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 ± 104.7 and 119.7 ± 17.6 respectively. This difference was statistically significant (P, 0.001). Their clonogenicity was 13.7% ± 1.4% and 1.6% ± 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. 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.

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. The contribution of the Shh pathway in hepatocarcinogenesis has been proposed. Recently, some research has suggested that activation of this pathway occurs mainly in cancer stem cells.

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% CO2.

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 (Ptc1-1) (forward), 5′-CTC ACT CGG GTG GTC CCA TAA 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 TGG GAA 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 TGG TGG-3′; and (reverse), 5′-GAT GGT TGC TCT TGA TGG-3′; GAPDH

splice variants of Smoh and Gli-1 were detected by specific PCR primers: (mutant) Shh receptor patched homolog 1 (Ptch-1) (forward), 5′-GGC GTG AAT AGG ACT TCC GAC AG-3′; and (reverse), 5′-GGC GTG AAT AGG ACT TCC GAC AG-3′; glioma-associated oncogene homolog 1 (Gli-1) (forward), 5′-ATC ACC TGG GAA 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 TGG TGG-3′; and (reverse), 5′-GAT GGT TGC TCT TGA TGG-3′; GAPDH
Sonic hedgehog pathway, CD133+ Hepa 1–6 cells, and liver cancer

(0.90–0.98) respectively. Among the factors of the Shh pathway, expression of the nucleus and cytoplasm of CD133– cells were 1.41 ± 0.24 (0.43–1.10), 1.13 ± 0.13 (0.86–1.28), 1.00 ± 0.12 (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

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 ± 0.58 (0.86–2.58), 1.00 ± 0.12 (0.87–1.28), 1.05 ± 0.38 (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
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
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

<table>
<thead>
<tr>
<th></th>
<th>Shh/GAPDH</th>
<th>Ptch-1/GAPDH</th>
<th>Gli-1/GAPDH</th>
<th>Smoh/GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD133+</td>
<td>CD133−</td>
<td>CD133+</td>
<td>CD133−</td>
</tr>
<tr>
<td>Median</td>
<td>0.79</td>
<td>1.26</td>
<td>1.13</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean</td>
<td>0.78</td>
<td>1.41</td>
<td>1.13</td>
<td>1.00</td>
</tr>
<tr>
<td>±SD</td>
<td>0.24</td>
<td>0.54</td>
<td>0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>P-value</td>
<td>0.005</td>
<td>0.103</td>
<td>0.051</td>
<td>0.043</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>−1.023 to −0.237</td>
<td>−0.023 to 0.283</td>
<td>−0.558 to −0.002</td>
<td>0.026 to 0.414</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Shh/actin</th>
<th>Ptch-1/actin</th>
<th>Gli-1/actin</th>
<th>Smoh/actin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD133+</td>
<td>CD133−</td>
<td>CD133+</td>
<td>CD133−</td>
</tr>
<tr>
<td>Median</td>
<td>0.32</td>
<td>0.64</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean</td>
<td>0.64</td>
<td>0.89</td>
<td>1.14</td>
<td>0.09</td>
</tr>
<tr>
<td>±SD</td>
<td>0.32</td>
<td>0.44</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>P-value</td>
<td>0.037</td>
<td>0.675</td>
<td>0.45</td>
<td>0.586</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.21 to 3.81</td>
<td>−0.031 to 0.264</td>
<td>0.196 to 0.408</td>
<td>0.080 to 0.321</td>
</tr>
</tbody>
</table>

Abbreviations: Shh, sonic hedgehog; Ptch-1, patched homolog 1; Gli-1, glioma-associated oncogene homolog 1; Smoh, smoothened homolog.
CD133+  CD133−
102 bp  Shh
335 bp  Ptch-1
291 bp  Gli-1
100 bp  Smoh
334 bp  GAPDH

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. established our hypothesis as CD133 which has been suggested as a marker for liver cancer stem cells. We demonstrated that freshly isolated CD133+ cells possess characteristics similar to those of stem/progenitor cells. In addition, Piao et al, found that CD133+ liver cancer stem cells may modulate radio-resistance in human HCC. Suetsugu et al, found that only HuH7 cells with CD133+ could form tumors in SCID mice. 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. 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. Some authors mentioned that the activation of the Shh pathway may occur in human HCC. Che et al found that Shh, Ptch-1, and Gli-1 are overexpressed in human HCC. The correlation between Shh pathway factors and the invasiveness of HCC has been variably reported.

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. 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. 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. 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. The high expressions of Gli-1 mRNA in other cancers adversely affect recurrence. 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.

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 Ptc-1, Gli-1, and Smoh were too small to compare. Ptc-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.

Acknowledgment
The authors would like to acknowledge support, including a research grant, from the Far Eastern Memorial Hospital (New Taipei City, Taiwan) (FEMH-98-SCRM-A-004).

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.

References


