ORIGINAL RESEARCH

Activation of the sonic hedgehog signaling pathway occurs in the CD133 positive cells of mouse liver cancer Hepa 1–6 cells

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Background: The important role of cancer stem cells in carcinogenesis has been emphasized in research. CD133+ cells have been mentioned as liver cancer stem cells in hepatocellular carcinoma (HCC). Some researchers have proposed that the sonic hedgehog (Shh) pathway contributes to hepatocarcinogenesis and that the pathway activation occurs mainly in cancer stem cells. We investigated whether the activation of the Shh pathway occurs in CD133+ cells from liver cancer.

Materials and methods: We used magnetic sorting to isolate CD133+ cells from mouse cancer Hepa 1–6 cells. To examine the clonogenicity, cell culture and soft agar colony formation assay were performed between CD133+ and CD133− cells. To study the activation of the Shh pathway, we examined the mRNA expressions of *Shh*, *patched homolog 1* (*Ptch-1*), *glioma-associated oncogene homolog 1* (*Gli-1*), and *smoothened homolog* (*Smoh*) by real-time polymerase chain reaction of both CD133+ and CD133− cells.

Results: The number (mean ± standard deviation) of colonies of CD133+ cells and CD133− cells was $1,031.0 \pm 104.7$ and 119.7 ± 17.6 respectively. This difference was statistically significant ($P < 0.001$). Their clonogenicity was $13.7\% \pm 1.4\%$ and $1.6\% \pm 0.2\%$ respectively with a statistically significant difference found ($P < 0.001$). CD133+ cells and CD133− cells were found to have statistically significant differences in *Shh* mRNA and *Smoh* mRNA ($P = 0.005$ and $P = 0.043$ respectively).

Conclusion: CD133+ Hepa 1–6 cells have a significantly higher colony proliferation and clonogenicity. The Shh pathway is activated in these cells that harbor stem cell features, with an underexpression of *Shh* mRNA and an overexpression of *Smoh* mRNA. Blockade of the Shh signaling pathway may be a potential therapeutic strategy for hepatocarcinogenesis.

Keywords: sonic hedgehog, hepatocellular carcinoma, stem cells, CD133+ cells, liver cancer, Hepa 1–6 cells

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of death in many Asian countries. Its prevalence has also been increasing in the West in recent years.[1–3] The prognosis for those who are not candidates for resection or transplantation is poor.[1] To create new treatment strategies, research on some pathways of hepatocarcinogenesis is required.

Stem cells in healthy tissues have an ability to perpetuate themselves through self-renewal and to generate mature cells through differentiation.[4] According to some hypotheses, similar to normal stem cells, a small number of tumor cells, defined as "cancer stem cells", are capable of initiating and sustaining tumors.[5–10] CD133+ has been suggested as a marker of stem cells of various cancers including HCC though

some controversy exists.[11–17] The important role of cancer stem cells in carcinogenesis has been emphasized. To inhibit cancer stem cells, blockade of the contributing pathway may be an effective strategy.[18–19]

Hepa 1–6 cells are a well-known mouse liver cancer cell line. We hypothesize that CD133+ cells are the cancer stem cells of Hepa 1–6 cells. The aim of this study was to investigate whether CD133+ cells of mouse liver cancer Hepa 1–6 cells are clonogenic, and to elucidate which part of the sonic hedgehog (Shh) pathway participates in affecting these cells.

The Shh signaling pathway plays an important role in organizing cell growth and differentiation during embryonic tissue patterning.[20,21] The contribution of the Shh pathway in hepatocarcinogenesis has been proposed.[22–24] Recently, some research has suggested that activation of this pathway occurs mainly in cancer stem cells.[25]

Materials and methods
Sorting of CD133+ Hepa 1–6 cells
Cell culture

Mouse liver cancer Hepa 1–6 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone Laboratories, Logan, Utah, USA). The medium contained 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) and 100 unit/mL penicillin and 100 g/mL streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere that contained 5% CO_2.

Magnetic sorting and culture of CD133+ Hepa 1–6 cells

Cells were labeled with anti-prominin-1 microbeads of mouse (Miltenyi Biotec, Bergisch Gladbach, Germany) (1 μL per million cells) and separated on a MACS LS column (Miltenyi Biotec). All procedures were carried out according to the manufacturer's instructions. The purity of sorted cells was evaluated by flow cytometry and western blot. The flow cytometry was carried out with a BD FACS Calibur Flow Cytometer, using anti-prominin-1 PE conjugate (Miltenyi Biotec). The isolated CD133+ cells were cultured before assay in DMEM/10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 200 mM L-glutamine (Life Technologies), 25 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) (Sigma-Aldrich, St Louis, MO, USA), 10 mM nonessential amino acids (Life Technologies), 10 ng/mL leukemia inhibitory factor (Bioscience Research Reagents, Temecula,

CA, USA), 2% (v/v) 2-mercaptoethanol (Sigma-Aldrich) and antibiotics.

Soft agar colony formation (proliferation) assay and clonogenicity

Purified CD133+ and CD133− cells were suspended in 0.4% low melting point (LMP) agarose (SeaPlaque® Agarose, Lonza, Allendale, NJ, USA) in RPMI (Roswell Park Memorial Institute) 1640 media with 10% FBS and overlaid onto 0.8% LMP-agarose/RPMI 1640 (Hyclone Laboratories, Boston, MA, USA) in 6 cm culture plates at a density of 7,500 cells/well respectively. After 4 weeks, the cultures were fixed in methanol containing 0.5% crystal violet and colonies (≥10 cells) were counted under a microscope in 10 fields per well and photographed. For clonogenicity experiments, freshly isolated CD133− and CD133+ Hepa 1–6 cells were plated at a density of 7,500 cells/well in 6 cm culture plates and cultured for 4 weeks. Finally, the cells were stained crystal violet, photographed, and analyzed for their proliferation efficiency. Each experiment was performed three times.

Expression of messenger RNA (mRNA) of Shh signaling pathway genes between CD133+ Hepa 1–6 cells and CD133− Hepa 1–6 cells
Total RNA isolation, reverse transcription, and real-time polymerase chain reaction

Total RNA was isolated from CD133+ cells and CD133− cells using the REzol™ C&T reagent, according to the manufacturer's protocol (PROtech Technology, Taipei, Taiwan). Two micrograms of total RNA were reverse transcribed (RT) to cDNA templates using a high capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA).

The mRNA expression was analyzed using real-time polymerase chain reaction (PCR) (Roche LightCycler, Roche, Mannheim, Germany). Primer sequences used for real-time analysis were: *sonic hedgehog* (*Shh*) (forward), 5′-GGA AAA CAC TGG AGC AGA CC-3′; and (reverse), 5′-CCA CGG AGT TCT CTG CTT TC-3′; *patched homolog 1* (*Ptch-1*) (forward), 5′-CCG TTC AGC TCC GCA CAG A-3′; and (reverse), 5′-CTC ACT CGG GTG GTC CCA TAA A-3′; *glioma-associated oncogene homolog 1* (*Gli-1*) (forward), 5′-ATC ACC TGT TGG GGA TGC TGG AT-3′; and (reverse), 5′-GGC GTG AAT AGG ACT TCC GAC AG-3′; *smoothened homolog* (*Smoh*) (forward), 5′-GCC TGG TGC TTA TTG TGG-3′; and (reverse), 5′-GGT GGT TGC TCT TGA TGG-3′; *GAPDH*

(glyceraldehyde-3-phosphate dehydrogenase) (forward), 5′-CCA CCC ATG GCA AAT TCC-3′; and *GAPDH* (reverse), 5′-TGG GAT TTC CAT TGA TGA CAA-3′. Primers for *Shh*, *Gli-1*, *Ptch-1*, *Smoh* and *GAPDH* were used at temperature of 58°C, and gave expected band sizes of 308 bp, procedures for *Shh*, 333 bp for *Ptch-1*, 317 bp for *Gli-1*, 519 bp for *Smoh* and 69 bp for *GAPDH*. For real-time PCR, parameters were as follows: hot start at 95°C for 1 minute, followed by 45 cycles of denaturing at 95°C for 10 seconds, annealing at 58°C for 5 seconds, and extension at 72°C for 20 seconds. PCR products were detected using 2% agarose gel to confirm the expected sizes.

Triplicate crossing point values were analyzed in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) using the comparative quantitative method. The amount of target was obtained by normalization to an endogenous gene (*GAPDH*) and relative to a calibrator. Both CD133+ cells and CD133− cells of Hepa 1–6 cells were undertaken using the procedure guide. We repeated the procedure three times on independent samples.

Protein extraction and immunoblotting

Extracts equivalent to 5 micrograms of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) and transferred onto a polyvinylidene difluoride membrane. The primary antibodies employed were as follows: rat-anti-prominin-1 (MAB4310; 1:200; Millipore Corporation, Billerica, MA, USA), rabbit anti-Shh (sc-9024; 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Smoh (sc-13943; 1:500; Santa Cruz Biotechnology), rabbit anti-human Gli-1 (sc20687; 1:400; Santa Cruz Biotechnology) and goat anti-Ptch-1 (sc-6149; 1:400; Santa Cruz Biotechnology) used in Tris-buffered saline Tween-20 (TBST) containing 5% bovine serum albumin. The membranes were then incubated with goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in TBST containing 5% non-fat dry milk. Bound antibodies were detected with an enhanced chemiluminescence detection system (Millipore Corporation). After the 3,3′-Diaminobenzidine reaction, the specimens were counterstained with hematoxylin. For each antibody, negative controls were performed by omitting the primary. These controls revealed no or minimal background staining.

Nuclear localization of *Gli-1* between CD133+ cells and CD133− cells

To compare the *Gli-1* expression of the nucleus and cytoplasm between CD133+ cells and CD133− cells, we performed this study. Cell lysate was separated into nuclear part and cytoplasmic part according to the manufacturer's instructions (Thermo Fisher Scientific, Pittsburgh, PA, USA). Cells were added 10 × volume of lysis buffer, vortexed on ice, and centrifuged at 500*g*, 4°C, for 7 minutes. We collected the supernatants and centrifuged at 20,000*g*, 4°C, for 15 minutes, then collected and stored the supernatants at −70°C (ie, the cytoplasmic part). The nuclear pellets were washed by nuclear washing buffer twice and centrifuged at 500*g*, 4°C, for 7 minutes. We removed the supernatants and add nuclear storage buffer, then pipetted 5–10 times. We added 1/10 volume of nuclei lysis reagent and vortexed at 4°C for 15 minutes. We centrifuged at 20,000*g*, 4°C for 5 minutes and collected supernatants (nuclear part). Then we used supernatants of both cytoplasm and nucleus to perform the western blot respectively.

Statistical analysis

Biostatistical analyses were performed using SPSS software (IBM Corporation, Armonk, NY, USA). The nonparametric Kruskal–Wallis rank test was used to detect differences among different experimental groups. Some findings were statistically significant and compared using Fisher's exact test for evaluation in a two-group experiment. $P < 0.05$ was regarded as statistically significant.

Results

The mean purity of CD133+ cells sorted from Hepa 1–6 cells (sample size n = 10) was 41.05% ± 15.33% (mean ± standard deviation [SD]) with a range of 17.89% to 62.87%.

From our experiment, the mean ± SD of the number of colonies of CD133+ cells and CD133− cells were 1031.0 ± 104.7 and 119.7 ± 17.6, respectively (Figure 1A and B). The difference was found to be statistically significant ($P < 0.001$).

The comparison of clonogenicity between CD133+ Hepa 1–6 cells and CD133− Hepa 1–6 cells was made at the end of 20 days following the initial plating. The clonogenicity of CD133+ cells and CD133− cells was 13.7% ± 1.4% and 1.6% ± 0.2% respectively. The difference was also found to be statistically significant ($P < 0.001$) (Figure 2A and B).

The values of means ± SD (range) of *Shh* mRNA, *Ptch-1* mRNA, *Gli-1* mRNA, and *Smoh* mRNA of CD133+ cells were 0.78 ± 0.24 (0.43–1.10), 1.13 ± 0.19 (0.88–1.43), 0.77 ± 0.28 (0.01–0.99) and 1.16 ± 0.29 (0.91–1.60) respectively. Those of CD133− cells were 1.41 ± 0.54 (0.86–2.58), 1.00 ± 0.13 (0.87–1.28), 1.05 ± 0.31 (0.71–1.73) and 0.94 ± 0.03 (0.90–0.98) respectively. Among the factors of the Shh pathway, there was a statistically significant difference between

A

B
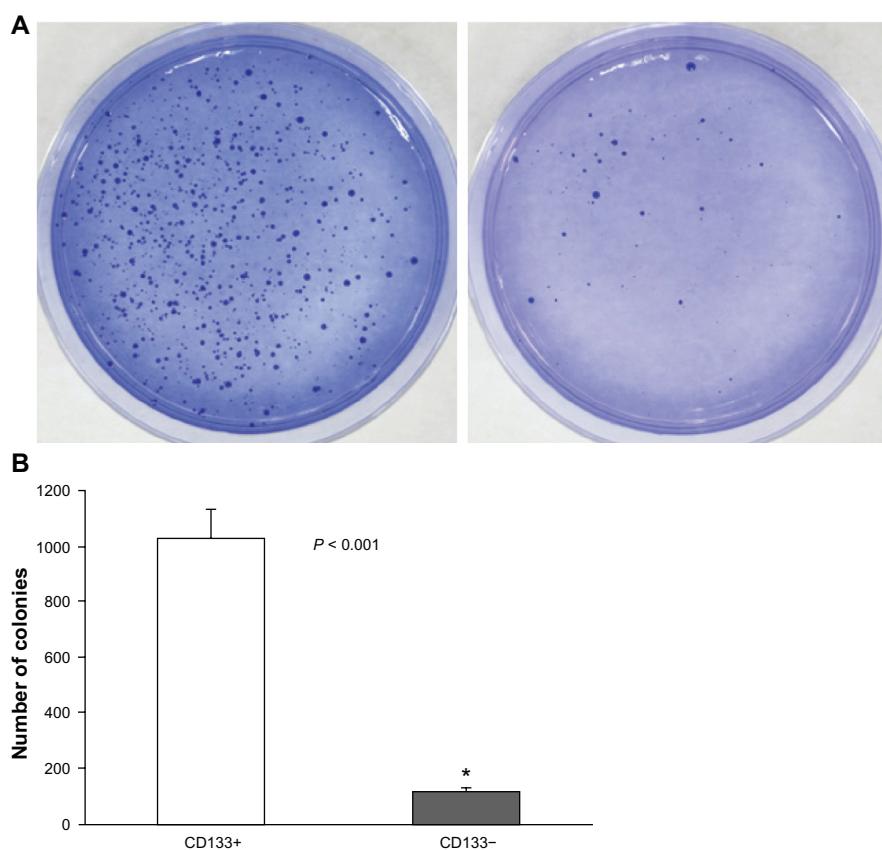
Figure 1 Colony proliferation experiments (colony numbers). (**A**) For clonogenicity experiments, freshly isolated CD133+ Hepa 1–6 cells and CD133– Hepa 1–6 cells were plated at a density of 7,500 cells/well in 6 cm culture plates and cultured for 4 weeks. At the end, cells were stained with crystal violet, photographed, and analyzed for their proliferation efficiency. Each experiment was performed three times. The left shows the result of CD133+ Hepa 1–6 cells and the right shows the result of CD133– Hepa 1–6 cells. (**B**) The comparison of the number of colonies (mean ± SD) between CD133+ Hepa 1–6 cells and CD133– Hepa 1–6 cells is 1031.0 ± 104.7 and 119.7 ± 17.6. *The difference was found to be statistically significant ($P < 0.001$).

CD133+ and CD133– cells in *Shh* mRNA ($P = 0.005$) and *Smoh* mRNA ($P = 0.043$), whereas the difference of *Ptch-1* mRNA and *Gli-1* mRNA between CD133+ cells and CD133– cells had no or borderline statistical significance($P = 0.103$, and 0.051 respectively) (Table 1).

From the western blot of the protein expression, the value of means ± SD of Shh protein expression of CD133+ cells was 0.64 ± 0.44, whereas that of CD133– cells is 0.89 ± 0.32. The difference was found to be statistically significant ($P = 0.037$). However, the difference of the expressions of Gli-1 protein, Ptch-1 protein, and Smoh protein between CD133+ cells and CD133– cells had no statistical significance (Table 2).

Figure 3 demonstrates the RT-PCR expression of the four genes. The expressions of *Shh* and *Smoh* between these two kinds of cells has statistical significance. Figure 4 shows the expression of proteins with western blotting. Only the difference of Shh between CD133+ Hepa 1–6 cells and CD133– Hepa 1–6 cells was found to be statistically significant.

Figure 5 demonstrates the Gli-1 protein expression of CD133+ cells and CD133– cells in the nucleus and the

cytoplasm respectively. The Gli-1 expression was higher in the CD133– cells in both nucleus and cytoplasm, not similar to the result of the real-time PCR (Table 1) and that of the western blot of the whole cells (Table 2).

Discussion

From this study, activation of the Shh pathway occurs in CD133+ Hepa 1–6 cells that harbor the stem cell feature. Among the factors of the pathway, Shh and Smoh play significant contributory roles in the activation.

In our study, the sorting rate of CD133+ cells from all Hepa 1–6 cells is not high (under 2%). From the amount perspective, it means that these "cancer stem cells" may be only a small population among all cancer cells. This finding is similar to some investigators' findings.[26–30]

The existence of cancer stem cells has been demonstrated in various organ malignancies including breast, blood, brain, prostate, colon, and pancreatic.[29–35] Some authors believe that cancer stem cells may be derived from normal stem cells through mutations.[10] There are many various cancer stem

A

B
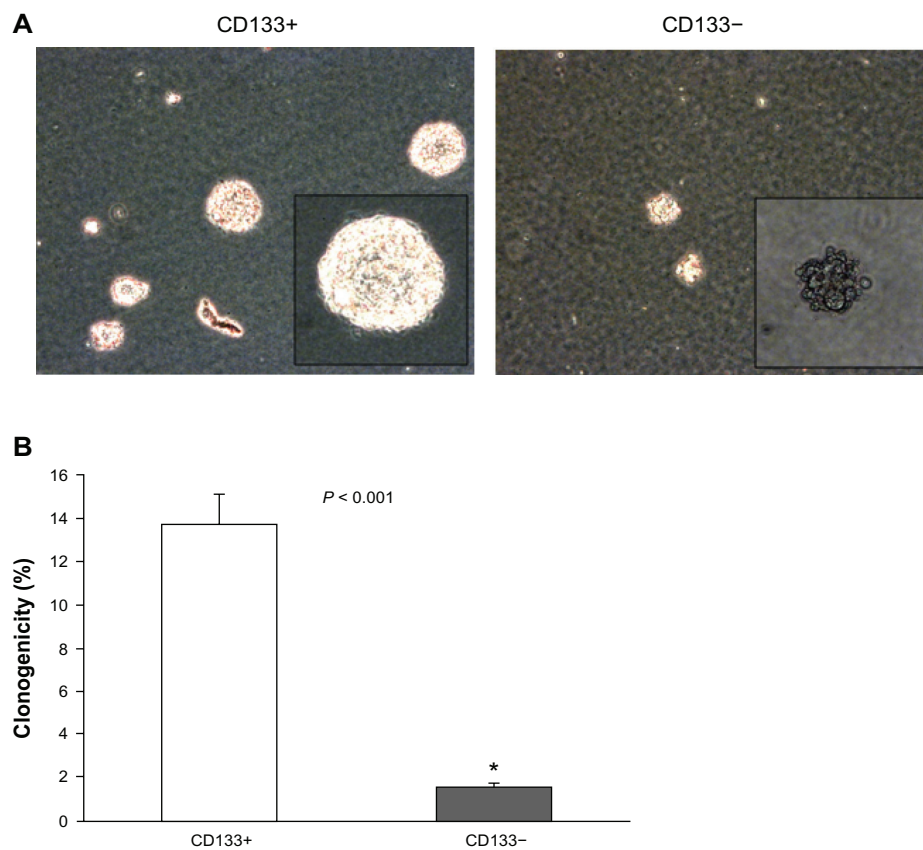
Figure 2 Clonogenicity between CD133+ Hepa 1–6 cells and CD133– Hepa 1–6 cells. (**A**) The results at the 20th day after initial plating are shown (50×). The smaller panels in (**A**) show representative examples of clonogenic assays magnified at 100× : the left shows the result of CD133+ Hepa 1–6 cells and the right shows that of CD133– Hepa 1–6 cells. Some blebs were present, probably the apoptic change. (**B**) The comparison of clonogenicity (%) (mean ± SD) between CD133+ Hepa 1–6 cells and CD133– Hepa 1–6 cells. The clonogenicity of CD133+ cells and CD133– cells is 13.7% ± 1.4% and 1.6% ± 0.2% respectively. *The difference was found to be statistically significant ($P < 0.001$).

Table 1 Comparison of median and mean crossing point (CP) values from real-time PCR of target genes of Shh pathway between CD133+ and CD133– of Hepa 1–6 cells

	Shh/GAPDH		Ptch-1/GAPDH		Gli-1/GAPDH		Smoh/GAPDH	
	CD133+	CD133–	CD133+	CD133–	CD133+	CD133–	CD133+	CD133–
Median	0.79	1.26	1.13	1.00	0.86	0.98	1.11	0.94
Mean	0.78	1.41	1.13	1.00	0.77	1.05	1.16	0.94
±SD	0.24	0.54	0.19	0.13	0.28	0.31	0.29	0.03
P-value	0.005		0.103		0.051		0.043	
95% confidence interval	−1.023 to −0.237		−0.023 to 0.283		−0.558 to −0.002		0.026 to 0.414	

Abbreviations: *Shh, sonic hedgehog; Ptch-1, patched homolog 1; Gli-1, glioma-associated oncogene homolog 1; Smoh, smoothened homolog; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation; PCR, polymerase chain reaction.*

Table 2 Comparison of median and mean protein values from western blot of target genes of Shh pathway between CD133+ and CD133– of Hepa 1–6 cells

|	Shh/actin		Ptch-1/actin		Gli-1/actin		Smoh/actin	
	CD133+	CD133–	CD133+	CD133–	CD133+	CD133–	CD133+	CD133–
Median	0.32	0.64	0.14	0.09	0.26	0.36	0.23	0.24
Mean	0.64	0.89	1.14	0.09	0.27	0.34	0.17	0.23
±SD	0.32	0.44	0.15	0.02	0.10	0.11	0.12	0.13
P-value	0.037		0.675		0.45		0.586	
95% confidence interval	0.21 to 3.81		−0.031 to 0.264		0.196 to 0.408		0.080 to 0.321	

Abbreviations: *Shh, sonic hedgehog; Ptch-1, patched homolog 1; Gli-1, glioma-associated oncogene homolog 1; Smoh, smoothened homolog.*
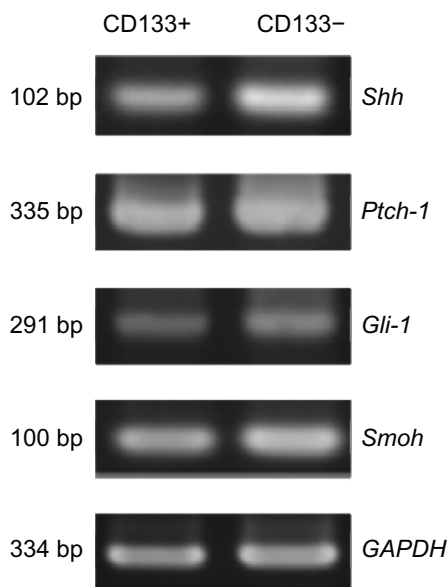
Figure 3 Semi quantitative reverse transcription polymerase chain reaction analysis of mRNA expression of Shh pathway of CD133+ and CD133− of Hepa 1–6 cells. The difference of *Shh* mRNA and *Smoh* mRNA expressions between CD133+ Hepa 1–6 cells and CD133− Hepa 1–6 cells is statistically significant.
Abbreviations: mRNA, messenger ribonucleic acid; *Shh*, sonic hedgehog; *Ptch-1*, patched homolog 1; *Gli-1*, glioma-associated oncogene homolog 1; *Smoh*, smoothened homolog; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cell markers such as CD133, Oct 4, EpCAM, etc.[5,7,8,11–19] We established our hypothesis as CD133 which has been suggested as a marker for liver cancer stem cells.[13–15,17] Ma et al, demonstrated that freshly isolated CD133+ cells possess characteristics similar to those of stem/progenitor cells.[26]
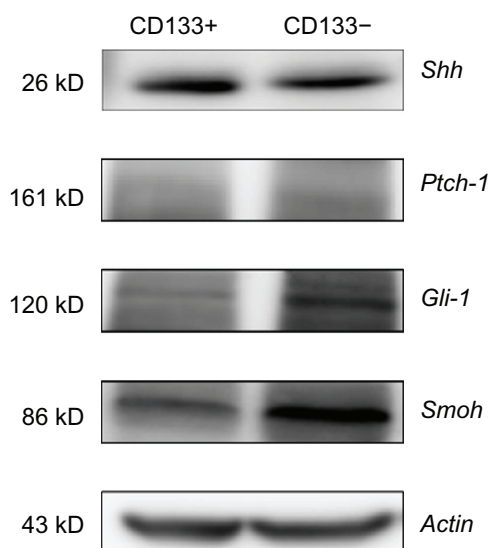

Figure 4 Western blot showing the Shh pathway related protein expression of CD133+ Hepa 1–6 cells and CD133− Hepa 1–6 cells. The difference of Shh protein expression between CD133+ Hepa 1–6 cells and CD133− Hepa 1–6 cells is statistically significant.
Abbreviations: Shh, sonic hedgehog; Ptch-1, patched homolog 1; Gli-1, glioma-associated oncogene homolog 1; Smoh, smoothened homolog.

In addition, Piao et al, found that CD133+ liver cancer stem cells may modulate radio-resistance in human HCC.[17] Suetsugu et al, found that only HuH7 cells with CD133+ could form tumors in SCID mice.[13,14] Kim et al, reported that CD133+ cells sorted from either HuH7 or PLC5 cell lines showed increased tumorigenicity and clonogenicity when compared to CD133− cells.[36] Similarly, from our soft agar study, CD133+ cells have significantly higher colony proliferation and the clonogenicity (Figures 1 and 2), which may suggest a higher tumorigenicity.

The Shh pathway implicating hepatocarcinogenesis has been reported.[22–24] Some authors mentioned that the activation of the Shh pathway may occur in human HCC.[1,22–24,37] Che et al found that Shh, Ptch-1, and Gli-1 are overexpressed in human HCC.[22] The correlation between Shh pathway factors and the invasiveness of HCC has been variably reported.[1,22,23,37] Some researchers found Shh signaling pathway activation correlated with tumor size, capsular invasion, and vascular invasion. The correlation of the Shh pathway factors (Shh, Ptch-1, and Gli-2) and portal venous invasion or poor differentiation has also been reported.[37]

From our study, the expressions of *Shh* mRNA and *Smoh* mRNA are significantly different between CD133+ and CD133− cells. According to the definition of Huang et al, activation of the shh pathway occurring in CD133+ Hepa 1–6 cells may be defined.[24] These two factors of the pathway may participate in the activation. To explain the lower expression of *Shh* mRNA and the higher expression of *Smoh* mRNA in CD133+ cells, we proposed some possible mechanisms. The first is the complex interactions among the factors of the Shh pathways. Ptch-1 activation predisposes a cell to proliferative and expansive behavior.[38,39] Some interactions between Smoh and Ptch-1 are not fully understood. In our study, expression of *Ptch-1* mRNA of CD133+ cells is higher than CD133− cells without statistical significance. Smoh is an intracellular substrate that migrates to the cellular membrane where it becomes active after engagement of Ptch-1 by Shh. At the cellular membrane, the activated Smoh triggers the downstream transcription of Gli-1 proteins.[38,39] Aberrant activation of the pathway leads Gli-1 into the nucleus to promote gene transcriptions and to maintain the biological behaviors of cancer cells. *Gli-1* is a well-known target gene of the Shh pathway.[1,22–24] The high expressions of *Gli-1* mRNA in other cancers adversely affect recurrence.[1,40] Whereas, from our study, a lower expression of Gli-1 of CD133− cells is noted (Table 1 and 2). Nuclear Gli-1 activity of CD133+ cells is significantly lower than that of CD133− cells (Figure 5). Gli-1 plays a role in proliferation,
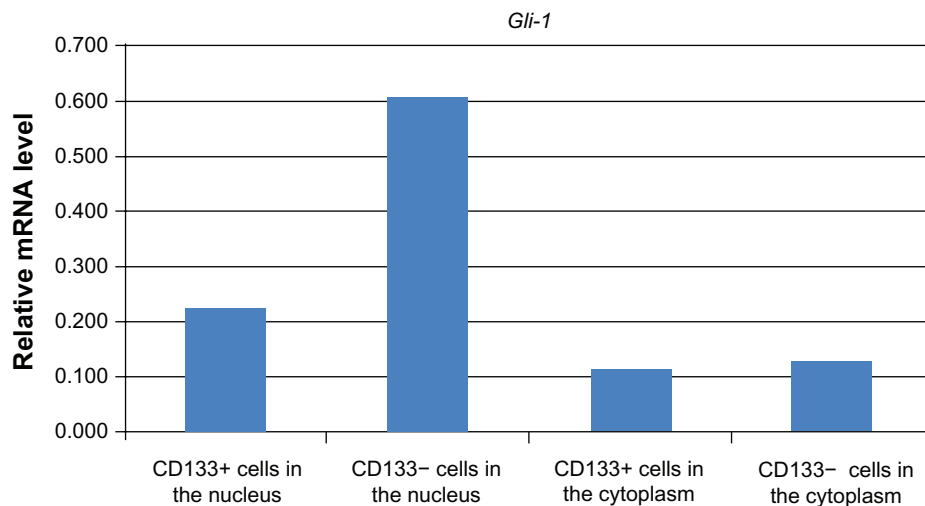
Figure 5 Comparison of *Gli-1* mRNA expression of CD133+ and CD133− cells in the nucleus and the cytoplasm respectively. The *Gli-1* expression was higher in CD133− cells than in CD133+ cells in both the nucleus and the cytoplasm.
Note: n = 4.
Abbreviation: *Gli-1*, glioma-associated oncogene homolog 1; mRNA, messenger ribonucleic acid.

survival, and migration of some cancers.[41] However, the Shh expression varied not only among different cancers but also between mouse hepatoma and human hepatoma.[40–44] To analyze the activation of Smoh, hyperphosphorylation study may be another option.

The second possibility is that the changes of the mRNA expression may be dynamic. The timing of tumor cell sorting study may affect the sorted cell varieties. Huang suggested that the activation occurs in the early stage of HCC.[24] The degree of the Shh pathway activation may differ among different stages of the same cancer and among different malignancies at the same stage.[22–24,42,44–47] This idea remains controversial. Kim et al reported that the expression of Shh and Gli-1 was independent of tumor stage and cell differentiation in extrahepatic biliary tract cancer cells.[46] Thayer et al, considered that the activation of the Shh pathway occurs as both an early and late mediator in pancreatic carcinogenesis.[44] Whereas, the Shh pathway activates in advanced stages of gastric cancer or prostate cancer, etc.[42,44–47]

The third possibility affecting our expression of the Shh pathway is the hypothesis that the Shh pathway activation occurs only in "cancer stem cells". The lung cancer study of Tian et al, showed that the Shh pathway is activated mainly in the "cancer stem cells" and not in every cancer cell.[25] Shackleton et al emphasized the heterogeneity of cancer cells.[28]

According to the study by Che et al, in HCC tissues, protein expressions are compatible with mRNA.[22] Our protein study showed a significantly lower expression of Shh of CD133+ Hepa 1–6 cells. In our study, the protein expressions of Ptch-1, Gli-1, and Smoh were too small to compare.

Ptch-1 is a membrane protein. Western blot is not sensitive in detecting the membrane protein. Therefore, the real-time PCR may measure a greater amount than western blot, which results in a discrepancy between the mRNA expression (real-time PCR) and protein expression (western blot). Probably, the real-time PCR is more sensitive than western blot in detecting the small amount of protein. Further experiments on cell growth of CD133+ and CD133− cells using siRNA of *Smoh* should be studied in the future.

Conclusion

From our study, CD133+ Hepa 1–6 cells have a significantly higher clonogenicity, and are probably more tumorigenic. The Shh pathway is activated in these cells, which harbor stem cell features, with an underexpression of *Shh* mRNA and an overexpression of *Smoh* mRNA. Blockade of the Shh signaling pathway has been tried in various previous in vitro studies.[48–51] It may be a potential clinical therapeutic strategy for hepatocarcinogenesis.

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Author contributions

Conception and design, KS Jeng, IS Sheen, WJ Jeng; provision of study materials, MC Yu, HI Hsiau, FY Chang, HH Tsai; acquisition of data, MC Yu, HI Hsiau, FY Chang, HH Tsai; analysis and interpretation of data, MC Yu, HI Hsiau, IS

Sheen, WJ Jeng; manuscript writing, KS Jeng; critical revision of manuscript, IS Sheen, WJ Jeng, MC Yu, HI Hsiau, FY Chang, HH Tsai; final approval of manuscript, all authors.

Disclosure

The authors declare that they have no conflicts of interest in this work.

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