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Hedgehog signaling pathway affects the sensitivity of hepatoma cells to drug therapy through the ABCC1 transporter

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The poor response to drug therapy often seen in hepatocellular carcinoma requires insight into the molecular interplay responsible for intrinsic or acquired drug resistance. We previously demonstrated that the CD133−/EpCAM− subpopulation of the Huh-7 hepatoma cell line features aberrant activation of the hedgehog signaling (Hh) pathway and chemoresistance. The prevailing hypothesis of the present study is that hedgehog signaling may govern expression of ATP-binding cassette (ABC) transporters, which are responsible for drug resistance in the CD133−/EpCAM− subpopulation. Our aim is to reveal the molecular interplay in the mediation of drug resistance with a newly established Huh-7 subpopulation featuring high Hh signaling activity and drug resistance. In this study, chemoresistance was determined in a newly established Huh-7-DN subpopulation featuring the CD133−/EpCAM− surface marker profile, aberrant expression of Hh pathway, and epithelial–mesenchymal transition (EMT). Expression of ABC transporter proteins (ABCB1, ABCC1, and ABCG2) and Hh transcription factor Gli-1/2 was evaluated with and without Hh signaling antagonists LDE225 or itraconazole. We found that hedgehog signaling activity as determined by transfection with a Gli-Lux reporter cassette and gene expression levels tended to increase from Huh-7 CD133+/EpCAM+ to CD133−/EpCAM−, and the highest levels were found in Huh-7-DN cells. The Huh-7-DN subpopulation exhibited characteristics of EMT as evidenced by increased expression of vimentin and loss of E-cadherin. Sorafenib significantly inhibited the viability of all subpopulations except the Huh-7-DN subpopulation. Compared with other sorafenib-sensitive subpopulations, the Huh-7-DN subpopulation showed enhanced expression of Hh transcription factor Gli-2 and ABCC1 transporter protein. Silencing Gli-2 by lentivirus harboring shRNA against Gli-2 or LDE225 significantly suppressed expression of Gli-2 and ABCC1 genes in Huh-7-DN subpopulation. In conclusion, aberrant hedgehog signaling activation is linked to poor differentiation, epithelial–mesenchymal transition, and chemoresistance in the Huh-7-DN subpopulation. Hedgehog signaling transcription factor Gli-2 appears to be the primary regulator for drug sensitivity of hepatoma through the ABCC1 transporter.

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As the third most common death of malignancy, more than 700 000 new cases of hepatocellular carcinoma (HCC) occur worldwide every year.1 Despite the steadily increasing incidence, only 25% of patients benefit from surgical intervention.2 The advanced stage at the time of diagnosis, aggressive behavior of HCC progression, the high recurrence after resection (60–80% in 5 years), and ablation (40–70%) are major causes of a very low 5-year survival rate.3 Limited adjuvant therapies are available for advanced HCC patients.4,5 Sorafenib, a Raf-1 kinase inhibitor, was reported to extend patient survival for nearly 3 months.6 However, a recent multicenter, double-blinded trial concluded that sorafenib did not extend recurrence-free survival, but increased adverse effects of the treatment,3 and the findings are consistent with a growing list of negative reports from primary or adjuvant use after resection, ablation, or transarterial chemoembolization.7

Primary or acquired multidrug resistance (MDR) is one of the reasons leading to HCC treatment failure and progression.8 It is known that MDR increases drug efflux by

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Hedgehog signaling and drug resistance
J Ding et al

Materials and Methods

Cell Culture and Reagents
Hepatoma cell lines, Huh-7 and Hep3B, were obtained from the American Type Culture Collection (Manassas, VA, USA). The Hep3B cells were incubated in minimum essential medium (MEM, Gibco, Grand Island, NY, USA), and Huh-7 cells were incubated in Dulbecco’s modified essential medium (DMEM, Gibco), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin–streptomycin.

Fluorescence Activation Cell Sorting Enrichment and Transwell Matrigel Invasion Assay for Subpopulation Selection
CD133+/EpCAM+ and CD133+/EpCAM− subpopulations were enriched from Huh-7 cells by fluorescence activation cell sorting (FACS) using allophycocyanin (APC)-conjugated monoclonal antibodies against human CD133 and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human EpCAM as we previously described. Briefly, the Huh-7 cells were detached by 0.05% Trypsin-EDTA (Gibco) and incubated with corresponding antibodies (CD133/EpCAM) for 45 min. After washing, the cells were suspended in DMEM containing 10% FBS until sorting. All the cells were sorted in a high-speed MoFlo XDP Cell Sorter (Beckman, Indianapolis, IN, USA). The Huh-7-DN subpopulation, which stably retained negative CD133/EpCAM expression profile was isolated by FACS enrichment plus culture selection from CD133+/EpCAM− subpopulation. The Huh-7-trans subpopulation was isolated using Matrigel invasion transwell selection as we previously described.

Immunofluorescent Staining
CD133+/EpCAM+, CD133−/EpCAM−, Huh-7-DN, and Huh-7-trans subpopulations were seeded on coverslips and fixed in 4% buffered paraformaldehyde. Then cells on coverslips were incubated with primary antibodies against Gli-1, E-cadherin, vimentin, Gli-2, and ABC1 overnight at 4°C, and were stained with secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit IgG) as we previously described. Briefly, the cells were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear visualization. All electronic images were captured under a Leica TCS SP8 Confocal laser scanning microscope. The sources of all antibodies were listed in the Supplementary Table 1.

Hh Signaling Activity by a Gli-Lux Reporter System
The Gli-Lux reporter system, in which the firefly luciferase gene is driven by the Gli promoter, was used to determine Hh signaling activity in different subpopulations. pRL-thymidine kinase (TK) renilla luciferase control reporter (Promega, Madison, WI, USA) was used for transfection normalization. The Gli-Lux reporter system was kindly provided by Dr Hiroshi Sasaki from the RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan. FACS-enriched Huh-7 CD133+/EpCAM+ or CD133−/EpCAM− subpopulations were transfected with both Gli-Lux and pRL-TK reporter gene plasmids by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To determine the inhibitory effect of Hh antagonists, culture medium was replaced with medium containing itraconazole or LDE225 24 h after transfection. Firefly and renilla luciferase activity in transfected cells was determined by a dual-luciferase reporter system (Promega).
Assays of Cell Viability
CD133+/EpCAM+, CD133−/EpCAM−, Huh-7-DN, and Huh-7-trans subpopulations were seeded at 10^4/well in 96-well plates and allowed to adhere overnight. The cells were treated with Sorafenib (Selleck, TX, USA), Itraconazole (Biovision, Milpitas, CA, USA) and LDE225 (Cellagen Technology, San Diego, CA, USA) at various concentrations for 24 h. The cell viability was assayed with thiazolyl blue tetrazolium bromide (MTT) assay. The MTT assay was also used for the determination of IC50 in various cell types in exposure to sorafenib as shown in the Supplementary Information.

RNA Isolation and Quantitative RT-PCR
Total RNA was extracted from various cells using RNAprep Pure Cell kit (TIANGEN, Beijing, China) according to the manufacturer’s instruction. cDNA was synthesized using PrimeScript RT reagent Kit (TAKARA, Dalian, China). Quantitative RT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in the Eppendorf AG 22331 RT-PCR system (Eppendorf, Hamburg, Germany). Primer pair sequences for Gli-1, Gli-2, ABCB1, ABCC1, ABCC2, Twist, Snail, Bcl-2, and Bax are listed in Supplementary Table 2. Relative gene expression was normalized to the housekeeping gene, human glyceraldehyde phosphate dehydrogenase (GAPDH) and expressed as 2^{−\Delta\Delta Ct} as previously described. C DNA generated from human primary hepatocytes was provided by Dr Ping Zhou, Stem Cell Program, UC Davis Medical Center, Sacramento, CA, USA, and used as a control for human gene expression level. Human primary hepatocytes were obtained from the Liver Tissue Procurement and Distribution System (LTPDS), Pittsburgh, PA, USA.

Western Blot Analysis
Total protein was extracted from different cell subpopulations using RIPA buffer (Ruian BioTechnologies, Shanghai, China). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce Biotech, Rockford, USA). Protein lysate (40 μg) was loaded and separated by 4–12% gradient Tris-glycine gels and was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA) at 80 V for 1.5 h. Then, membrane of protein extracts was blocked with 5% skim milk and incubated with monoclonal anti-Gli-1, ABCG2, ABCC1, and β-actin or polyclonal anti-Gli-2 in Tris-buffered saline containing 0.1% bovine serum albumin (BSA) overnight at 4 °C. Blotted membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Specific protein bands were visualized by the enhanced chemiluminescent reagent (Tanon, Shanghai, China) as previously reported.

Statistical Analysis
All the experiments were performed three times with a minimum of triplicates. Statistical analysis was performed with SPSS 20.0. The data were expressed as means ± standard deviation (s.d.), and analyzed by independent-samples T-test between two groups or one-way ANOVA for difference between more than two groups, and further by Newman–Keuls or Bonferroni tests for multiple comparisons between given two groups. P < 0.05 was considered as statistically significant.

RESULTS

Stable CD133+/EpCAM− Expression Profile of Huh-7-DN and Huh-7-Trans Subpopulations
Well-differentiated hepatoma Huh-7 cells were separated into CD133+/EpCAM+ and CD133−/EpCAM− subpopulations by FACS enrichment. As shown in Figure 1a, CD133+/EpCAM+ and CD133−/EpCAM− subpopulations constituted 48.7 and 21.0% of unsorted Huh-7 cells. Flow cytometric analysis verified that more than 80% of freshly sorted CD133+/EpCAM+ and CD133−/EpCAM− cells possessed their own surface marker profile (Figures 1b and c). However, freshly sorted CD133+/EpCAM+ and CD133−/EpCAM− subpopulations gradually lost characteristics of their surface marker profile, and tended to return to an unsorted state after a few passages. To obtain a subpopulation, which stably retained CD133+/EpCAM− expression profile, Huh-7-DN and Huh-7-trans subpopulations were established by FACS enrichment plus culture selection or Matrigel invasion transwell selection. Both Huh-7-DN and Huh-7-trans subpopulations remained CD133−/EpCAM− profile (> 98%) after more than 10 passages (Figures 1d and e).

Huh-7-DN Cells were Positive for Hh Transcription Factors Gli-1/2 and EMT Markers
As shown in Figure 2, Gli-1 and Gli-2 were positive in CD133+/EpCAM+ and CD133−/EpCAM− subpopulations, especially in Huh-7-DN and Huh-7-trans subpopulations. Staining of EMT markers demonstrated that E-cadherin was only observed in CD133+/EpCAM+ subpopulation, whereas other three subpopulations with a negative CD133/EpCAM expression profile exhibited obvious vimentin positivity and loss of E-cadherin expression, indicating that these subpopulations with CD133+/EpCAM− expression acquired an EMT status.

Enhanced Hh Signaling Activity in CD133+/EpCAM− Subpopulation
To evaluate the Hh signaling activity, freshly sorted CD133+/EpCAM+ and CD133−/EpCAM− subpopulations were transfected with the Gli-Lux firefly reporter cassette, and co-transfected with pRL-TK renilla luciferase reporter control vector for the normalization of transfection efficiency. Two days after transfection, normalized luciferase activity of CD133+/EpCAM− subpopulation was 1.8-fold higher than CD133+/EpCAM+ subpopulation (P < 0.01). These two
Figure 1 Huh-7-DN and Huh-7-trans subpopulations retained CD133−/EpCAM− cell surface marker profile over multiple passages. (a) Unsorted Huh-7; (b) Freshly sorted CD133+/EpCAM+ subpopulation; (c) Freshly sorted CD133−/EpCAM− subpopulation; (d) Huh-7-DN subpopulation at passage 16; (e) Huh-7-trans subpopulation.
subpopulations did not show any significant difference in Hh signaling activity when transfected with a mutated Gli-Lux reporter cassette (Figure 3a). Moreover, both itraconazole and LDE225, two Hh signaling SMO antagonists, significantly suppressed luciferase activity of CD133−/EpCAM− subpopulation (Figures 3b and c). Notably, LDE225 was effective at 100 nM in suppressing Hh signaling activity in this subpopulation. This data demonstrated that CD133−/EpCAM− subpopulation displayed higher Hh signaling activity than CD133+/EpCAM+ subpopulation, and that specific antagonists targeting the SMO molecule were effective in suppressing Hh signaling activity in this subpopulation.

**Intrinsic Chemoresistance of Huh-7-DN Subpopulation**

To investigate the influence of Hh signaling activity on drug sensitivity in Huh-7 subpopulations with varying Hh signaling activity, CD133+/EpCAM+, CD133+/EpCAM−, Huh-7-DN, and Huh-7-trans subpopulations were treated with
itraconazole, LDE225 and a Raf kinase inhibitor, Sorafenib for 24 h. The cell viability was determined by an MTT reagent. As shown in Figures 4a and b, treatment with itraconazole at 5–10 μM resulted in a decrease in cell viability by 13.15 and 14.57% in CD133+/EpCAM+ subpopulation, but no difference was observed in the other three subpopulations. Treatment with sorafenib at 10 μM significantly compromised cell viability in CD133+/EpCAM+, CD133−/EpCAM−, and Huh-7-trans subpopulations, but did not affect Huh-7-DN subpopulation (Figures 4c–f). Neither itraconazole, LDE225, nor sorafenib (10 μM) suppressed cell viability of Huh-7-DN subpopulation. Moreover, Huh-7-DN and Huh-7-trans subpopulations were treated with various concentrations of sorafenib to determine the effects of hedgehog signaling activity on drug sensitivity. Hep3B cells that are well differentiated in hepatic gene expression profile and exhibit positive CD133/EpCAM expression profile, were used as a control.10 The IC50 was calculated to reflect resistance to sorafenib in Hep3B, Huh-7-DN, and Huh-7-trans cell types, and was higher in Huh-7-DN (21.58 μM) than 11.6 μM, and 17.87 μM in Hep3B and Huh-7-trans cells (Supplementary Figures 2A–D). Taken together, the cell viability assay documented that Huh-7-DN possessed intrinsic chemoresistance to sorafenib.

### Increased Expression of Hh Signaling Molecules Contributed to Chemoresistance of Huh-7-DN Subpopulation

In order to reveal the controlling mechanisms of chemoresistance of Huh-7-DN subpopulation, expression of Hh signaling transcription factors Gli-1/2 and MDR-related ABC transporters (ABCB1, ABCC1, and ABCG2) was determined in various hepatoma subpopulations. Abnormal activation of Hh signaling in Huh-7-DN and Huh-7-trans subpopulations resulted in an increase in mRNA levels of Gli-1/2 and Bcl-2 genes (Figures 5a–c). Compared with relatively sensitive CD133+/EpCAM+ subpopulation, Gli-1 mRNA levels of Huh-7-DN and Huh-7-trans subpopulations were increased more than 12- and 22-fold. Gli-2 mRNA levels of Huh-7-DN and Huh-7-trans subpopulations were increased more than 31- and 35-fold. Of note, ABCC1 expression levels were higher in Huh-7-DN and Huh-7-trans cells than the other cell types tested (Figure 5d). Therefore, higher expression of Gli-1 and Gli-2 was accompanied with upregulation of ABCC1 expression in Huh-DN and Huh-7-trans subpopulations (2- and 3.5-fold). In contrast, expression levels of ABCB1 and ABCG2 in Huh-7-DN and Huh-7-trans subpopulations were much lower than unsorted Huh-7 and CD133+/EpCAM+ subpopulations (Figures 5e and f). Although...
Gli-1 and Gli-2 mRNA levels were much higher in Huh-7-DN and Huh-7-trans subpopulations than other hepatoma cells, only Gli-2 protein level was strikingly increased in both Huh-7-DN and Huh-7-trans subpopulations (Figure 6a).

Consistent with real-time PCR results, immunofluorescent staining exhibited a similar increase in ABCC1 protein level in Huh-7-DN subpopulation (Figure 6b). Moreover, the ABCC1 protein level of Huh-7-DN was confirmed to be significantly
higher than Huh-7-trans subpopulation (Figures 6a and f). These findings indicate that the Hh signaling has a crucial role in the development of chemoresistance in Huh-7-DN subpopulation through the ABCC1 transporter.

Pharmacological Suppression of the Hh Signaling Activity Decreased ABCC1 Expression
To further investigate whether Hh signaling is involved in the regulation of ABCC1 transporter gene expression, Huh-7-DN
and Huh-7-trans subpopulations were treated with Hh signaling SMO antagonists, LDE225 or itraconazole. As shown in Figures 7 and 8, neither itraconazole nor LDE225-affected Gli-1 mRNA levels in all concentrations tested in both Huh-7-DN and Huh-7-trans subpopulations, whereas Gli-2 expression was suppressed by a high concentration of...
LDE225 (10 μM) in both Huh-7-DN (P = 0.016) and Huh-7-trans subpopulations (P = 0.017; Figures 7b and 8b). A significant inhibitory effect on Gli-2 expression was observed by itraconazole at 1 μM, and a dose-dependent decrease of Gli-2 was seen at high concentrations (5–10 μM) in Huh-7-trans subpopulation (Figure 8e). Both LDE225 and
Hedgehog signaling and drug resistance

J Ding et al.

Figure 7 The effects of LDE225 and itraconazole on expression of Gli-1, Gli-2, and ABCC1 in Huh-7-DN subpopulation. Huh-7-DN cells were exposed to LDE225 or itraconazole at various concentrations as indicated for 24 h. mRNA levels of Gli-1, Gli-2, and ABCC1 in Huh-7-DN subpopulation were determined by quantitative RT-PCR, using untreated Huh-7 cells as a control. Protein levels of Gli-2 and ABCC1 were determined by western blot analysis, using β-actin as a loading control. Neither LDE225 nor itraconazole effectively suppressed Gli-1 mRNA expression (a and d), whereas LDE225 (10 μM) significantly reduced Gli-2 mRNA expression (b and e). Both LDE225 and itraconazole treatment inhibited ABCC1 mRNA expression in a dose-dependent manner, and a maximum inhibitory effect was observed at 10 μM (c and f). Gli-2 protein levels started to decrease from 5 to 10 μM when exposure to LDE225 or itraconazole in Huh-7-DN subpopulation (g and h). *P < 0.05, **P < 0.01 compared with untreated Huh-7-DN subpopulation.

irtraconazole suppressed expression of ABCC1 in Huh-7-DN subpopulation starting at 1 μM in a dose-dependent manner, and the most obvious decrease of ABCC1 mRNA expression was observed at 10 μM (Figures 7c and f). Consistent with mRNA levels, ABCC1 protein levels were significantly reduced in Huh-7-DN by LDE225 or itraconazole at 10 μM, whereas the reduction of Gli-2 protein levels occurred when the concentration of LDE225 and itraconazole was increased to 5 μM (Figures 7g and h). Both LDE225 and itraconazole (1–10 μM) significantly reduced ABCC1 mRNA expression in Huh-7-trans subpopulation, and subsequently western blot analysis confirmed that ABCC1 protein levels decrease from 1.0 μM (Figures 8g and h). These data further verified that Hh signaling transcription factor Gli-2 appeared to be the primary regulator for drug sensitivity of Huh-7-DN and Huh-7-trans subpopulations through ABCC1 transporter.

DISCUSSION

In the present study, we compared the phenotypic features of a newly established subpopulation of Huh-7-DN with the previously reported Huh-7-trans subpopulation, and other subpopulations or hepatoma cells, and characterized their sensitivity to sorafenib, LDE225 and another Hh inhibitor, itraconazole. The latter is an antifungal agent, and was defined as a potent Hh inhibitor that targets SMO and is a newly established subpopulation of Huh-7-DN with the most obvious decrease of ABCC1 mRNA expression in Huh-7-DN cells, we first examined levels of the ABC transporter molecules, and found that ABCC1 expression was significantly increased in both mRNA and protein levels in Huh-7-DN compared with other subpopulations. However, the other two ABC transporters (ABCB1 and ABCG2) were decreased in both Huh-7-DN and Huh-7-trans cells compared with other subpopulations. At the same time, Gli-2 expression levels in both Huh-7-DN and Huh-7-trans cells were much higher (61- and 68-fold compared with Huh-7 cells) than other subpopulations, though there was a moderate increase in Gli-1 level in Huh-7 DN (6.8-fold), and a larger increase in Huh-7-trans cells (12.2-fold) compared with Huh-7 cells. The aberrant Gli-1 expression, especially the occurrence of truncated Gli-1, was thought to be responsible for the highly metastatic property in Huh-7-trans subpopulation as we previously reported. Based on these results, it is reasonable to believe that Gli-2 has a more dominant role than Gli-1 in the mediation of chemoresistance in Huh-7-DN subpopulation. Our findings are consistent with a notion that ABCC1 is not expressed in mature hepatocytes (Figure 5d), and its expression levels are negatively correlated with differentiation grade in untreated HCC. Moreover, constitutive activation of the Hh pathway maintains chemoresistance through increasing drug efflux of ABC transporters (ABCB1 and ABCG2), and Gli-1 maintained multidrug-resistant phenotype of myeloid leukemia by ABCB1. These findings are further supported by the observation that ABCB1 and ABCG2 promoter regions have the consensus sequence of Gli-binding site, and Gli-1 directly bound to the consensus GACCACCCA-like motif located in the promoter regions of ABCB1 and ABCG2 to regulate their transcription in B-cell lymphoma and ovarian cancer cells. However, our results demonstrate that ABCC1 was highly expressed in Huh-7-DN and Huh-7-trans cells;
In contrast, ABCB1 and ABCG2 were downregulated in these two subpopulations. Accordingly, the protein levels of Gli-2 were remarkably increased in Huh-7-DN and Huh-7-trans subpopulations. Given the fact that both Gli-1 and Gli-2 may bind to the same motif in Gli-binding site and initiate transcription of target genes, we speculate that Gli-2, rather...
than Gli-1, is the major transcription factor controlling the resistance of Huh-7-DN subpopulation to sorafenib through the ABCC1 transporter, which is obviously different from what was observed in myeloid leukemia.

To further confirm the regulation of Gli-2 on ABCC1 expression, itraconazole and LDE225 were separately used to treat various subpopulations. As shown in Figures 7a and d and 8a and d, both LDE225 and itraconazole did not display any inhibition on Gli-1 gene expression in either Huh-7-trans or Huh-7-DN subpopulations. The results demonstrated that inhibition of Gli-2 expression by either itraconazole or LDE225 simultaneously suppressed ABCC1 expression in Huh-7-DN or Huh-7-trans subpopulations (Figures 7 and 8), although the inhibition on Gli-2 expression took place when the concentration of LDE225 was reached to 10 μM. The discrepancy in suppressing Gli-1 and Gli-2 gene expression by SMO antagonists, LDE225 or itraconazole, may be partially attributed to the fact that hedgehog signaling activation is under the control of canonical and noncanonical pathway, and in noncanonical pathway, hedgehog activation is operated in SMO-independent manner. This is intriguing, and more experimentation is needed to confirm our speculation. It is also possible that LDE225 was less sensitive in indirectly suppressing Gli expression when compared with its direct inhibition on Hh signaling activity in CD133+/EpCAM+ Huh-7 cells. Drug resistance has apparently developed in these cells because LDE225 has been shown to be effective at nM levels.

To further verify the effect of Gli-2 on ABCC1 expression, we used RNA interference (RNAi) and suppressed Gli-2 expression with transduction of a lentiviral-particle-harboring shRNA against Gli-2 gene in both Huh-7-DN and Huh-7-trans cells, and used cells transduced with lentiviral particles harboring scrambled shRNA as a control (Supplementary Figure 3). Our data demonstrate that shRNA against Gli-2 reduced Gli-2 gene expression by 50–70% at mRNA levels in two clones, and nearly 25–30% reduction at protein levels in both Huh-7-trans and Huh-7-DN cells (P < 0.05–0.01). Subsequently, a 10–20% decrease (P < 0.05) in ABCC1 mRNA and protein levels was seen in cells transduced with a lentiviral particles harboring shRNA against Gli-2 compared with those transduced with a lentiviral vector harboring scrambled shRNA. Therefore, these data partially support that ABCC1 is the target gene of the Hh signaling in both Huh-7-DN and Huh-7-trans cells, and Gli-2 has a major role in the development of drug resistance through modulating ABCC1 gene expression, although other factors, such as EMT transcription factors, may also participate in this process as discussed below.

Hh signaling has a critical role during embryonic development, and is often silent in adult tissue. It is activated to participate in tissue repair in chronic damage. With Hh pathway activation, hedgehog ligands (Sonic, Indian or Desert) bind to the PTCH1 receptor to release the G-protein-coupled receptor (GPCR)-family protein smoothened (SMO), and the latter leads to stabilization and nuclear translocation of GLI family members. Gli-1/2 then transact target genes, such as B-cell lymphoma 2 (Bcl-2), twist, snail, Gli-1 and PTCH-1. SMO, one of critical molecules in the Hh signaling pathway, has been a major target for pharmaceutical intervention in cancer therapy, and has been clinically proved to be effective in basal cell cancer and other malignancies although drug resistance has been reported in basal cell cancer against vismodegib. As one of Gli-1/2 target genes, BCL-2 exerts its anti-apoptosis effects by binding to pro-apoptotic proteins and preventing the release of cytochrome c from mitochondria. Consistent with the expression trend of ABCC1 protein, Huh-7-DN subpopulation had the highest BCL-2 expression level, which was followed by Huh-7-trans subpopulation. Thus, Hh signaling maintains chemoresistance of hepatoma cells through increasing drug efflux mediated by ABCC1 transporter and promoting cell survival by overexpression of Bcl-2.

Huh-7-DN and Huh-7-trans cells exhibited EMT features by overexpression of relevant transcription factors, twist and snail. The occurrence of EMT has been implicated as another mechanism of hedgehog signaling in the mediation of MDR (Supplementary Figure 1). Hh pathway activation directly contributes to the expression of twist and snail. The expression levels of twist in Huh-7-DN and Huh-7-trans cells were 300–400-fold higher than in Huh-7 cells, which indicates that this transcription factor may participate in modulating drug resistance through regulating the EMT status. Hence, it was postulated that aberrant hedgehog signaling activation promotes the transacting activity of twist and snail, which may in turn increase the activity of ABC transporters in the downstream. It remains to be seen whether Gli-2 directly transacts ABCC1 or indirectly transacts it through activation of twist and/or snail at a transcriptional level in Huh-7-DN cells.

In conclusion, the present study established a poorly differentiated and chemoresistant CD133+/EpCAM−

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Figure 8 The effects of LDE225 and itraconazole on expression of Gli-1, Gli-2, and ABCC1 in Huh-7-trans subpopulation. Huh-7-trans cells were exposed to LDE225 or itraconazole at various concentrations as indicated for 24 h. mRNA levels of Gli-1, Gli-2, and ABCC1 in Huh-7-trans subpopulation were determined by quantitative RT-PCR, using untreated Huh-7 as a control. Protein levels of Gli-2 and ABCC1 were determined by western blot analysis, using β-actin as a loading control. Similar to Huh-7-DN subpopulation, LDE225 or itraconazole did not reduce Gli-1 mRNA expression (a and d). LDE225 or itraconazole could not only significantly inhibit Gli-2 mRNA, but also ABCC1 mRNA expression (b, c, e and f). ABCC1 protein levels started to decrease when LDE225 or itraconazole concentration reached to 1.0 μM (g and h). *P < 0.05, **P < 0.01 compared to untreated Huh-7-trans subpopulation.
Huh-7-DN subpopulation exhibiting EMT and upregulation of the ABCG1 transporter. These characteristics are attributed to increased expression of Hh transcription factor Gli-2, which may govern expression of the ABCG1 transporter and contribute to sorafenib resistance of hepatoma cells. Our results reveal the molecular mechanism underlying HCC chemoresistance, and may aid in improving the outcome of chemotherapy in HCC.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.