Keratin 19, a cancer stem cell marker in human hepatocellular carcinoma

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Running Title

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Translational relevance

Cancer stem cells (CSCs) are strongly associated with various malignant properties of carcinomas including rapid tumor growth and the epithelial-mesenchymal transition (EMT). Therefore, CSCs have been attracting increasing attention as a new target for cancer therapies. In human hepatocellular carcinoma (HCC), keratin 19 (K19), a marker of hepatic progenitor cell, is known to be a marker of poor prognosis and a key player in tumor invasion. However, the relationship between K19 and HCC-CSCs is not clear. Therefore, we hypothesized that K19+ cells possess CSC characteristics and that K19 is a therapeutic target in HCC. Our study indicates that K19+ HCC cells possess CSC properties in vitro and in vivo, which are closely related to transforming growth factor beta (TGFβ)/Smad signaling and EMT, and would be sensitive to a TGFβ receptor 1 inhibitor. Further studies on K19+ HCC will provide novel therapeutic approaches for HCC treatment.
Abstract

Purpose

Keratin 19 (K19) is a known marker of poor prognosis and invasion in human hepatocellular carcinoma (HCC). However, the relationship between K19 and cancer stem cells (CSCs) is unclear. Here, we determined whether K19 can be used as a new CSC marker and therapeutic target in HCC.

Experimental Design

HCC cell lines were transfected with a K19 promoter-driven enhanced green fluorescence protein gene. CSC characteristics, epithelial-mesenchymal transition (EMT), and transforming growth factor beta (TGFβ)/Smad signaling were examined in fluorescence-activated cell sorting (FACS)-isolated K19+/K19− cells. K19 and TGFβ receptor 1 (TGFβR1) expression in 166 consecutive human HCC surgical specimens was examined immunohistochemically.

Results

FACS-isolated single K19+ cells showed self-renewal and differentiation into K19− cells, whereas single K19− cells did not produce K19+ cells. K19+ cells displayed high proliferation capacity and 5-fluorouracil resistance in vitro. Xenotransplantation into immunodeficient mice revealed that K19+ cells reproduced, differentiated into K19−
cells, and generated large tumors at a high frequency in vivo. K19+ cells were found to be involved in EMT and the activation of TGFβ/Smad signaling, and these properties were suppressed by K19 knockdown or treatment with a TGFβR1 inhibitor. The TGFβR1 inhibitor also showed high therapeutic effect against K19+ tumor in the mouse xenograft model. Immunohistochemistry of HCC specimens showed that compared to K19− patients, K19+ patients had significantly poorer recurrence-free survival and higher tumor TGFβR1 expression.

Conclusion

K19 is a new CSC marker associated with EMT and TGFβ/Smad signaling, and it would thus be a good therapeutic target for TGFβR1 inhibition.
Introduction

Recent developments in stem cell biology have revealed the existence of cancer stem cells (CSCs) in various cancers, including leukemia, breast cancer, and colon cancer.\(^{(1-6)}\) CSCs are a subset of cells with the ability to self-renew, generate a heterogeneous population of cancer cells, and initiate tumor formations.\(^{(7, 8)}\) These stem-cell-like features of CSCs contribute to rapid tumor growth, tumor resistance to chemotherapy/radiotherapy, and the epithelial-mesenchymal transition (EMT).\(^{(9-11)}\) Therefore, CSCs have been attracting increasing attention as a new target for cancer therapies.

Liver cancer, including hepatocellular carcinoma (HCC), is the second leading cause of cancer-related deaths worldwide. Although various therapies have been established and many others are under development, the prognosis of HCC is still poor. In HCC, epithelial cell adhesion molecule (EpCAM), cluster of differentiation (CD) 90, CD133, and sal-like protein 4 have been reported to be useful as CSC surface markers.\(^{(12-16)}\) However, molecular therapeutic targets for HCC-CSCs remain unestablished. During normal hepatic development, hepatic progenitor cells express both the hepatocyte marker albumin and the cholangiocyte marker keratin 19 (K19).\(^{(17, 18)}\) K19 is known to be a marker of poor prognosis in HCC in several studies.\(^{(19-21)}\) Especially, K19 is
reported as a key player in tumor invasion in HCC. However, the relationship between K19 and HCC-CSCs is not fully understood. Therefore, based on the similarity between normal tissue stem cells and CSCs, we hypothesized that K19+ cells possess CSC characteristics and that K19 is a therapeutic target in HCC.

The aims of this study were to demonstrate that K19+ cells have CSC properties in HCC, and to investigate whether K19+ cells could be a new therapeutic target. In this study, a transgene vector that expressed green fluorescence protein (EGFP) under the control of the human K19 promoter was transfected into four HCC cell lines to characterize K19+ cells as HCC-CSCs. Using fluorescence-activated cell sorting (FACS)-isolated K19+/K19− cells, we explored the relationship between K19 and known HCC-CSC markers, the involvement of K19+ cells in transforming growth factor beta (TGFb)/Smad signaling and EMT, and the therapeutic potential of a TGFb receptor 1 (TGFbR1) inhibitor. Moreover, the expression of K19 and TGFbR1 was investigated in human HCC surgical specimens.

**Materials and methods**

**Patients**
This study included 166 consecutive patients with HCC confirmed by pathologic analyses who had undergone a hepatic resection between January 2005 and December 2006 (resection group, \( n = 104 \)) or a liver transplantation between January 2005 and December 2008 (transplantation group, \( n = 62 \)) at Kyoto University Hospital. Thirty-four patients fulfilled the Milan criteria for transplantation, and 28 did not. The clinicopathological characteristics of the subjects are summarized in Supplemental Table 1. Tumor recurrence was investigated until the patient’s death or the end of this study (March 31, 2013). No patient was lost to follow-up. The follow-up period from surgery until death or the endpoint of this study was 58 to 2808 days (mean 1581 days) in resection group and 223 to 2857 days (mean 1915 days) in transplantation group.

Written informed consent for the use of resected tissue samples was obtained from all patients in accordance with the Declaration of Helsinki, and this study was approved by the institutional review committee of our hospital.

**Construction of the transgene vector**

We generated a transgene plasmid vector that expressed EGFP under the control of the human K19 promoter. The promoter activity of the 2952-bp 5′-flanking region of the human K19 gene (from −2952bp to the first ATG) was previously reported.\(^{(23)}\) The
plasmid vector pHCK-2952, in which the 2952-bp 5′-flanking region of the human K19 gene was inserted into the pGL3-Basic (Promega, Madison, WI, USA) vector, was kindly provided by Professor Shuichi Kaneko (Kanazawa University, Kanazawa, Japan). We obtained the human K19 promoter region from pHCK-2952 by using XhoI and HindIII (Takara Bio, Otsu, Japan), and we then ligated it with XhoI and HindIII-digested plasmid EGFP-1 (pEGFP1; BD Biosciences, Franklin Lakes, NJ, USA).

**Generation of transgenic HCC cell lines**

The human HCC cell lines (Huh7, HLF, PLC/PRF/5, and Hep3B) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were authenticated by short tandem repeat (STR) profiling before receipt and were propagated for less than 6 months after resuscitation. These cells were cultured at 37°C under 5% CO2 in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (ICN, Aurora, OH, USA), and penicillin/streptomycin (Meiji Seika, Tokyo, Japan).

The transgenic vector was transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer’s protocol. Stably transfected cells were selected in the
presence of 200 µg/mL G418 (Sigma, St Louis, MO, USA) over 30 days. We confirmed the proper transgene insertion by polymerase chain reaction (PCR) and immunocytochemistry.

**Immunohistochemistry and immunocytochemistry**

Immunohistochemistry and immunocytochemistry were performed as previously reported.(24, 25) Anti-human K19 mouse antibodies (Dako, Glostrup, Denmark) diluted at 1:100, anti-human Ki-67 mouse antibodies (BioLegend, San Diego, USA) diluted at 1:200, anti-TGFbR1 rabbit antibodies (Abcam, Tokyo, Japan) diluted at 1:50, and anti-GFP rabbit antibodies (Invitrogen) diluted at 1:200 were used as the primary antibodies. Alexa 488-conjugated or Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) for K19 staining and Alexa 594-conjugated donkey anti-rabbit IgG (Molecular Probes) for GFP or TGFbR1 staining were used as the secondary antibodies. All secondary antibodies were diluted at 1:500. Two investigators (T.K. and K.Y.) independently evaluated the slides.

**PCR, quantitative PCR, reverse transcription-PCR (RT-PCR), and quantitative RT-PCR**

(qRT-PCR)
The total RNA was extracted with RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) and RNase-free DNase (Qiagen). The genomic DNA was extracted with QuickGene-SP Kit (FUJIFILM, Tokyo, Japan). The Omniscript Reverse Transcription Kit (Qiagen) was used according to the manufacturer's protocol to reverse transcribe 1 µg total RNA into cDNA. Primers were generated for the following genes: K19, pEGFP1, CD90, EpCAM, TGFβR1, snail1, slug, E-cadherin, vimentin, multidrug-resistance protein-5 (MRP5), and actin-beta. Their sequences are summarized in Supplemental Table 2. PCR and RT-PCR assays were performed as previously described. We performed qPCR and qRT-PCR assays using SYBR-green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the ABI 7500 system (Applied Biosystems). Each target was run in triplicate, and expression levels were normalized to those of actin-beta.

**Flow cytometry and single-cell culture analysis**

We prepared the cultured cells as described previously. Dead cells were eliminated using 7-amino-actinomycin D (Beckman Coulter, Brea, CA, USA) staining. To determine the proportion of EpCAM+ or CD90+ cells, the cells were incubated with phycoerythrin (PE)-conjugated anti-human EpCAM antibody (BioLegend) or PE-conjugated anti-human CD90 antibody (BioLegend) on ice for 30 min.
PE-conjugated mouse IgG2b (BioLegend) was used as an isotype control for the
PE-conjugated EpCAM antibody. PE-conjugated mouse IgG1 (BioLegend) was used as
an isotype control of the PE-conjugated CD90 antibody. The cells were analyzed and
isolated using a FACSaria cell sorter (BD Biosciences).

We performed single-cell culture analyses as previously described.(27, 28) The
individual isolated cells were each sorted into 96-well culture plates using FACSaria
(BD Biosciences). We used a light microscope 10–16h after cell sorting to confirm that
each well contained only one cell. Following cell expansion after isolation of each clone,
we subjected the cells to flow cytometry.

**Cell proliferation assay, anchorage-independent growth assay, and sphere-forming
assay**

We inoculated the isolated EGFP+ and EGFP− cells differentiated from one EGFP+ cell
at a density of 1×10^3 cells per well in 96-well culture plates, which were then allowed to
grow for 7 days. The cell numbers were determined using the
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazori
um, inner salt (MTS) assay (Cell Titer 96 Aqueous One Solution Reagent, Promega),
according to the manufacturer’s protocol. After 1 h of incubation, the absorbance value was measured using a plate reader at 490 nm.

To examine the anchorage-independent growth, $1 \times 10^4$ EGFP$^+$ and EGFP$^-$ cells were suspended in 2.0 mL of 0.3% agar (Wako) supplemented with culture medium. The cell suspension was layered over the bottom layer of 2.0 mL of 0.6% agar. We counted the colonies 14 days after cell sorting.

To investigate the ability to form cell spheres, $1 \times 10^5$ EGFP$^+$ and EGFP$^-$ were seeded in 6-well ultra-low attachment plates (Corning Inc., NY, USA) in serum-free medium. We observed the spheres 5 days after cell sorting.

**Reagents and drug resistance assay**

5-Fluorouracil (5-FU) was purchased from Wako (Osaka, Japan) and was diluted directly with RPMI-1640 to the desired concentration. The TGFβR1 inhibitor LY2157299 was obtained from Axon Medchem (Groningen, NL). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) and diluted with RPMI-1640 or saline to the desired concentration with a final DMSO concentration of under 0.5%.

We investigated the resistance of the cells to 5-FU or LY2157299. EGFP$^+$ and EGFP$^-$ cells were cultured with 5-FU for 96h at concentrations of $1 \times 10^{-9}$, $1 \times 10^{-7}$, $1 \times$
10^{-5}, 1 \times 10^{-3}, and 1 \times 10^{-1} \text{ M, or with LY2157299 for 72h at concentrations of } 1 \times 10^{-9}, 1 \times 10^{-8}, 1 \times 10^{-7}, 1 \times 10^{-6}, \text{ and } 1 \times 10^{-5} \text{ M. After the culture, MTS assays were performed to determine the half-maximal inhibitory concentrations (IC50).}

Wound healing assay and migration assay

Wound healing assays were used to assess capacity for cell motility. We seeded the isolated EGFP+ and EGFP− cells differentiated from one EGFP+ cell at a density of 1 \times 10^6 \text{ cells per well in 35-mm culture dishes. On reaching full confluency, the cell layer was scratched with a 10-µL plastic tip and then cultured with low serum (2% fetal bovine serum) culture medium with or without 0.5 µM LY2157299. Micrographs were taken at 24 or 48 h after the scratch.}

For migration assays, 8-µm-pore 24-well cell culture plates (Corning Inc.) coated with type I collagen were used. We plated 2.5 \times 10^4 \text{ EGFP+ and EGFP− cells in the upper chamber with serum-free medium; in the lower chamber, normal culture medium containing 10% fetal bovine serum was added. After 48 h of incubation, the cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed with 4% paraformaldehyde and stained using the Diff-Quick staining kit (Sysmex, Kobe, Japan).}
Western blot analysis

Western blot analysis was performed as previously reported. (29) Primary antibodies recognizing phospho-smad2 (pSmad2) (Ser465/467, #3108; Cell Signaling, Tokyo, Japan), Smad2 (#5339, Cell Signaling), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-25778, Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used at 1:1000 dilution.

K19 knockdown with RNA interference

We transfected K19-siRNA (#4427037-s7998 or #4427037-s7999, Invitrogen) or control-siRNA (#4390843, Invitrogen) into HCC cells using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. The final concentrations were 10nM. K19 expression was significantly downregulated by both K19 siRNAs (Supplemental Fig. 1D). According to the same result acquired with both siRNAs, K19-siRNA (#4427037-s7999) was shown as representative data. For western blot analysis, HCC cells were harvested 48 h post-transfection.

Xenotransplantation
Male 6 to 10-week-old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Charles River Laboratories, Inc., Kanagawa, Japan) were used as recipients for xenotransplantation. The isolated $1 \times 10^4$ EGFP$^+$ or EGFP$^-$ cells derived from a single EGFP$^+$ cell were suspended in 200 µL of a mixture of serum-free medium and Matrigel (BD Biosciences) (1:1 volume). The mixture was injected subcutaneously through a 26-gauge needle into the right and left dorsal areas of anesthetized NOD/SCID mice. We monitored tumor formation and tumor size twice a week, and dissected out the tumors 11 weeks after engraftment.

To investigate the differentiation ability \textit{in vivo}, we performed serial transplantation. Tumors generated from single-cell-derived EGFP$^+$ or EGFP$^-$ cells were harvested, and then digested with collagenase solution for 30 min at 37°C. After rinsing the tumors in phosphate-buffered saline (PBS), we analyzed and sorted the cells using the FACSAria. Prior to the second transplantation, the harvested cells were cultured in G418-containing medium for at least 7 days to eliminate cells that originated from the host mice. Thereafter, we sorted these cells according to the EGFP fluorescence, and transplanted $1 \times 10^4$ EGFP$^+$ or EGFP$^-$ cells into NOD/SCID mice in the same way as in the first transplantation.
Experiments on a TGFbR1 inhibitor in a mouse xenograft model

To investigate the therapeutic effect of the TGFbR1 inhibitor \textit{in vivo}, we performed experiments using LY2157299 in a mouse xenograft model. Isolated $1 \times 10^6$ EGFP$^+$ or EGFP$^-$ cells were injected subcutaneously into the right and left dorsal areas of anesthetized NOD/SCID mice. Thereafter, 24 days after the xenotransplantation, we treated bid with either saline (control group; $n = 5$) or 75 mg/kg LY2157299 (treated group; $n = 5$) for 10 consecutive days. Tumor size was measured every 3 days for 36 days after engraftment; afterwards, the animals were sacrificed.

All animal experimental procedures were performed according to the Animal Protection Guidelines of Kyoto University.

Statistical analysis

The statistical analyses were performed using SPSS version 17.0 (SPSS Inc., IL, USA) and GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Student’s $t$ test, $F$ test, Mann-Whitney $U$ test, Fisher’s exact test or chi-squared test, and repeated-measures analyses of variances were used for assessment. The mean $\pm$ SD of three or more independent experiments is reported.
Recurrence-free survival (RFS) and overall survival (OS) after the operation were calculated using the Kaplan-Meier method and analyzed with the log-rank test. Significant variables from the univariate analysis were entered in the multivariate analysis using a Cox regression model with forward stepwise selection. Statistical significance was defined as $P < 0.05$.

Results

K19+ cells in HCC cell lines

K19 expression was evaluated in the Huh7, PLC/PRF/5, and Hep3B HCC cell lines (Supplemental Fig. 1A). The K19-EGFP reporter vector was transfected into the four HCC cell lines. Huh7, PLC/PRF/5, and Hep3B cells expressed EGFP fluorescence (Supplemental Fig. 1B), whereas HLF cells did not, even though they possessed the transgene in their genomic DNA (Supplemental Fig. 1B). We also confirmed, by immunocytochemistry, that the cells expressing K19 corresponded to the cells expressing EGFP and that the ratio of K19+EGFP+ to K19+ cells was over 95% (Supplemental Fig. 1B). FACS analyses showed that 20.6% ± 3.9% of the Huh7 cells, 14.8% ± 2.7% of the PLC/PRF/5 cells and 26.7% ± 5.9% of the Hep3B cells ($n = 3$) expressed EGFP (Supplemental Fig. 1C). The sorted EGFP+ cells expressed both K19
and EGFP, whereas the sorted EGFP− cells did not express either molecule (Supplemental Fig. 1C). Additionally, qPCR assays showed that the K19-EGFP reporter vector was almost equally transfected into both EGFP+ and EGFP− cells (Supplemental Fig. 1C). These results indicated that the EGFP+ cells corresponded to the K19+ cells and that cell sorting could divide the Huh7, PLC/PRF/5, and Hep3B cells according to their K19 production. Therefore, we performed further investigations of the K19+ cells in Huh7, PLC/PRF/5, and Hep3B cells to determine whether they were cancer stem cells.

**Single-cell culture analysis**

We performed single-cell culture analyses to examine self-renewal activity and multipotency. Notably, single K19+ Huh7 cells generated both K19+ and K19− cell fractions, although single K19− Huh7 cells produced only a K19− cell fraction (Fig. 1A). These findings were reproducible in three trials (three EGFP+/EGFP− clones). Similar results were obtained with PLC/PRF/5 and Hep3B cells (Supplemental Fig. 2A). These results indicate that K19+ cells have self-renewal activity and the ability to differentiate into K19− cells *in vitro.*
Cell proliferation assay, anchorage-independent growth assay, and sphere-forming assay

Compared to the K19− Huh7 cells, K19+ Huh7 cells showed higher proliferation activity (P < 0.01, n = 3; Fig. 1B) and exhibited a higher ability to form colonies in soft agar (P < 0.05, n = 3; Fig. 1C). In addition, the sphere assays revealed that K19+ Huh7 cells formed cell spheres 5 days after cell sorting, whereas the K19− Huh7 cells did not (Fig. 1C). Similar results were obtained with PLC/PRF/5 and Hep3B cells (Supplemental Fig. 2B, 2C). These findings suggest that the K19+ cells have stronger tumorigenic and malignant potential than K19− cells in vitro.

Drug resistance to 5-FU

Because CSCs are known to show strong resistance to chemotherapy, we examined 5-fluorouracil (5-FU) resistance of K19+ and K19− cells. The half-maximal inhibitory concentrations (IC50) values for 5-FU were 3.7 × 10−4 M for K19+ Huh7, 2.9 × 10−5 M for K19− Huh7, 1.7 × 10−4 M for K19+ PLC/PRF/5, 2.8 × 10−5 M for K19− PLC/PRF/5, 2.8 × 10−4 M for K19+ Hep3B, and 2.2 × 10−5 M for K19− Hep3B. In all three cell lines, K19+ cells were significantly more resistant to 5-FU than K19− cells (Fig. 1D, Supplemental Fig. 2D, P < 0.01).
To investigate the mechanism responsible for 5-FU resistance, we analyzed the mRNA expressions of multidrug-resistance protein-5 (MRP5), a key drug transporter for 5-FU. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays of the three cell lines showed that the MRP5 expression of K19+ cells was three or more fold higher than that of K19− cells (Fig. 1D, Supplemental Fig. 2D, *P* < 0.05).

**Xenotransplantation into immunodeficient mice**

To explore cancer stem cell properties *in vivo*, we transplanted 1 × 10^4 K19+ or K19− cells derived from one K19+ cell into the backs of NOD/SCID mice. K19+ Huh7 cells produced larger, hypervascular tumors in 10/10 mice, whereas K19− Huh7 cells produced smaller, pale-looking tumors in 4/10 mice (Fig. 2A, 2B). FACS analyses and immunohistological assays revealed that the tumors from K19+ cells had both K19+ and K19− cell fractions, whereas the tumors from K19− cells had only K19− cell fractions (Fig. 2C, 2D). In serial transplantation, we transplanted 1.0 × 10^4 K19+ or K19− cells isolated from tumors produced by K19+ cells. The tumors derived from K19+ cells also contained K19+ and K19− cell fractions. In contrast, the tumors derived from K19− cells consisted of only K19− cell fractions (Fig. 2D). Similar results were obtained with PLC/PRF/5 and Hep3B cells (Supplemental Fig. 3A–D). These results demonstrate that K19+ cells...
possess the capacity to replicate themselves, to generate heterogeneous lineages of cancer cells, and to initiate tumors \textit{in vivo}.

\textbf{Relationship between K19 and reported HCC-CSCs markers}

In the three cell lines that expressed K19, EpCAM expression was observed in Huh7 and Hep3B cells, whereas CD90 expression was observed in HLF and PLC/PRF/5 cells (Fig. 3A). qRT-PCR assays demonstrated significantly higher expression of EpCAM in K19\(^+\) Huh7 and Hep3B cells than in K19\(^-\) cells \((P < 0.05, \text{ Fig. } 3B)\). K19\(^+\) PLC/PRF/5 cells showed higher CD90 expression than K19\(^-\) cells, although the difference was not statistically significant \((P = 0.061, \text{ Fig. } 3B)\). In FACS analyses, the proportion of EpCAM\(^+\) cells in K19\(^+\) Huh7/Hep3B cells tended to be larger than that in K19\(^-\) cells (Fig. 3C). Only a few CD90\(^+\) cells were seen in K19\(^+\) PLC/PRF/5 cells or in K19\(^-\) PLCPRF/5 cells (Fig. 3C).

We further explored the growth potential of K19\(^+\)EpCAM\(^+\), K19\(^-\)EpCAM\(^+\), and K19\(^-\)EpCAM\(^-\) cells. Cell proliferation assays indicated that K19\(^+\)EpCAM\(^+\) Huh7/Hep3B cells had greater proliferation capability than K19\(^-\)EpCAM\(^+\) cells \((P < 0.01, \text{ Fig. } 3D)\). K19\(^-\)EpCAM\(^+\) cells showed slightly faster growth than K19\(^-\)EpCAM\(^-\) cells, although the difference was not statistically significant \((\text{Huh7, } P = 0.39; \text{ Hep3B, } P = 0.75; \text{ Fig. } 3D)\).
These data suggest that the pattern of K19 expression in K19+ HCC cells may be similar to that of EpCAM, and that K19 may play a stronger role than EpCAM in the proliferative capacity of HCC-CSCs.

**Involvement of K19 expression in EMT and TGFβ/Smad signaling**

To investigate the involvement of K19 in EMT and TGFβ/Smad signaling, we evaluated the expression pattern of EMT-related markers in HCC cell lines (Fig. 4A). qRT-PCR assays revealed that K19+ Huh7 cells showed an EMT gene expression profile: down-regulation of E-cadherin and up-regulation of TGFβR1, snail1, slug, and vimentin (P < 0.05, Fig. 4A). Wound healing and migration assays revealed that K19+ cells possessed significantly greater motility than K19- cells (Fig. 4A). This high motility of K19+ cells was suppressed by K19 knockdown via RNA interference (Supplemental Fig. 1D). These data indicate that K19+ HCC cells have a strong association with EMT.

With the primary focus on strong TGFβR1 expression of K19+ cells, we explored TGFβ/Smad signaling in K19+ cells. Western blot analysis of pSmad2/Smad2 showed that K19+ Huh7 cells expressed pSmad2 more strongly than did K19- cells (Fig. 4B). Furthermore, K19 knockdown in Huh7 K19+/K19- cells resulted in significant suppression of pSmad2 expression and proliferation ability in K19+ Huh7 cells, whereas
these effects were not observed in K19− cells (Fig. 4B). These results indicate that TGFβ/Smad signaling is activated in K19+ cells and might confer the high proliferation ability to K19+ cells.

The effectiveness of the TGFβR1 inhibitor LY2157299 as a new target therapy against K19+ cells

We next focused on activation of TGFβ/Smad signaling in K19+ cells; for this purpose, we examined the effectiveness of LY2157299 to K19+ cells in vitro and in vivo. The IC_{50} values for the TGFβR1 inhibitor were 6.2 × 10^{-8} M for K19+ Huh7 and 1.7 × 10^{-7} M for K19− Huh7 cells. K19+ Huh7 cells showed significantly higher sensitivity to LY2157299 than did K19− cells (P = 0.02, Fig. 4C). Additionally, LY2157299 abolished the motility advantage of K19+ cells and suppressed pSmad2 expression in K19+ cells (Fig. 4C).

Notably, in these experiments, K19+ tumors treated with LY2157299 were significantly smaller than K19+ tumors treated with saline, whereas LY2157299 showed no significant effectiveness against K19− tumors (K19+, P< 0.01; K19−, n.s.; not significant, Fig. 4D). Immunohistochemistry analyses showed that Ki-67 positive cells in LY2157299-treated K19+ tumors were significantly fewer than those in saline-treated
K19+ tumors. Collectively, these data indicate that LY2157299 would be useful for the treatment of K19+ HCC in vitro and in vivo.

**K19 expression in human HCC surgical specimens**

To examine K19 expression in human HCC clinical samples, 166 surgically resected HCC tumors were subjected to immunohistochemistry. K19 expression in HCC nodules was observed in 11/104 (11%) patients in the resection group and in 7/62 (11%) patients in the transplantation group (Fig. 5A). K19 expression was detected in a large proportion or small proportion of HCC tissues (Fig. 5A). In the resection group, the median recurrence-free survival (RFS) and overall survival (OS) were, respectively, 417 and 1099 days for K19+ patients, and 1038 and 1857 days for K19− patients (Fig. 5B). In the transplantation group, the median RFS and OS were, respectively, 1616 and 1700 days for K19+ patients, and 2740 and 2657 days for K19− patients (Fig. 5B). In both groups, K19+ patients had significantly lower RFS (resection group, \( P < 0.001 \); transplantation group, \( P = 0.001 \)) and OS (resection group, \( P = 0.002 \); transplantation group, \( P = 0.016 \)).

In the resection group, the log-rank test revealed that K19 expression, preoperative low albumin concentration, portal vein invasion, and liver cirrhosis were associated
with worsened RFS (Supplemental Table 3). Multivariate analysis demonstrated that K19 expression and portal vein invasion were independent predictors of postoperative recurrence (Table 1). K19 expression, low albumin concentration, portal vein invasion, and liver cirrhosis were associated with OS by log-rank test (Supplemental Table 3).

Multivariate analysis showed that low albumin concentration, portal vein invasion and liver cirrhosis were independent predictors of OS, whereas K19 expression was not (Table 1). In the transplantation group, K19 expression, tumor differentiation, and portal invasion significantly decreased both RFS and OS (Supplemental Table 4).

Multivariate analysis revealed that K19 expression predicted postoperative recurrence (Table 1). Regarding OS, K19 expression and portal vein invasion were independent predictors of postoperative survival in multivariate analysis (Table 1). Additionally, K19 expression tended to relate to tumor size in the resection (Fig. 5C, \( P = 0.086 \)) and transplantation groups (Fig. 5C, \( P = 0.095 \)).

**Correlation between K19 and TGF\( \beta \)R1 expression in human HCC surgical specimens**

To investigate the therapeutic potential of a TGF\( \beta \)R1 inhibitor for K19+ HCC, we performed double immunofluorescence staining for K19 and TGF\( \beta \)R1 in HCC surgical specimens. TGF\( \beta \)R1 expression in HCC nodules was observed in 9/11 (82%) K19+ and
18/93 (19%) K19− patients in the resection group, and in 5/7 (71%) K19+ and 15/55 (27%) K19− patients in the transplantation group. In K19+TGFβR1+ patients, TGFβR1 expression was detected in K19+ and K19− HCC cells (Fig. 5D). Many K19+TGFβR1+ cells were seen in the invasive front of HCC (Fig. 5D, upper panel). In both groups, K19 expression was significantly correlated with TGFβR1 expression (resection group, \(P < 0.01\); transplantation group, \(P = 0.019\)).

**Discussion**

HCC has one of the poorest prognoses among carcinomas, despite advances in treatment such as surgical resection, liver transplantation, radiofrequency ablation (RFA), and regional/systemic chemotherapy. Identification of CSCs and CSC-related therapeutic targets is necessary for improving HCC treatment outcome. Our results showed that K19 can serve as a marker of HCC-CSCs with stem cell characteristics and tumor-initiating capability. Single-cell culture analyses showed that K19+ cells had self-renewal ability and differentiation potency whereas K19− cells could only replicate. Cell proliferation and anchorage-independent growth assays revealed the higher malignant potency of K19+ cells. K19+ cells were more resistant to 5-FU based on their higher MRP5 expression. Additionally, xenotransplantation experiments showed that
K19+ cells could self-renew, differentiate into K19− cells, and generate larger tumors with a higher incidence in vivo. These findings strongly suggest that K19+ cells in HCC possess CSC characteristics.

Immunohistochemical analyses of 166 patients revealed that K19 expression was an independent predictor of postoperative recurrence in both resection and transplantation patients, and it was also an independent predictor for OS in the transplantation group. However, in the resection group, K19 expression tended to relate to poor postoperative survival although the difference was not statistically significant (P = 0.088). This result may be attributed to the fact that patients in the resection group have more HCC treatment choices than those in the transplantation group after HCC recurrence, e.g., RFA, transarterial chemoembolization, and systemic chemotherapy. Additionally, K19 expression tended to relate to tumor size in both groups, portal invasion in the resection group (P = 0.09) and tumor differentiation in the transplantation group (P = 0.012). These results indicate that K19+ HCC cells with CSC properties could be deeply involved in postoperative early tumor recurrence.

EpCAM and CD90 have been used to identify HCC-CSCs. Our results revealed that K19+ cells displayed significantly higher EpCAM gene expression in quantitative RT-PCR analyses and that the K19+ cell fraction tended to contain more EpCAM+ cells.
in FACS analyses. Similar to recent studies reporting that EpCAM was associated with
tumor size,(31) our xenotransplantation assays revealed that K19+ cells generated
larger tumors. Moreover, a proliferation assay of K19+EPCAM+, K19−EpCAM+, and
K19−EpCAM− cells suggested that K19 may be more strongly associated with rapid
tumor growth than EpCAM.

We found that TGFb/Smad signaling was activated in K19+ cells and suppressed by
K19 knockdown. Moreover, K19 knockdown also suppressed the aggressiveness of K19+
cells. These results indicate that TGFb/Smad signaling is involved in conferring the
high proliferation ability to K19+ cells.

K19 has been reported by Govaere et al (22) as a key player in HCC invasion. They
reported that K19-positive HCCs highly express invasion or metastasis-related
markers.(22) Our results also showed a close correlation between K19 and the EMT
which is known as a major mediator of tumor migration/invasion. During EMT, cancer
cells shed their epithelial characteristics and acquire more migratory mesenchymal
cell-like properties. In this study, we confirmed that K19+ cells express several genes
associated with EMT and have higher motility and migration than K19− cells.

TGFb/Smad signaling is known to increase the growth of cancer cells with mesenchymal
properties. Our results suggest that K19+ cells acquire mesenchymal characteristics
through EMT and exhibit high proliferation ability owing to the activation of TGFβ/Smad signaling.

Notably, we found that the TGFβR1 inhibitor LY2157299 would be useful as a new therapy against K19+ HCC. K19+ cells exhibited high LY2157299 sensitivity in IC$_{50}$ drug resistance assays. Combined with the result that LY2157299 suppressed the activation of TGFβ/Smad signaling in K19+ cells, LY2157299 should suppress the aggressiveness of K19+ cells thorough the suppression of TGFβ/Smad signaling. The high antitumor effect of LY2157299 to K19+ cells was also observed in the mouse xenograft model examined in the present study. Furthermore, immunohistochemistry of samples from HCC patients showed that K19 expression is significantly correlated with TGFβR1 expression. These results suggest that HCC patients with K19 expression could be sensitive to LY2157299. A TGFβR1 inhibitor was reported to block HCC migration, vascular invasion, and growth through regulation of neo-angiogenesis regulation.(32-34) Currently, LY2157299 is in phase II clinical trials.

In this study, we were able to isolate K19+ and K19- HCC cells. A comprehensive analysis of these cells may detect surrogate marker for a K19 that could be easily evaluated by laboratory tests. Such a marker should be useful in HCC treatments.
because we could then identify K19+ patients without immunohistochemistry or RT-PCR analysis of HCC tissues.

In conclusion, our study indicates that K19+ HCC cells possess CSC properties, are closely associated with TGFβ/Smad signaling and EMT, and would be sensitive to a TGFβR1 inhibitor. We believe that further studies of K19-related mechanisms will provide novel therapeutic approaches in HCC treatment.

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Differential Inhibition of the TGF-β Signaling Pathway in HCC Cells Using the Small 

Molecule Inhibitor LY2157299 and the D10 Monoclonal Antibody against TGF-β 

Table 1

**Multivariate analysis of factors predicting postoperative prognosis**

<table>
<thead>
<tr>
<th>Postoperative recurrence in the resection group</th>
<th>Hazard Ratio (95% CI)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Variable</td>
<td></td>
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<tr>
<td>K19 expression</td>
<td>2.58 (1.16–5.76)</td>
<td>0.021</td>
</tr>
<tr>
<td>Portal invasion</td>
<td>2.01 (1.24–3.25)</td>
<td>0.004</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>1.47 (0.90–2.39)</td>
<td>0.121</td>
</tr>
<tr>
<td>Albumin (&lt; 3.5 g/dl)</td>
<td>1.39 (0.72–2.66)</td>
<td>0.325</td>
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</table>

<table>
<thead>
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<th>Postoperative survival in the resection group</th>
<th>Hazard Ratio (95% CI)</th>
<th>P value</th>
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<tr>
<td>Variable</td>
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<tr>
<td>Portal invasion</td>
<td>2.15 (1.21–3.83)</td>
<td>0.009</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>1.96 (1.09–3.55)</td>
<td>0.026</td>
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<tr>
<td>Albumin (&lt;3.5 g/dl)</td>
<td>2.00 (1.01–3.94)</td>
<td>0.046</td>
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<tr>
<td>K19 expression</td>
<td>2.04 (0.90–4.63)</td>
<td>0.088</td>
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<tr>
<th>Postoperative recurrence in the transplantation group</th>
<th>Hazard Ratio (95% CI)</th>
<th>P value</th>
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</thead>
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<tr>
<td>Variable</td>
<td>Hazard Ratio (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------</td>
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<tr>
<td>K19 expression</td>
<td>18.4 (1.97–171.3)</td>
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<tr>
<td>Poorly differentiated</td>
<td>0.75 (0.10–5.58)</td>
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<tr>
<td>Portal invasion</td>
<td>NA</td>
<td>0.909</td>
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Postoperative survival in the transplantation group

<table>
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<th>Variable</th>
<th>Hazard Ratio (95% CI)</th>
<th>P value</th>
</tr>
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<tr>
<td>Portal invasion</td>
<td>7.60 (1.63–35.6)</td>
<td>0.01</td>
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<tr>
<td>K19 expression</td>
<td>5.72 (1.21–27.1)</td>
<td>0.028</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>1.29 (0.28–5.96)</td>
<td>0.75</td>
</tr>
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</table>

Abbreviation: K19, cytokeratin 19; CI, confidence interval; NA, not available.
Figure legends

**Fig. 1** Cancer stem cell properties of K19+ Huh7 cells *in vitro*. (A) Single-cell culture analyses of K19+ and K19− Huh7 cells. Single K19+ cells sorted from P5 quadrangle generated both K19+ and K19− cell fractions (middle panel), whereas single K19− cells sorted from P4 quadrangle produced only K19− cell fractions (right panel). The vertical axis indicates 7-amino-actinomycin D fluorescence and the horizontal axis indicates the intensity of enhanced green fluorescence protein-K19. (B) Cell proliferation assays of K19+ and K19− Huh7 cells (repeated-measures ANOVA, *P* < 0.01). (C) Colony numbers and light microscopic images in the anchorage-independent growth assay (left and middle panels, Student’s *t*-test, *P* < 0.05), and phase-contrast images in the sphere-forming assay (right panel). The original magnification was 20×. (D) Half-maximal inhibitory concentration (IC50) of 5-fluorouracil (5-FU) in K19+ and K19− Huh7 cells (left panel, *F*-test, *P* < 0.01) and qRT-PCR analyses of K19+ and K19− Huh7 cells for multidrug resistance protein-5 (right panel, Student’s *t*-test, *P* < 0.05).

**Fig. 2** Cancer stem cell properties of K19+ Huh7 cells *in vivo*. (A) Tumors produced by K19+ and K19− Huh7 cells. (B) Sequential tumor size generated from K19+ or K19− Huh7 cells in NOD/SCID mice (repeated-measures ANOVA, *P* < 0.01). Data are shown
as the mean ± SD (K19⁺ Huh7: n = 10, K19⁻ Huh7: n = 4). (C) Hematoxylin-eosin and K19 staining of tumors. Scale bar represents 100 µm. (D) FACS analyses of initial and serial transplantation. In initial transplantation, tumors were generated from 1 × 10⁴ sorted K19⁺ (upper right panel) or K19⁻ (upper left panel) Huh7 cells derived from a single K19⁺ Huh7 cell. In serial transplantation, tumors were generated from sorted 1 × 10⁴ K19⁺ (lower right panel) or K19⁻ (lower left panel) Huh7 cells derived from tumors produced by K19⁺ cells.

**Fig. 3 Relationship between K19 and reported HCC-CSC markers.** (A) PCR analysis of HCC cell lines for reported HCC-CSC markers. (B) qRT-PCR analysis of K19⁺ and K19⁻ cells for EpCAM (Student’s t-test, Huh7, P < 0.05; Hep3B, P < 0.05) and CD90 (Student’s t-test, P = 0.061). Data are shown as the mean ± SD. (C) FACS analyses of K19⁺ and K19⁻ cells. The vertical axis indicates the cell number and horizontal axis indicates the intensity of PE-EpCAM or PE-CD90. (D) Cell proliferation assays of K19⁺EpCAM⁺, K19⁻EpCAM⁺, and K19⁻EpCAM⁻ cells (repeated-measures ANOVA, ***P < 0.01, **P = 0.39, ****P = 0.75).

**Fig. 4 Involvement of K19 expression in the epithelial mesenchymal transition (EMT)**
and transforming growth factor beta (TGFβ)/Smad signaling, and the therapeutic effect of LY2157299 against K19+ cells. (A) qRT-PCR analysis of K19+ and K19− Huh7 cells for EMT-related genes (upper panel, Student’s t-test, P < 0.05). Data are shown as the mean ± SD. Phase-contrast images obtained at 0 h or 24 h after the scratch and of the remained wounds at 24 h after the scratch in wound-healing assays of K19+ and K19− cells without LY2157299 treatment (right upper panel, Student’s t-test, P < 0.05). Original magnification was 20×. Right lower panel shows migration assays and migrated cell numbers of K19+ and K19− cells (Student’s t-test, P < 0.05). Scale bar represents 100 µm. (B) Western blot analysis for phospho-Smad2 (pSmad2) and Smad2 (upper panel, KD: knockdown by K19-siRNA, control: negative control by control-siRNA). Lower panel shows cell proliferation assays of K19+ and K19− Huh7 cells with K19-siRNA or control-siRNA (repeated-measures ANOVA, K19+, P < 0.05; K19−, n.s.: not significant). (C) IC50 values of K19+ and K19− cells for LY2157299 (left panel, F-test, P = 0.02). Middle panel shows phase-contrast images obtained at 0 h or 48 h after the scratch and the remained wounds at 48 h after the scratch in wound-healing assays with 0.5 µM LY2157299 treatment (Student’s t-test, n.s.: not significant). Original magnification was 20×. Right panel shows western blot analysis of K19+ and K19− cells with 24 h LY2157299 or control treatment for pSmad2 and Smad2. (D)
Tumors produced by K19+ and K19− Huh7 cells and sequential tumor size with LY2157299 or control treatment in NOD/SCID mice (repeated-measures ANOVA, K19+, $P < 0.01$; K19−, n.s.; not significant). Data are shown as the mean ± SD (n = 5). Ki-67 staining of tumors and Ki-67 positive cell numbers (lower panel, Student’s $t$-test, K19+, $P < 0.01$; K19−, n.s.; not significant).

Fig. 5 Immunohistochemistry analyses of human HCC surgical specimens. (A) K19 staining in samples from two patients in the resection group (left panel) and two patients in the transplantation group (right panel). Scale bar represents 100 µm. (B) Recurrence-free survival and overall survival days in the resection group and in the transplantation group according to K19 expression in the HCC tissue. (C) Tumor size of K19+ and K19− patients (Mann-Whitney $U$ test, resection group: $P = 0.086$, transplantation group: $P = 0.095$). (D) K19 and TGFbR1 staining in samples from two HCC patients. Many K19+TGFbR1+ cells were observed in the invasive front. Scale bar represents 100 µm.
Figure 1

A

Huh7

one K19+ cell derived

one K19− cell derived

single-cell culture

7-AAD

EGFP-K19

B

C

K19+

K19−

P<0.01

P<0.05

D

K19+

K19−

Relative mRNA expression of MRPs

Percent survival

P<0.01

P<0.05
Figure 2

A. Huh7

B. Tumor incidence

- K19+ 10/10 mice
- K19- 4/10 mice

P < 0.01

C. Initial transplantation

K19- tumor

K19+ tumor

D. Serial transplantation

Initial transplantation

K19+ tumor

7-AAD

EGFP-K19

Serial transplantation

K19+ tumor

7-AAD

EGFP-K19
Figure 3

A

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<tr>
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<th>HLF</th>
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<th>Hep3B</th>
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<td>EpCAM</td>
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<tr>
<td>ACTb</td>
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B

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<th>Hep3B</th>
<th>PLC/PRF/5</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>CD90</td>
<td></td>
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<td></td>
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</tbody>
</table>

P < 0.05

P < 0.05

P = 0.061

C

Huh7 control

PLC/PRF/5 control

Hep3B control

PLC/PRF/5 control

D

Huh7

Hep3B
**Figure 4**

**A**

- **TGFr1**
  - $P<0.05$
- **snail1**
  - $P<0.05$

**E-cadherin**
- $P<0.05$

**Vimentin**
- $P<0.05$

**B**

- **Huh7**
  - **K19**
  - **K19-**

- **0h**
- **24h**

- **pSmad2**
- **Smad2**
- **GAPDH**

- **Absorbance** (480nm)

**C**

- **Huh7**
  - **LY2157299**

- **0h**
- **48h**

- **pSmad2**
- **Smad2**
- **GAPDH**

- **n.s.**

**D**

- **Control**
- **LY2157299**

- **K19**
- **K19-**

- **K19**
- **K19-**

- **K19+ control**
- **K19+ LY2157299**
- **K19- control**
- **K19- LY2157299**

- **Tumor size (mm)**
- **Days after transplantation**

- **Ki-67 positive cell number**

- **P<0.01**
- **n.s.**
Figure 5

A  
Resection group  
Transplantation group

B  
Resection group  
Transplantation group

C  
Resection group  
Transplantation group

D  
K19 TGFbR1 DAPI

K19 TGFbR1 DAPI

P = 0.001  
P = 0.002

P = 0.002  
P = 0.016

P = 0.086  
P = 0.095

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Takayuki Kawai, Kentaro Yasuchika, Takamichi Ishii, et al.

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