*, 2010). Recent advances in surgical treatment and the development of chemotherapeutic agents have improved its prognosis, but the 5-year recurrence rate after curative surgery nonetheless remains high, at 70% (Forner *et al*, 2012). To understand its molecular pathogenesis, the cancer stem cell concept has been discussed in relation to hepatocellular carcinoma (HCC). Several markers, such as CD133 (Ma *et al*, 2007), Bmi1 (Chiba *et al*, 2007, 2008, 2010), EpCAM (Yamashita *et al*, 2007, 2008, 2009), CD90 (Yang *et al*, 2008), CD13 (Haraguchi *et al*, 2010), and CD24 (Lee *et al*, 2011), have been suggested as promising molecules for the identification of TICs. CD44s was introduced as a TIC marker with CD90 by Yang *et al* (2008). We previously demonstrated that CD44s works downstream of TGF-β as a regulator of the mesenchymal phenotype in HCC and that CD44s is highly related to the EMT phenomenon and to poor prognoses in patients with HCC (Mima *et al*, 2012). However, whether or not CD44s-expressing cells obtain aspects of cancer stem cells remains elusive.

In this study, we aimed to clarify the mechanism by which CD44s bridges the two major concepts, TIC and EMT. In addition, we confirmed that CD44s-expressing cells acquire the mesenchymal phenotype during metastatic step in clinical settings.

MATERIALS AND METHODS

Cell lines and antibodies. The cell lines and respective culture media used in this study are listed in Supplementary Table 1. All cultures were maintained in a 5% CO_2 air-humidified atmosphere at 37 °C. The antibodies used in this study are listed in Supplementary Table 2.

RNA extraction, quantitative reverse transcription-PCR, immunoblotting, and immunohistochemistry. RNA extraction, reverse transcription, and quantitative reverse transcription-PCR were performed as previously described (Okabe *et al*, 2009, 2012). Immunoblotting and immunohistochemistry were performed as previously described (see Supplementary Information).

Apoptosis assay. Phosphatidylserine externalisation was detected by Annexin V staining. From the cell suspension, 5.0×10^5 cells were resuspended in 100 μl of PBS and then incubated with 5 μl of Annexin V-FITC (Millipore, Tokyo, Japan) followed by PI staining (Sigma-Aldrich, Tokyo, Japan). The samples were analysed using a FACS Aria II flow cytometer (BD Biosciences, Tokyo, Japan). Results from FACS were analysed by FlowJo software (TOMY Digital Biology, Tokyo, Japan). Analysis was performed 48 h after siRNA treatment.

Proliferation assay. Cells were plated at a density of 3000 cells per well in a 96-well plate, and cell proliferation was assessed in triplicate at 24, 48, and 72 h using the Cell Counting Kit-8 containing WST-8 (Dojin Laboratories, Kumamoto, Japan), as described previously (Okabe *et al*, 2009).

Sphere formation assay. A total of 5.0×10^4 cells were suspended in ultralow attachment 24-well plates (Corning Inc., Tokyo, Japan) and grown in Hanks' balanced salt solution medium (Sigma-Aldrich), supplemented with 10 mM HEPES, 2% fetal bovine serum, 20 ng ml^{-1} EGF (Invitrogen, CA, USA) and 20 ng ml^{-1} FGF (Sigma-Aldrich). Fresh media was changed every other day. Tumour spheres were counted and photographed at day 5. Number of spheres per well and size of spheres over 100 μm per well were documented.

Anoikis assay. Plates were coated with 10 ml of 20 mg ml^{-1} poly-HEMA (Sigma-Aldrich) and air-dried. A total of 5.0×10^5 cells were plated on the poly-HEMA-coated plates. The number of cells was counted at day 5.

Single cell separation from human liver tissue and blood collection from patients. Tumour tissues from the liver were minced by chopping for cell isolation. After digestion with type II collagenase (200 units per ml; Worthington, Lakewood, NJ, USA) and hyaluronidase (500 units per ml; Sigma) at 37 °C for 30 min with gentleMACS Dissociator (Miltenyi Biotec, Tokyo, Japan) according to the manufacturer's instructions, the tissue cell suspension was passed through a 40 μm nylon mesh. Cells were then counted and subjected to staining for cell sorting.

Before operating, 7 ml of EDTA blood were collected from patients. Mononuclear cells were isolated from the EDTA blood using Ficoll-Paque PLUS (GE Healthcare, Tokyo, Japan) density gradient centrifugation before proceeding to flow cytometry analysis or cell sorting.

FACS analysis and cell sorting. Antibodies used in the flow cytometric analysis are listed in Supplementary Table 1. Cells were incubated with PE, PerCP-, or FITC-conjugated antibodies for 60 min on ice. Isotype-matched mouse immunoglobulins served as controls. The samples were analysed using a FACS Aria II flow cytometer (BD Biosciences). Viable cells from a single cell suspension of cultured cells, cells from blood samples, and dissociated cells from HCC tissue samples were sorted by a FACS Aria II cell-sorter equipped with an automated cell deposition unit. After exclusion of leukocytes by CD45$^+$ cells, CD44$^+$CD90$^+$ cells were determined for collection.

Statistical analysis. Statistical analysis was performed using the JMP programme v.10.0 (SAS Institute, Cary, NC, USA). Comparisons were carried out by a Wilcoxon's test and P-values < 0.05 were considered to be significant. To compare multiple samples, we used ANOVA followed by a Tukey–Kramer *post-hoc* test with a significance level of 0.05.

RESULTS

CD44$^+$CD90$^+$ cells showed EMT acquisition in patients with HCC. In the second step of metastasis, the invaded tumour cells in the vasculature separate from a tumour cluster and circulate throughout the body. CD90$^+$CD44$^+$ cells in patients' blood were introduced to reveal the tumour-initiating capacity and relate to the patients' prognoses (Fan *et al*, 2011). HLE was found to have a rich CD90^{high}CD44^{high} population and it demonstrates the mesenchymal phenotype; therefore, we hypothesised that CD90$^+$CD44$^+$ cells would show the mesenchymal phenotype in human subjects. Major population, CD90$^-$CD44$^-$ cells, and CD90$^+$CD44$^+$ cells were separated from HCC tumour tissues.

We obtained CD90$^+$CD44$^+$ cells from 5 ml blood and liver tumour tissues from two HCC patients (Figure 1A). Figure 1B shows a representative outcome of CD90 and CD44 expression in tumour tissue and in the blood of HCC patient, as well as the expression pattern from the blood of healthy volunteer. Results from one of the two HCC patients are shown in Figure 1C. To confirm that the cancer cells in this analysis were hepatocytes, we examined the expression of *Albumin* in addition to the EMT markers and *CD44*. The HCC cells showed *Albumin* expression that was relatively decreased in CD90$^+$CD44$^+$ cells compared with that in CD90$^-$CD44$^-$ cells. Confirming our expectations, substantial downregulation of *E-cadherin* and upregulation of *Vimentin* were observed in CD90$^+$CD44$^+$ cells compared with that in CD90$^-$CD44$^-$ cells. Among the CD90$^+$CD44$^+$ cancer cells, this change in expression was more extreme in the circulating tumour cells (CTCs) than in the tissue. In addition, *CD44*

expression increased in CD90$^+$CD44$^+$ cells and this pattern was strikingly similar to that of *Vimentin* expression. The results from the other HCC patient are shown in Figure 1D. Although results were almost identical to the first patient (Figure 2C), in this patient the difference in expression between CD90$^-$CD44$^-$ cells and CD90$^+$CD44$^+$ cells was observed to be most extreme in the tumour tissue.

CD44s is a dominant isoform in HCC patients and is related to the mesenchymal phenotype in vitro. CD44 is known to have several isoforms in gastroenterological tumours. We previously reported that CD44s is the dominant isoform in HCC cell line (Mima *et al*, 2012). To confirm that this isoform has an HCC-specific characteristic, CD44 isoforms were examined in gastroenterological cancer cell lines. Although CD44v8-10 (489 bp) is dominantly expressed in gastroenterological adenocarcinomas
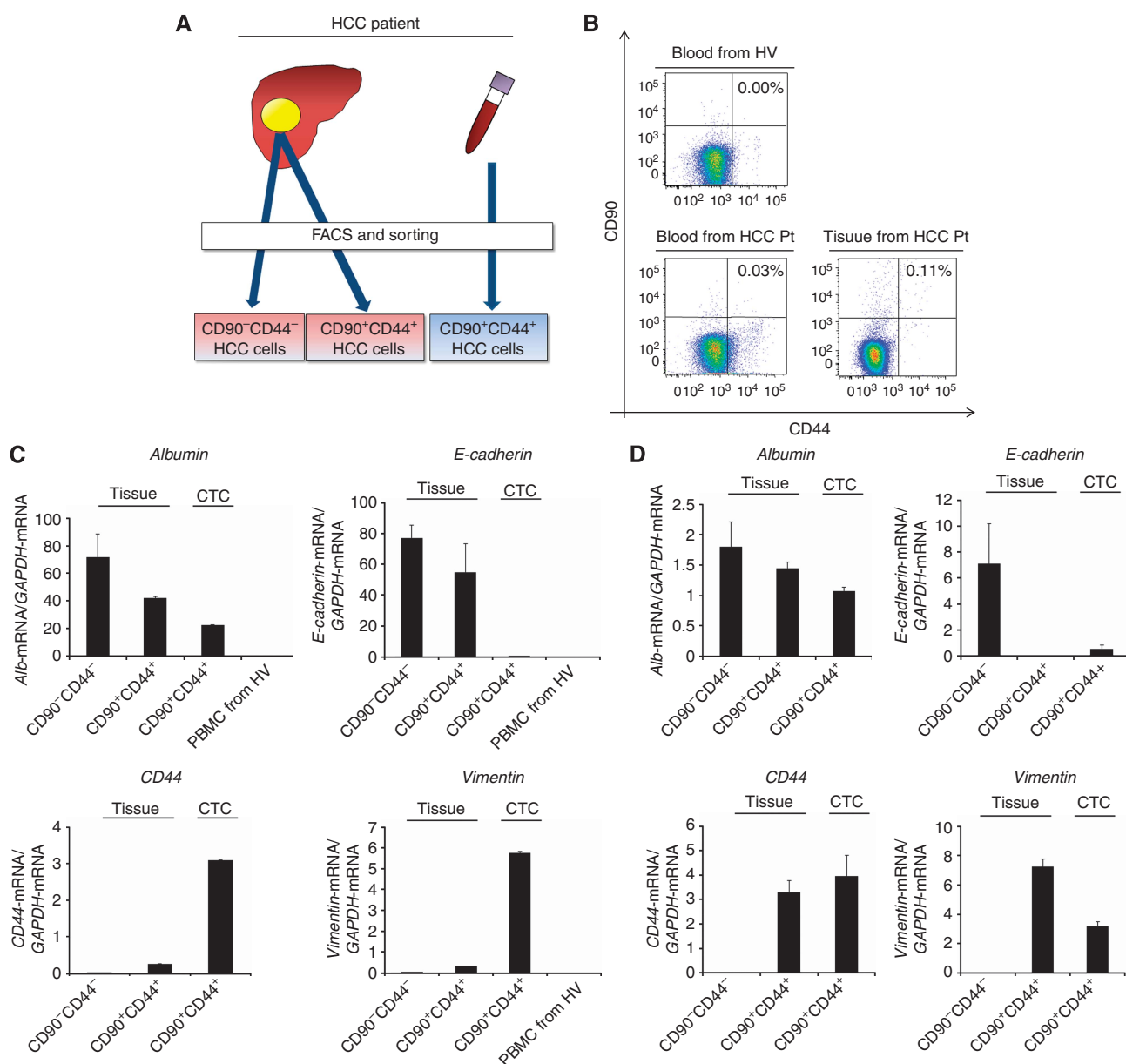

Figure 1. CD90$^+$CD44$^+$ cells from human HCC show EMT. (A) Schema of sample collection from human tissue or blood. **(B)** Representative results of flow cytometric analyses from human blood of a healthy volunteer (HV) and an HCC patient, and from human tumour tissue. CD90$^+$CD44$^+$ cells are 0.00%, 0.03%, and 0.11%, respectively. **(C)** Comparison of mRNA expressions in cells from an HCC patient using quantitative reverse transcription-PCR (qRT-PCR). Peripheral blood mononuclear cells (PBMCs) from HV are used as the negative control. **(D)** Comparison of mRNA expressions of cells from another HCC patient using qRT-PCR. PBMCs from HV are used as the negative control.
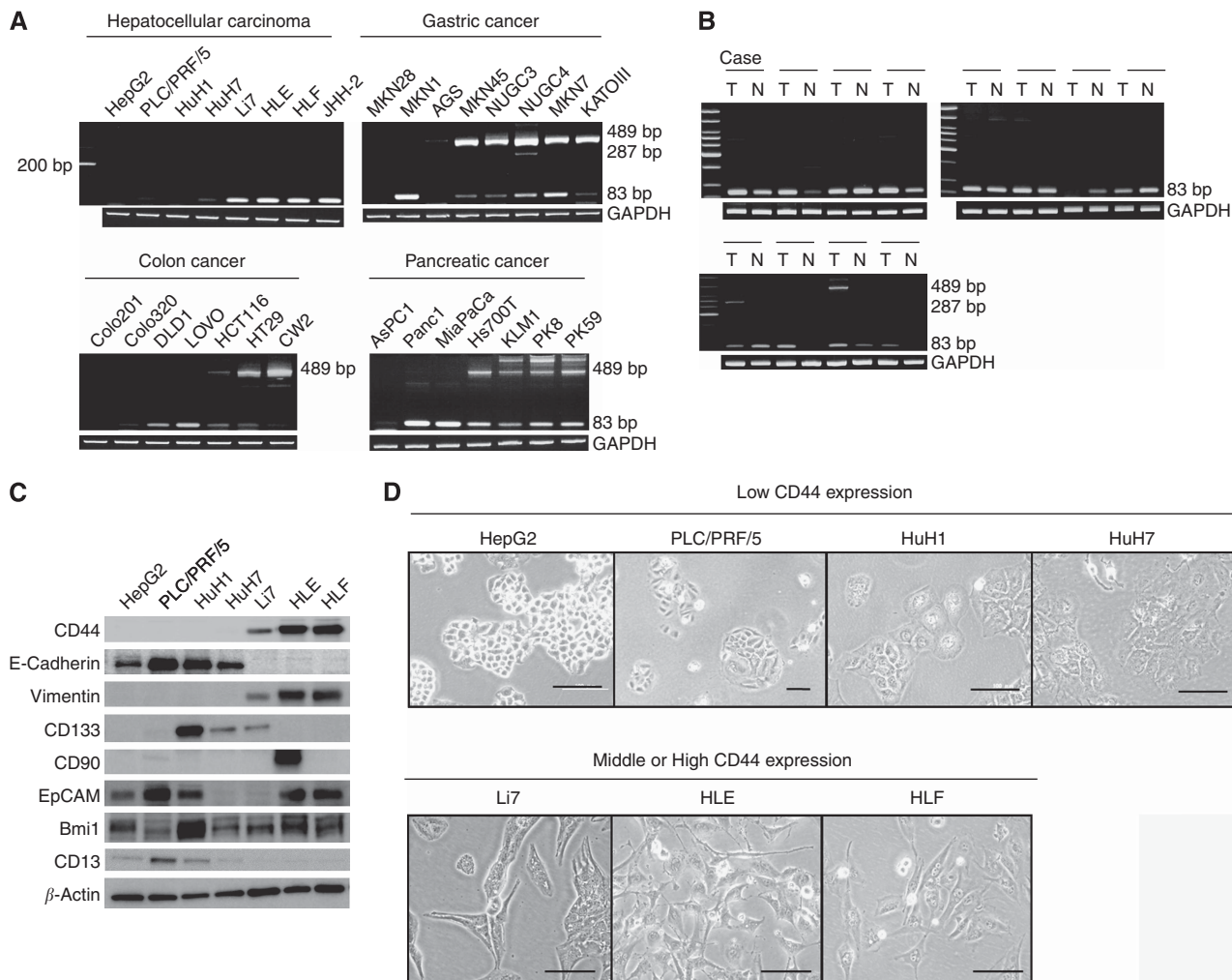
Figure 2. HCC cells are characterised by expressing CD44 standard isoform related to the mesenchymal phenotype. (A) Expression of CD44 variant isoforms in gastrointestinal cancer cell lines using reverse transcription-PCR (RT-PCR). (B). Expression of CD44 variant isoforms in patients with HCC using RT-PCR. Samples from tumour tissue (T) and non-cancerous tissue (N) are shown. Each PCR product (83, 287, and 489 bp) was subject to sequencing analysis (Supplementary Figure 1). (C) Comparison of EMT-related markers and tumour-initiating markers by western blot analysis. (D) Comparison of morphologies of seven HCC cell lines cultured under normal conditions. Bars, 100 μm.

such as gastric cancer and pancreatic cancer, CD44s was the dominant isoform in HCC cells (Figure 2A). We also examined CD44 isoforms in human tissues and found that CD44s is a major isoform along with several other isoforms (Figure 2B). Two variant isoforms were confirmed by sequencing analysis, CD44v8-10 and CD44v10 (Supplementary Figure 1). As a result, we performed further functional analyses focusing on CD44s in HCC.

Among the many TIC markers previously reported in HCC, the expression of CD44s was the only marker that was significantly positively correlated with Vimentin expression and negatively correlated with E-cadherin expression (Figure 2C). This relationship was also supported based on morphological features. Cells in the four HCC cell lines with low CD44s expression are round-shaped and clustered, whereas those in the three HCC cell lines with middle or high CD44s expression are spindle shaped (Figure 2D). Overall, CD44s-expressing cells have a mesenchymal phenotype.

CD44s-expressing cells with mesenchymal phenotype showed high proliferating, sphere-forming, and anoikis-resistant potential. As CD44$^+$CD90$^+$ cells from human blood sample have been shown to have high potential for initiating tumour cells (Yang et al, 2008), we hypothesised that CD44-expressing cells would have

anoikis-resistant properties. We used two cell lines: Li7, expressing an intermediate level of CD44s, and HLE, expressing a high level of CD44s. We then divided these lines into a high CD44-expressing population and low CD44-expressing population (Figure 3A). CD44^{high} cells in both cell lines showed higher sphere-forming ability than did the CD44^{low} cells ($n = 3$, $P < 0.05$; Figure 3B). In addition, CD44^{high} cells in both lines showed higher anoikis resistance than did CD44^{low} cells ($n = 3$, $P < 0.05$; Figure 3C). As cancer cells highly expressing CD44v were shown to have high proliferating potential (Ishimoto et al, 2011), we investigated whether cells highly expressing CD44s showed a similar property in HCC. Indeed, CD44^{high} cells in both cell lines showed higher proliferation than did CD44^{low} cells ($P < 0.05$; Figure 3D). We further focused on the difference in intracellular signals between CD44^{high} and CD44^{low} cells. Tyrosine receptor kinase array revealed that phosphor-Akt was more activated in CD44^{high} cells compared with that in CD44^{low} cells (Figure 3E). We confirmed that CD44^{high} cells, in both Li7 and HLE cell lines, showed higher mesenchymal phenotype (Figure 3F).

To clarify whether these differences in phenotype between the CD44^{high} and CD44^{low} populations were produced by CD44s, we overexpressed CD44s in PLC cells with low CD44s expression, or depleted CD44s expression in HLE cells with high CD44s
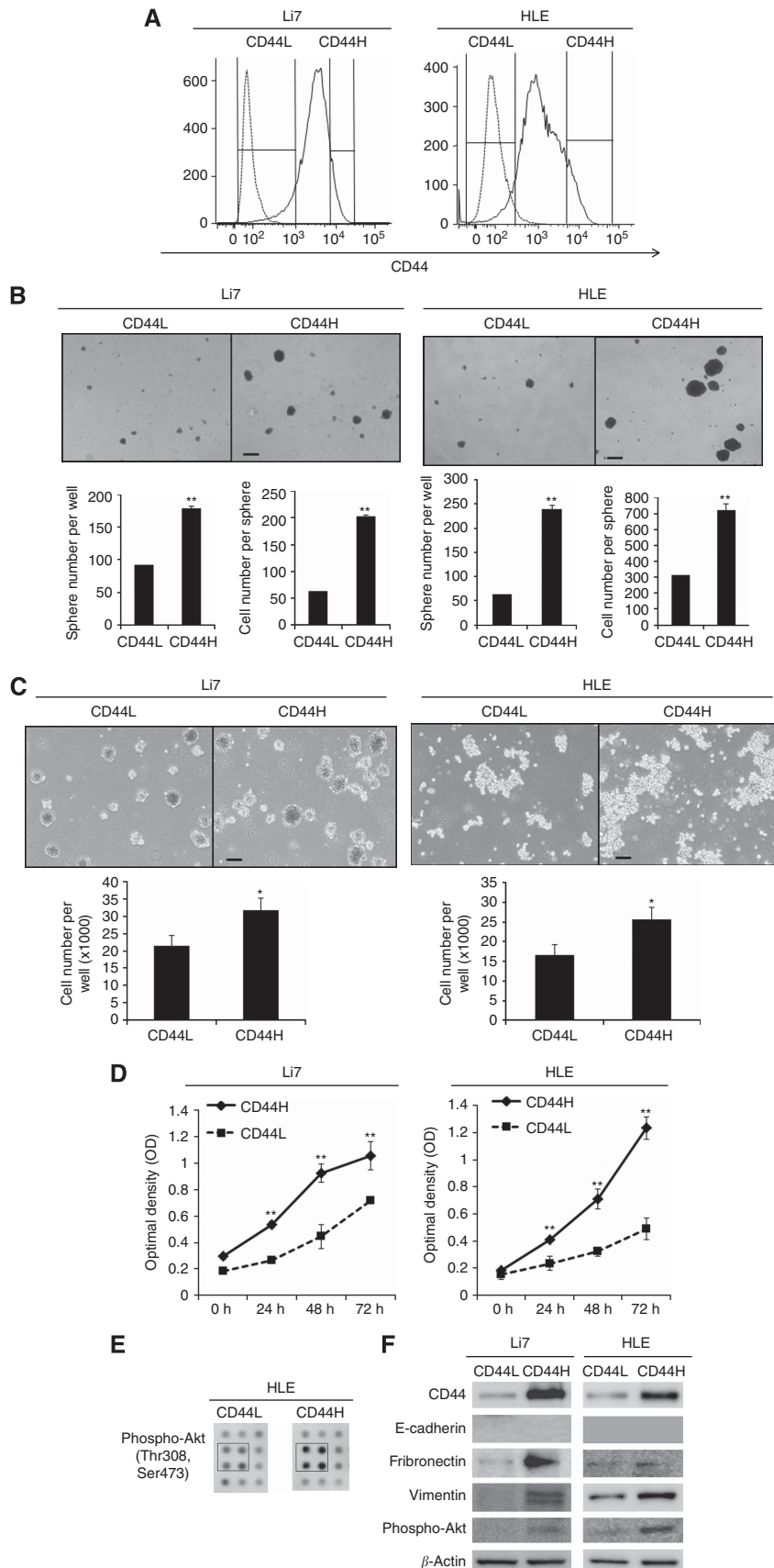
Figure 3. CD44-expressing cells have high sphere-forming, high proliferative, anoikis-resistant capacities and show the mesenchymal phenotype. (A) Ten per cent of the CD44^{high} population and 10% of the CD44^{low} population were sorted for further analyses. Solid line shows CD44 expression and dotted line shows the isotype control, IgG. (B and C) Sphere formation assay (B) and anoikis assay (C) were analysed on day 5. Bars, 200 μm. *$P < 0.05$ and **$P < 0.01$. (D) Growth assay was performed. **$P < 0.01$. (E) Tyrosine-phosphorylated RTKs and some downstream signals were compared between the CD44 high and low populations. (F) Expressions of CD44, pAkt, and EMT-related markers are shown, based on western blot analysis.

expression, and examined the effect on sphere-forming ability. Neither CD44s overexpression nor depletion contributed to sphere formation or to the activation of the Akt signal (Supplementary Figure 2A and 2B). These results strongly suggest that it is not CD44s but rather other factors, which regulate the anchorage-independent phenotype.

Twist knockdown decreased the phenotype and CD44s expression, resulting in loss of high proliferating, sphere-forming, and anoikis-resistant potential. To find out the factor responsible for the regulation of anchorage-independent cell survival, mesenchymal phenotype, and CD44 expression, we examined the expression patterns of transcription factors that are well-known EMT regulators in seven HCC cell lines. The expression pattern of *Twist1* was similar to that of *CD44s* and *Vimentin* (Supplementary Figure 3). Twist1 has been shown to be a regulator of CD44s, as an inducer of the standard isoform and the EMT phenotype, by regulating ESRP1 in breast cancer (Brown *et al*, 2011). We examined whether the mesenchymal phenotype of HCC cells is inhibited by a Twist1 knockdown. The CD44^{high} population showed higher Twist1 status in Li7 and HLE (Figure 4A). The Twist1 knockdown, obtained by siRNA, resulted in downregulation in the expression of Vimentin and phosphor-Akt in HLE with high CD44s expression, but not in Li7 with intermediate CD44s expression (Figure 4B). E-cadherin expression was not rescued by the Twist1 knockdown in either cell line. CD44s expression was downregulated by the Twist1 knockdown (Figure 4C). Furthermore, the Twist1 knockdown significantly decreased cell proliferation (Figure 4D), sphere formation (Figure 4E) and anoikis resistance (Figure 4F). These effects of the Twist1 knockdown on the phenotype and on CD44s expression was observed more clearly in HLE cells with high CD44s expression than in the Li7 cells with intermediate CD44s expression. Furthermore, the Twist1 knockdown increased the early apoptotic cells in the HLE line but not in the Li7 line (Figure 4G). These results suggest that HLE is largely dependent on the mesenchymal phenotype regulated by Twist1 and thereby shows remarkable catastrophic changes from a Twist1 knockdown.

Co-expressing tumour cells of Twist1 and CD44s can be seen in human HCC. To confirm the relationship between CD44s expression and some mesenchymal markers in humans, we investigated it by immunohistochemistry. The results of this analysis revealed that cancer cells expressing Twist1, but not all of them, co-expressed CD44s and Vimentin, and lost E-cadherin expression (Figure 5A–E). Leukocytes showed no staining for Twist1 or Vimentin (Figure 5F).

DISCUSSION

This study revealed that CD44-expressing cells acquire the mesenchymal phenotype and anchorage-independent survival. This is the point in which CD44-expressing cells represent a crucial population for invasion and metastasis. In a clinical setting, we confirmed our hypothesis that CD44$^+$CD90$^+$ TICs are characterised by a mesenchymal phenotype, and attempted to clarify why these TICs possessed a high capacity for circulating in the body and resisting anoikis. Previous reports and the current study have shown that most gastroenterological adenocarcinomas contain the CD44 variant isoform, CD44v8-10, as a dominant isoform (Ishimoto *et al*, 2011). In contrast, HCC cells contain the CD44 standard isoform CD44s. CD44s expression was observed on lymphocytes as one of the haematopoietic markers and was also observed on fibroblasts, although normal mature hepatocytes did not express it (data not shown). Hepatocellular carcinoma cells with CD44 upregulation are strikingly associated with the EMT
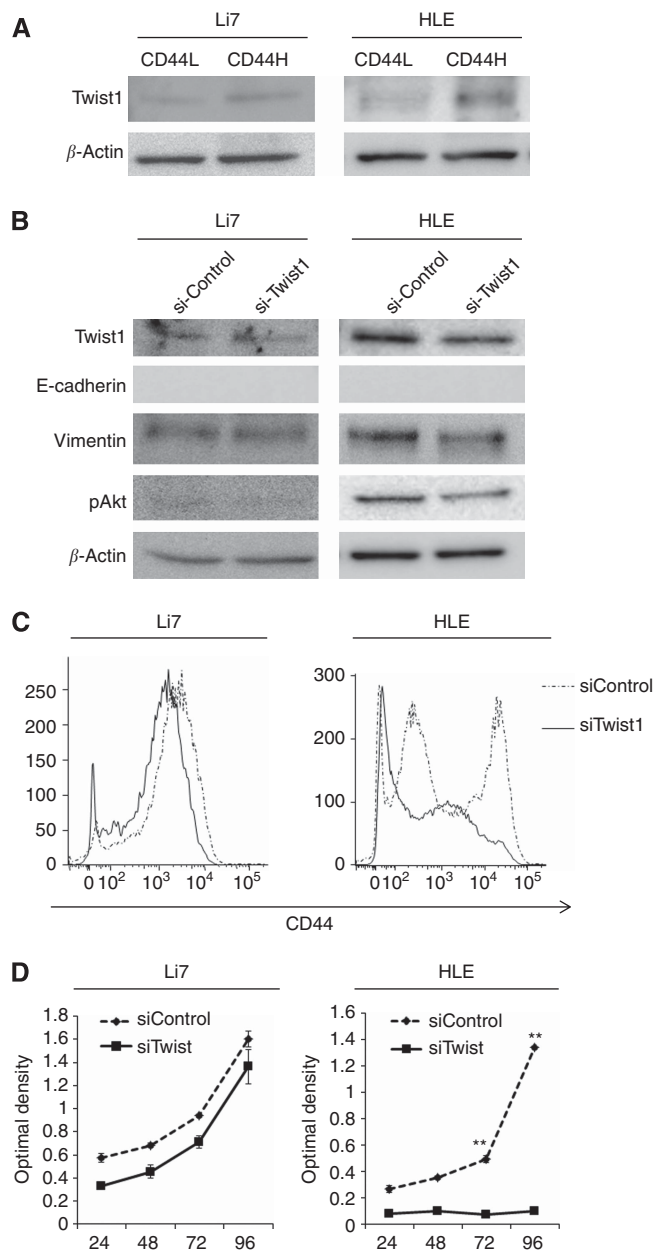
Figure 4. Twist knockdown decreases the mesenchymal phenotype, proliferation, sphere formation, and anoikis-resistant capacity. (**A**) Twist1 expression is compared between CD44^{high} cells and CD44^{low} cells by western blotting. (**B**) Protein expressions were examined by western blot analysis. (**C**) CD44 expression was analysed by flow cytometry 48 h after the siRNA treatment. (**D**) Growth assay was performed 48 h after siRNA treatment. (**E** and **F**) The sphere formation assay (**E**) and anoikis assay (**F**) were performed 5 days after siRNA treatment. (**G**) The percentages of early apoptotic cells are determined by flow cytometry 48 h after the siRNA treatment. *$P < 0.05$ and **$P < 0.01$.

phenotype and CD44s is the only TIC marker that is characterised by this trait in HCC (Figure 3C).

In a rodent model, hepatic stem/progenitor cells are well-characterised because they can be identified by their classical markers, OV6 and A6 (Akhurst *et al*, 2001; Roskams *et al*, 2003; Apte *et al*, 2008). So far, other markers have been used as their supportive markers, such as CD90, Oct3/4, EpCAM, and ALDH1 (Apte *et al*, 2008; Thenappan *et al*, 2010; Dolle *et al*, 2012). Interestingly, the mesenchymal markers, *CD44* and *Vimentin*, were

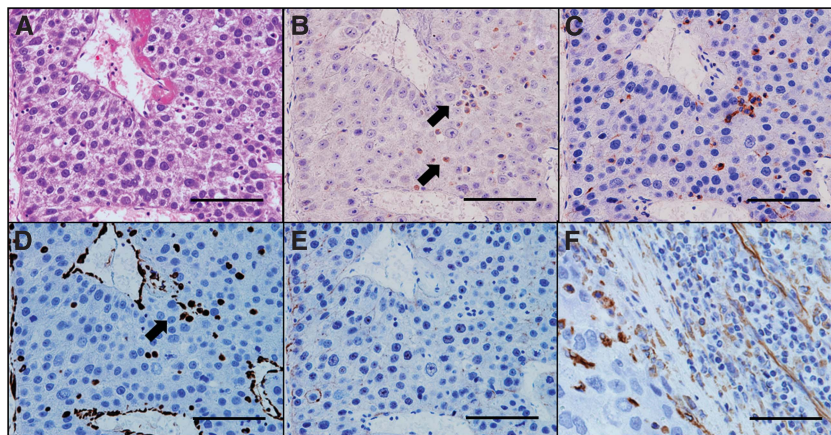**Figure 5. Co-expression of CD44s and mesenchymal markers expression in human tissue. (A–F)** Representative photographs of immunohistochemistry. Haematoxylin and eosin (**A**), Twist1 (**B**), CD44s (**C**), Vimentin (**D**), and E-cadherin expression (**E**) in tumour, and Vimentin expression (**F**) in leukocytes besides tumour cells are shown. Arrows show tumour cells clustering close to the vasculature and co-expressing Twist1, CD44, and Vimentin. Bars, 100 μm.

overexpressed in oval cells in the rat AAF/PH model (Yovchev *et al*, 2007). Oval cells are well known as amplifying transit cells and thereby have a high proliferating property under condition of hepatocyte damage in the liver (Hu *et al*, 2007). Therefore, the reason that the CD44^{high} population shows remarkably higher proliferative potential than the CD44^{low} cell population may be because of the properties of the progenitor cell. However, whether or not this population includes dormant stem cells remains an open question.

The results of the current study suggest, for the first time, that the CD44^{high} population has anoikis-resistant potential, a property that is especially important for CTCs. Furthermore, we found that Twist1 is involved in regulating not only the mesenchymal phenotype but also anoikis resistance. Twist1 depletion in HLE cells surprisingly decreased proliferation, anoikis-resistant potential and phosphorylation of Akt, suggesting that the survival of HLE was largely dependent on Twist1 regulation. On the other hand, there was no indication of any substantial induction of anoikis-resistant potential by Twist1 overexpression using PLC/PRF/5 and HuH7 (data not shown). A previous study showed that the induction of the mesenchymal phenotype or EMT was only due to ectopic Twist1 expression in HCC (Yang *et al*, 2009; Sun *et al*, 2010). We speculate that although Twist1 probably contributes to mesenchymal phenotype and anoikis-resistant potential, it may do so only partially, requiring a collaborating factor in order to strengthen these characteristics.

The detection of CTCs using an EpCAM antibody has been well established in some malignancies (Maheswaran *et al*, 2008), but the process of EMT that is signalled by the loss of the epithelial phenotype is well recognised as the metastatic step of cancer. Whether or not the loss of epithelial phenotype is essential for CTCs remains elusive. Using a mouse model of carcinogenesis in pancreatic cancer, it was demonstrated that CTCs can get mesenchymal properties by departing from the primary tumour, but this does not necessarily result in a loss of epithelial properties (Rhim *et al*, 2012). Although we confirmed that CD44$^+$CD90$^+$ cells in HCC showed mesenchymal characteristics, some TICs, or CTCs with other TIC markers, might retain epithelial characteristics regardless of their mesenchymal state. Sun *et al* (2012) demonstrated that EpCAM$^+$ CTCs in blood were related to poor prognoses in HCC. Interestingly, EpCAM$^+$ CTCs do not show any CD90 expression, suggesting that these expressions may be preferentially exclusive, although both are crucial markers for TICs in HCC. We are currently attempting to clarify the genetic characteristics among different subpopulations of TICs.

The immunohistochemical analysis demonstrated that some HCC cells acquire the mesenchynmal phenotype by expressing Vimentin, Twist1, and CD44s. These cells compacted their cytoplasm and some of them clustered close to the portal vein. Similar compaction of the cytoplasm and the round shape is observed in most Vimentin-expressing cells. We previously reported that the mesenchymal phenotype with CD44 upregulation is associated with a poorer prognosis in HCC (Mima *et al*, 2012). Furthermore, CD44$^+$CD90$^+$ TIC in the blood was shown to be an important indicator for the tumour recurrence (Fan *et al*, 2011). Taken together, the mesenchymal characteristic appears to be quite important in understanding tumour development in HCC.

In conclusion, we suggest that CD44s-expressing cell showed mesenchymal phenotype and anoikis-resistant properties. Twist1 might be partially involved in the regulation of these properties. The results of this study further indicate that mesenchymal traits should be emphasised for therapeutic targets in HCC, not only in primary tumours but also in circulating metastatic cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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