Cell-surface Vimentin: A mislocalized protein for isolating csVimentin\(^+\)CD133\(^-\) novel stem-like hepatocellular carcinoma cells expressing EMT markers

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Recent advances in cancer stem cell biology have shown that cancer stem-like cells with epithelial–mesenchymal transition (EMT) phenotypes are more aggressive and cause relapse; however, absence of a specific marker to isolate these EMT stem-like cells hampers research in this direction. Cell surface markers have been identified for isolating cancer stem-like cells, but none has been identified for isolating cancer stem-like cells with EMT phenotype. Recently, we discovered that Vimentin, an intracellular EMT tumor cell marker, is present on the surface of colon metastatic tumor nodules in the liver. In our study, we examined the potential of targeting cell surface Vimentin (CSV) to isolate stem-like cancer cells with EMT phenotype, by using a specific CSV-binding antibody, 84-1. Using this antibody, we purified the CSV-positive, CD133-negative (csVim\(^-\)CD133\(^-\)) cell population from primary liver tumor cell suspensions and characterized for stem cell properties. The results of sphere assays and staining for the stem cell markers Sox2 and Oct4A demonstrated that csVim\(^-\)CD133\(^-\) cells have stem-like properties similar to csVim\(^-\)CD133\(^+\) population. Our investigation further revealed that the csVim\(^-\)CD133\(^-\) cells had EMT phenotypes, as evidenced by the presence of Twist and Slug in the nucleus, the absence of EpCAM on the cell surface and basal level of expression of epithelial marker E-cadherin. The csVimentin-negative CD133-positive stem cells do not have any EMT phenotypes. csVim\(^-\)CD133\(^-\) cells exhibited more aggressively metastatic in livers than csVim\(^-\)CD133\(^+\) cells. Our findings indicate that csVim\(^-\)CD133\(^-\) cells are promising targets for treatment and prevention of metastatic hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) is the most fatal form of cancer and the third leading cause of cancer-related death worldwide.\(^1\) In the United States, the number of HCC cases is increasing, and the age-adjusted incidence has doubled compared to previously reported rates.\(^2\) The development of liver cancer can be considered as the downstream effect of cirrhosis and fibrosis which occur owing to chronic insults or viral infection that progress over decades. Even with aggressive treatments such as liver transplantation, chemoembolization or surgical resection of a tumor,\(^3\) massive recurrence still occurs with HCC patients during their life span.\(^4\) Several studies have found an association between recurrence and cancer stem cells, which have shown chemoresistance in different types of tumors.\(^5\),\(^6\) Characterization of these slow-growing, dormant and drug-resistant cells may be useful for the development of treatments to improve HCC outcomes.

Phenotypic heterogeneity is one of the hallmarks of cancer stem cells. Increasing evidence suggests that liver cancer stem cells (LCSCs) are a highly heterogeneous population expressing different markers such as CD133,\(^7\) CD24,\(^4\) CD13,\(^8\) CD90\(^9\) and EpCAM.\(^10\) Our laboratory recently identified the well-known EMT marker Vimentin as a novel cell surface marker while screening metastatic liver nodules of patients with primary colorectal cancer. Previously, our laboratory showed that Vimentin is expressed on the surface of circulating tumor cells having EMT phenotypes.\(^11\),\(^12\) On the basis of these observations, we examined the potential of targeting Vimentin to isolate stem-like cancer cells with EMT phenotype, by using a cell surface Vimentin-binding antibody. We isolated from primary liver tumor a pure population of LCSCs that expressed Vimentin on their surface and were CD133 negative (csVim\(^-\)CD133\(^-\)). Upon characterization of this novel population, we discovered that csVim\(^-\)CD133\(^-\) cells have stem-like properties, differentiation ability and tumorigenic properties similar to csVim\(^-\)CD133\(^+\). However, unlike csVim\(^-\)CD133\(^+\) cells, csVim\(^-\)CD133\(^-\) cells had the epithelial–mesenchymal transition (EMT) phenotype and metastasized aggressively.

The study described in this article demonstrates the use of Vimentin for isolating a putative stem cell population that...
What’s new?
While cancer stem–like cells with epithelial–mesenchymal transition (EMT) phenotypes are known to be more aggressive and cause relapse, further advances are hampered by the absence of a specific marker. This study identifies for the first time the existence of Vimentin on the surface of liver cancer stem cells (LCSCs) and presents a separation technique to enrich EMT-positive LCSCs directly from primary tumor cells. csVim⁺CD133⁻ cells thus display stem-like properties, differentiation ability, and tumorigenic properties, have the EMT phenotype, and metastasize aggressively. The findings indicate that csVim⁺CD133⁻ cells are promising targets for treatment and prevention of metastatic hepatocellular carcinoma.

Material and Methods
Isolation and culture of primary tumor cell lines
A tumor-bearing MST⁻/+ mouse was euthanized and tumors were resected from liver. A single-cell suspension was prepared as described previously.²⁻¹³ Both csVimentin-positive and -negative populations were sorted using α-Vimentin using MACS column. Then CD133-positive and -negative cells from those two populations were sorted in a similar fashion. csVim⁺CD133⁺, csVim⁺CD133⁻ and csVim⁻CD133⁻ cells were cultured with supplements as described previously.⁷ Liver metastasis samples resected from human colorectal cancer patients were obtained in accordance with a protocol approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

Differentiation assays
Freshly sorted csVim⁺CD133⁻ cells were cultured in hepatocyte-specific condition as described previously¹⁴ and to differentiate into cholangiocytes, we followed this protocol.¹⁵ Albumin staining (1:50) was performed in differentiated hepatocytic cells using the protocol as described previously.¹⁶

Western blot
Cell lysis and Western blots with antibodies to E-cadherin or GAPDH were performed as previously described.¹⁷ For all samples, total protein was determined by the BCA method (Pierce). Western blots were detected by enhanced chemiluminescence (Cell Signaling Technology: Beverly, MA).

Subcutaneous inoculation of sorted cells in NSG mice
A total of 10⁵ freshly sorted csVim⁺CD133⁻ or csVim⁻CD133⁺ cells were inoculated in 8- to 10-week-old NOD scid gamma chain knockout mice (NSG) by subcutaneous injections. Mice were euthanized in accordance with the guidelines approved by the IACUC.

Immunofluorescent staining of tissue sections
Frozen sections of liver tumors were stained with albumin antibody (1:50 dilution), and a secondary antibody conjugated with Alexa Fluor-488 (1:300 dilution) was used.¹⁸

Gross metastasis assay
Fifty thousand freshly sorted csVim⁺CD133⁻ or csVim⁻CD133⁺ cells were injected intraosseously into the right legs of NSG mice. Right legs were amputated 2 weeks after inoculation, and the mice were sacrificed 2 weeks after amputation. Gross metastases were quantified in each liver. Representative images were taken with a digital camera.

Results
Because the rate of HCC relapse remains very high, we sought to characterize EMT-positive LCSCs, as this cell population is known to promote relapse and drug resistance in other cancers.¹⁹ To date, no specific markers have been identified for isolating these EMT-positive LCSCs. By screening for mislocalized surface expression of proteins on primary tumor cells, we identified the well-known EMT marker Vimentin on the surface of cells from metastatic liver nodules from patients with colorectal cancer (Supporting Information Fig. 1). We fractionated a novel cell population from primary HCC tumors that we termed csVim⁺CD133⁻. These cells were cuboidal and formed colonies. Their purity was confirmed by cell surface staining for Vimentin and CD133 (Supporting Information Fig. 2). To characterize stem cell properties of csVim⁺CD133⁻, we used csVim⁻CD133− LCSCs as CD133 is considered to be the most widely accepted stem cell marker in different tissues. We then assessed the stem-like properties of these cells by Matrigel sphere formation assay and by staining spheres for stem cell-associated markers Sox2 and Oct4A (Figs. 1a and 1b). To further establish the stem cell phenotype, csVim⁺CD133⁻ cells were stained for pluripotency markers, such as SSEA4, on their surface (Supporting Information Fig. 3). The results indicated that csVim⁺CD133⁻ cells have a stem-like phenotype.

To determine the purity of stem cells among the csVim⁺CD133⁻ and csVim⁻CD133⁺ subpopulations, we performed limiting dilution assays. The assays revealed that csVim⁺CD133⁻ cells formed spheres at a rate similar to that for the csVim⁺CD133⁻ population at seeding densities from 5 to 50 cells/well (Fig. 1c). Additionally, cell cycle analysis revealed that many more cells were in G1-S-G2 phase in the csVim⁺CD133⁻ population than in the csVim⁻CD133⁺ population (Fig. 1d). Taken together, these data indicated that csVim⁺CD133⁻ cells have stem-like properties and actively traverse the cell cycle.

Liver stem cells are known to undergo differentiation into hepatocytes and cholangiocytes under suitable culture conditions. To determine their differentiation potential, csVim⁺CD133⁻ cells were cultured in hepatocyte-specific conditions. We found that csVim⁺CD133⁻ cells underwent differentiation to hepatocytes, as evidenced by albumin expression and positive periodic acid-Schiff staining, indicating glycogen storage.
These cells also differentiated into cholangiocytes by forming star-shaped structures with branching and lumens (Fig. 2d).

To determine tumorigenic potential, we evaluated both in vitro and in vivo tumorigenic potentials of csVim\(^+\)CD133\(^-\) cells (Supporting Information Fig. 4). Experimental studies showed that csVim\(^+\)CD133\(^-\) cells are slow-growing tumor compared to csVim\(^-\)CD133\(^+\) cells. Also, we tested tumorigenic potential of csVim\(^-\)CD133\(^+\) cells, and showed similar result as csVim\(^+\)CD133\(^-\) cells in vivo (Supporting Information Fig. 4). Previous studies exhibited that slow-growing HCC tumors have a high recurrence rate and are chemoresistant (10). Thus, the tumors produced by csVim\(^-\)CD133\(^+\) cells may have a greater chance of relapse and metastatic potential than the tumors produced by csVim\(^+\)CD133\(^-\) cells.

Given that EMT is associated with aggressive tumors and metastasis and that Vimentin is considered an EMT marker, our detection of the EMT marker Twist and Slug in freshly sorted csVim\(^+\)CD133\(^-\) cells inside the nucleus was as expected. Interestingly, no expression of the epithelial cell surface marker EpCAM was detected in csVim\(^+\)CD133\(^-\) cells (Fig. 3a). In contrast, csVim\(^-\)CD133\(^+\) and csVim\(^+\)CD133\(^-\) cells did not express Twist and Slug but did express EpCAM on their surface. Also, Western blot analysis of E-cadherin, an epithelial marker, confirmed that csVim\(^+\)CD133\(^-\) cells are more mesenchymal type compared to csVim\(^-\)CD133\(^+\) cells.
Figure 2. A novel csVim<sup>−</sup>CD133<sup>−</sup> subpopulation enriched from primary HCC showed differentiation. (a) csVim<sup>−</sup>CD133<sup>−</sup> cells under normal conditions (nontreated) and in hepatocyte-specific medium. (b) Immunofluorescent confocal microscopy was used to detect albumin expression in csVim<sup>−</sup>CD133<sup>−</sup> cells in both nontreated and hepatocyte-specific media. (c) Glycogen storage content of csVim<sup>−</sup>CD133<sup>−</sup> cells in nontreated and hepatocyte-specific media, determined by periodic acid-Schiff staining. (d) csVim<sup>−</sup>CD133<sup>−</sup> cells formed lumen- and asterisk-shaped structures in cholangiocyte-specific culture conditions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Figure 3. csVim\(^{+}\)CD133\(^{-}\) cells exhibit EMT-like phenotypes and metastasize at a higher rate than do csVim\(^{+}\)CD133\(^{-}\) and csVim\(^{-}\)CD133\(^{-}\) cells. (a) Freshly sorted csVim\(^{+}\)CD133\(^{-}\), csVim\(^{-}\)CD133\(^{-}\) and csVim\(^{-}\)CD133\(^{-}\) cells were stained for EpCAM, Twist and Slug. (b) Fifty thousand csVim\(^{+}\)CD133\(^{-}\) (four mice) or csVim\(^{-}\)CD133\(^{-}\) cells (five mice) or csVim\(^{-}\)CD133\(^{-}\) cells (three mice) were injected intraosseously into the right legs of NSG mice. Two weeks postinoculation, the right legs were amputated. The images are representative of liver metastases observed at week 4 postinoculation. (c) Numbers of gross metastatic liver nodules. p value is 0.0486. (d) Hematoxylin–eosin-stained sections of metastatic liver tumor tissues (original magnification \( \times 400\)). One-way ANOVAs: \(*p < 0.05; **p < 0.01; ***p < 0.001\). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
cells (Fig. 3b). These expression patterns clearly indicate the EMT nature of csVim\(^+\)CD133\(^+\) cells, in contrast to csVim\(^-\)CD133\(^-\) and csVim\(^-\)CD133\(^-\) cells. Furthermore, intrahepatic inoculation in NSG mice demonstrated that csVim\(^+\)CD133\(^+\) cells produced more liver metastases than did the csVim\(^-\)CD133\(^+\) and csVim\(^-\)CD133\(^-\) population (Figs. 3c and 3d). Histological examination of liver tumor tissues showed no gross differences between csVim\(^+\)CD133\(^+\) and csVim\(^-\)CD133\(^+\) tumors (Fig. 3d).

Our identification of the novel csVim\(^+\)CD133\(^-\) cell population sheds new light on EMT-positive LCSCs. An understanding of the biology of this cell population can be used to reduce HCC metastasis.

**Discussion**

The transition from primary CSCs to EMT-positive CSCs is a significant step in HCC progression. Recent studies suggest that cancer stem cells having EMT phenotypes are associated with sensitivity and resistance to chemotherapy in various tumor models. Identification of this novel population will greatly facilitate to understand their intrinsic resistance toward neoplastics, dormancy and effective therapeutic strategy. In our study, we isolated from primary HCC a novel cell population, csVim\(^+\)CD133\(^-\), which exhibited stem-like cell properties by forming spheres in Matrigel, expressing Oct4A and Sox2, and differentiating into hepatocytes and cholangiocytes.

The presence of LCSCs in HCC has been proven, and chemotherapeutic and other strategies have been used to reduce LCSCs. However, early and late intrahepatic relapses occur frequently. Several lines of evidence suggest that EMT-positive cancer stem cells regulate sensitivity and resistance to chemotherapy in various tumor models. Our study for the first time identifies the existence of Vimentin on the surface of LCSCs and presents a separation technique to enrich EMT-positive LCSCs directly from primary tumor cells. These csVim\(^+\)CD133\(^-\) cells are considered EMT-positive cells because these cells also express low E-cadherin and high nuclear Twist and Slug, two other critical EMT markers beside Vimentin. In agreement with this conclusion, these csVim\(^+\)CD133\(^-\) cells have superiority metastatic potential compared to the well-known csVim\(^-\)CD133\(^+\) stem cells in vivo. As Vimentin is a universal EMT marker in different types of cancer models, its surface expression could be utilized to enrich and characterize EMT-positive CSCs from different tissue-specific cancers. Further investigation of EMT-positive CSCs may unveil some of the key mechanisms of cancer relapse and to target selected pathways to prevent recurrence in different tissue-specific cancers.

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**References**