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Inhibition of NOTCH1 signaling in tumor-initiating cells overcomes chemoresistance and promotes apoptosis

Yang Rui, Zhan Gang, Zhou Jun, Jin Weidong and Jiang Hui*

Department of General Surgery, Central Theater Command General Hospital of PLA, Wuhan, 430000, PR China

***Corresponding author**: huijian69@gmail.com

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Abstract: The Notch signaling pathway is an evolutionarily conserved pathway essential for regulation of cell development and differentiation. Upregulation and activation of Notch signaling enhances the oncogenic potential of cancer cells through apoptosis resistance. The NOTCH1 expression pattern in hepatocellular cancer (HCC) and its role in apoptosis attenuation was determined. Immunohistostaining identified intensive positive staining of NOTCH1 in human HCC tissues as compared to control tissues. RT-PCR and Western blot quantification data showed that NOTCH1 and its downstream target transcription factor Hes1 were significantly upregulated in HCC cells. Based on these findings, we separated a population of $CD44^+$ tumor-initiating cells (HepG2: >7%; SNU449: >6%) from HCC cell lines to ascertain the role of NOTCH1 in tumorigenesis. After *NOTCH1*-specific small interfering RNA (siRNA) transfection of tumor-initiating cells (TICs), NOTCH1 was significantly downregulated, and efficient uptake of DNA-targeting chemotherapeutic drugs was observed. Meanwhile, by flow cytometry analysis we found that the rate of apoptosis induction was significantly higher ($P<0.01$) and that cell viability was reduced (HepG2<23%; SNU449<28%) in siRNA transfected cells. In addition, the release of cytochrome C and activation of caspase 9 in $CD44^+$ TICs was observed after siRNA transfection, confirming the induction of the mitochondrial-dependent intrinsic apoptotic pathway. Western blot analysis revealed inhibition of the PI3-Akt signaling pathway in siRNA-transfected TICs. These data suggest that activated NOTCH1 plays a significant role in liver cancer progression through apoptosis inhibition via regulation of PI3-Akt signaling. Therefore, pharmacological inactivation of NOTCH1 represents a clinically relevant therapeutic target for treating HCC.

Keywords: apoptosis; drug resistance; hepatocellular carcinoma; NOTCH1; tumor-initiating cells

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common digestive cancers with a third of the mortality rate globally [1]. HCC is categorized as a highly vascularized tumor that is highly invasive by angiogenesis and metastasis to distant sites [2]. Despite recent advances in clinical practice and therapeutic technologies, more than 70% cases were identified with tumor recurrence 5 years after resection [1]. Most patients undergoing operative resection of cancer tissues have a poor prognosis with a low survival rate, and tumor recurrence with a high mortality rate and metastasis [3,4]. According to cancer stem cell theory, a small population of cells exist within the tumor mass that are responsible for tumor recurrence and metastasis and are designated as cancer stem cells (CSCs) or tumor-initiating cells (TICs). The phenotypic characterization of TICs presents a wide array of features such as enhanced expression of stem cell proteins (CD44, CD133, CD24), high tumorigenicity, self-renewal and differentiation potential [5,6]. The persistence of enhanced CD44 levels plays a key role in cell migration and metastasis and thus CD44 is used as a prognostic biomarker for separating TICs from different solid tumors [7,8]. As a corollary, new therapeutic targets to attack and kill TICs of HCC cells should be identified. Unravelling the signaling pathways and molecular mechanism that underly HCC TIC-mediated tumorigenesis assumes an important place in the pursuit of anticancer therapy aimed at improving disease control.

NOTCH1 signaling has a crucial role in cancer biology as it is involved in the regulation of cell proliferation, differentiation, apoptosis and cell migration and cell fate determination during embryogenesis

[9]. NOTCH1 signaling is a complex mechanism as it performs different functions [10,11] at early and late stages of cancer. NOTCH1 can either act as a tumor suppressor or an oncogene in human cancers. As an oncogene, NOTCH1 signaling and its receptors are significantly activated, which contributes towards tumor cell survival, angiogenesis, treatment resistance and apoptosis inhibition [12,13]. Crosstalk with other oncogenes affects different signaling pathways that interfere with cell cycle regulation [14]. Increased NOTCH1 is associated with tumor progression, migration, invasion and promotion of vascular mimicry of several malignancies [15]. Thus, NOTCH1 could be a potential target for novel anticancer approaches to improve treatment outcome. Nonetheless, data regarding NOTCH1 involvement in HCC TICs remains scanty and the role of NOTCH1 in TIC-mediated treatment and apoptosis resistance of HCC are not well characterized. Herein we showed that downregulation of *NOTCH1* expression significantly improved the sensitivity of HCC cells for chemotherapeutic drugs and efficiently induced apoptosis.

MATERIALS AND METHODS

Cancer sample collection and ethical statement

HCC tumor samples (n=50) were obtained from patients during resection therapy at the Department of General Surgery, General Hospital of the Central Theater of the PLA, in accordance with the ethical procedures approved by the hospital (URY97981). All the patients were requested to sign a declaration consent form and no patient details are revealed in the manuscript as per the ethical standard procedure of the hospital. Hepatocellular carcinoma was obtained from patients during liver surgery; non-cancerous sections were obtained from the opposite portion of healthy liver in patients with HCC (n=30). Both tumor and control tissues were examined by the pathologist and were immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Cell culturing

Cancer tissues were subjected to enzymatic digestion with Type III collagenase/hyaluronidase for 30 min and incubated in a shaker at 37°C. Subsequently, 0.2% NaCl hypotonic solution was used for filtration by passing through sterile gauze (200 mesh pore diameter) to remove cell clumps. Cell monolayers were prepared for further culturing and passaging. The human hepatocellular carcinoma cell lines HepG2 and SNU449 were purchased from the Chinese Academy of Science, Shanghai, China. All the cells were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), penicillin (100 U/mL), streptomycin (100 mg/ml) and L-glutamine (2 mmol/L). Cells were maintained in T-75 flasks (Corning, USA) at 37°C, provided with humidified 5% CO_2 and 95% atmosphere.

siRNA transfection

The NOTCH1-specific small interfering RNA (siRNA) and scrambled negative siRNA (which serves as the control) were purchased from Life Technologies, Shanghai, China. Cells were transfected with 20 nM siRNA with the transfection reagent Lipofectamine 2000 (Life Technologies, USA) and incubated for 48 h. Cells were harvested and subjected to different *in vitro* cellular assays. The siRNA sequences used in this study were as follows: NOTCH1: GUCCAGGAAACAACUGCAATT, and Scrambled siRNA: UUCUCCGAACGUGUCACGUTT [11].

Quantitative real-time PCR analysis

The TRIzol® Plus RNA Purification Kit (Thermo Fisher Scientific, Invitrogen, USA) was used for RNA extraction. Complementary DNA synthesis was as per the manufacturer's protocol (Invitrogen SuperScript First Strand cDNA System). PCR amplification was performed as follows: 1 μL of cDNA mixed with SYBR Green Real-time PCR Master Mix and set for 35 cycles at 95°C for 30 s, at 58°C for 45 s and at 72°C for 30 s. The expression level of each amplicon was measured and normalized with the housing-keeping gene GAPDH with SDS2.3 and FIJI Software. The relative mRNA value was expressed according to the 2-ΔΔCt method. The RT-PCR primers used in this study are as described previously [1]. NOTCH1: Fwd: CACCCATGACCACTACCCAGTT, Rev: 5'-CCTCGGACCAATCAGAGATGTT; Hes1:

Fwd: TGATTTTGGATGCTCTGAAGAAAGATA, Rev: GCTGCAGGTTCCGGAGGT; GAPDH: Fwd: AATGAAGGGGTCATTGATGG, Rev: AAGGTGAAGGTCGGAGTCAA [1].

Immunohistochemistry (IHC)

HCC tumor and control tissues were fixed in formalin and paraffin buffer. Tissue sections were about 4-5 μm, deparaffinized in xylene and subsequently blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature (RT). After washing with phosphate-buffered saline (PBS), the tissue sections were incubated with primary antibody (Rabbit- Anti-NOTCH1 (1:500); Rabbit Anti-Hes1 (1:200), Sigma, USA) overnight at 4°C. After the PBS wash to remove the unbound primary antibody, the tissue sections were incubated with biotinylated goat anti-mouse IgG for 40 min at 37°C. The slides were treated with diaminobenzidine chromogen for 15 min, washed with PBS and counterstained with hematoxylin. The signal intensities were measured by Image J software and the values are represented as a quantification graph.

Flow cytometry analysis

Cells at the logarithmic growth phase were collected and stained with anti-CD44-FITC (1:500; Thermo Fisher) in PBS buffer for 1 h at RT, followed by 3 washes with PBS buffer. The cells were resuspended in 500 μL of PBS and further subjected to flow cytometry cell sorting (BD Accuri™ C6, BD Biosciences USA). A subset of the $CD44^+$ population of cells was sorted out from the P2 gated region in the dot plot analysis of the FACs profile. We quantified the percentage of $CD44^+$ cells obtained in the P2 gated region from three independent experiments and the values were presented as a quantitative graph.

Apoptosis assessment

The induction of apoptosis in liver cancer cells after downregulating *NOTCH1* was assessed by the Annexin V FITC and PI staining kit (Invitrogen, USA). Cells at 48 h post transfection were centrifuged and resuspended in 500 μL of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid))-binding buffer containing Annexin V FITC and PI, incubated for 15 min at RT and subjected to flow cytometry analysis (BD Biosciences, USA).

Chemoresistance assay

Cells grown for 24 h were treated with a series of DNA-targeting drugs (obtained from Jinan Trio Pharmatech, China): sorafenib (10 μM), cisplatin (20 μmol/L), paclitaxel (2 μmol/L), docetaxel (10 μM), 10 μg/mL 5-fluorouracil (5-FU), and incubated for 48 h at 37°C. Subsequently, 10 μL of CCK-8 solution was added to the cells and incubated for another 3 h. Resistance to the drugs was evaluated using the formula as described previously [16]:

Rate of cell resistance (%) = (experimental group OD_{450} value/control group OD_{450} value) x100.

Western blot

Cell lysate proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes and incubated with primary antibodies for Rabbit Anti-NOTCH1 (1:1000; Sigma), Rabbit Anti-Hes1 (1:1000; Thermo Fisher), Mouse Anti-GAPDH (1:2000; Thermo Fisher), Mouse Anti-PI3 and Anti-Akt (1:1000; Santa Cruz Biotechnology, USA); Mouse Anti-Caspase 9 and Anti-Cytochrome C (1: 500; Beijing Zhongshan Biotechnology, China). A secondary antibody of mouse or rabbit horseradish peroxidase (HRP) conjugated IgG was used, and protein bands were visualized by the enhanced chemiluminescent (ECL) kit from Bio-Rad Laboratories, USA. Blots were quantified using densitometric image analysis software and normalized with the loading control GAPDH protein.

Statistical analysis

SPSS 15.0 software was used to perform statistical analyses in MS Windows (SPSS Inc., USA). For the comparison between two groups, Student's T-test and one-way analysis of variance (ANOVA) were performed. The Kaplan-Meier method was employed to make the patients cumulative survival curves, and the log-rank test was used for univariate analysis. The data presented in the graphs are the mean±standard

error of the mean (SEM); P<0.05 was considered as statistically significant.

RESULTS

NOTCH1 overexpression in HCC tissues contributes to poor survival rates

To determine the NOTCH1 expression pattern in hepatocellular carcinoma and adjacent control tissues, we performed IHC in all tumor samples (n=50) and control tissues. We identified a positive intensive staining of NOTCH1 and its downstream target Hes1 staining in the HCC tissue samples (Fig. 1A). The corresponding control tissues exhibited very slight expression of NOTCH1 (Fig. 1A). These differences were further confirmed by RT-PCR analysis for the transcriptional evaluation of NOTCH1 and Hes1, one of the main downstream targets of NOTCH1. Consistent with the IHC results, mRNA expression of NOTCH1 and Hes1 was found to be significantly higher in all HCC samples compared to the non-tumor samples (Fig. 1B). We also performed an estimation of the patients' life span by co-relating the overexpression of NOTCH1 and Hes1. Consequently, the patients' life span graph indicated that individuals who were positive to both NOTCH1 and Hes1 had a significantly (p<0.01) higher mortality than patients that were either negative or mildly positive for NOTCH1 and Hes1 (Fig. 1C). These data suggest that overexpression of NOTCH1 and its target Hes1 are highly associated with the prognosis of a reduced survival rate in HCC patients.

NOTCH1 downregulation induces apoptosis in HCC TICs

Next, we wanted to investigate whether the inhibition of NOTCH1 influences apoptosis and prevents tumor growth in HCC. To that end, we decreased *NOTCH1* expression by the siRNA method and examined the cells for sensitivity towards different chemotherapeutic drugs and apoptosis induction. First, we tested the efficiency of *NOTCH1* depletion by RT-PCR and Western blot analysis. Fig. 2A and 2B confirm that *NOTCH1* is significantly downregulated (3-fold times less) by the *NOTCH1*-specific siRNA sequence when compared to scrambled siRNA. Interestingly, we found that *NOTCH1*-depleted cells responded well to DNA-targeting drugs such as docetaxel, sorafenib, 5-fluorouracil (5-FU), cisplatin and paclitaxel, and that cell viability was significantly reduced in *NOTCH1*-downregulated cells (Fig. 2C). There was a significant decrease in cell viability (<40% cells are viable) and induction of apoptosis (>16%) in *NOTCH1*-depleted cells when compared to non-

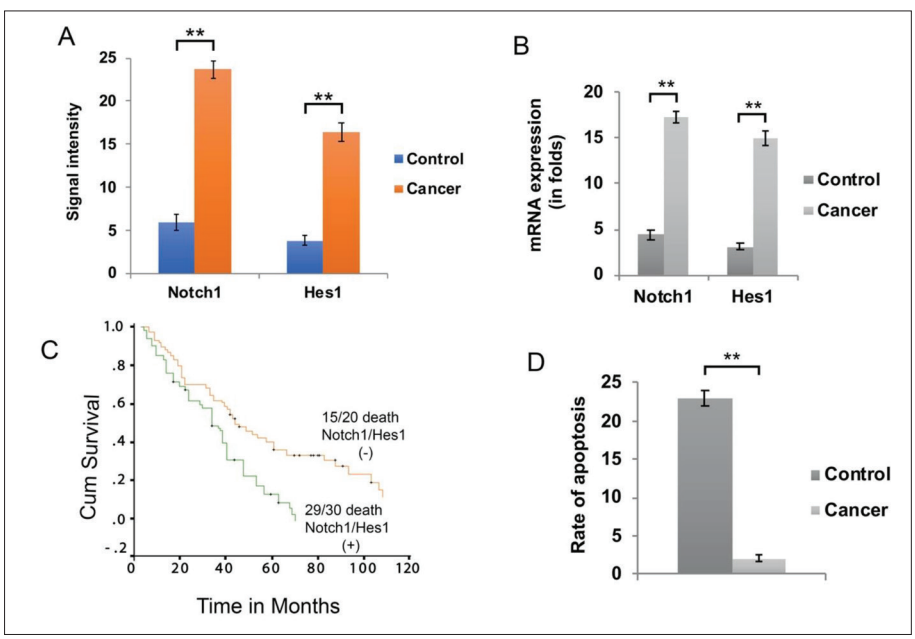

Fig. 1. Activation of NOTCH1 signaling in human hepatocellular carcinoma tissues. **A** – Quantification graph displaying the signal intensities of NOTCH1 and Hes1 from immunohistostaining of control and HCC tissues. **B** – RT-PCR analysis showing the enhanced rate of gene transcription for NOTCH1 and Hes1. ** P<0.01. **C** – Kaplan-Meier survival graph showing that the patients' cumulative life (in months) depends on NOTCH1 and Hes1 overexpression.

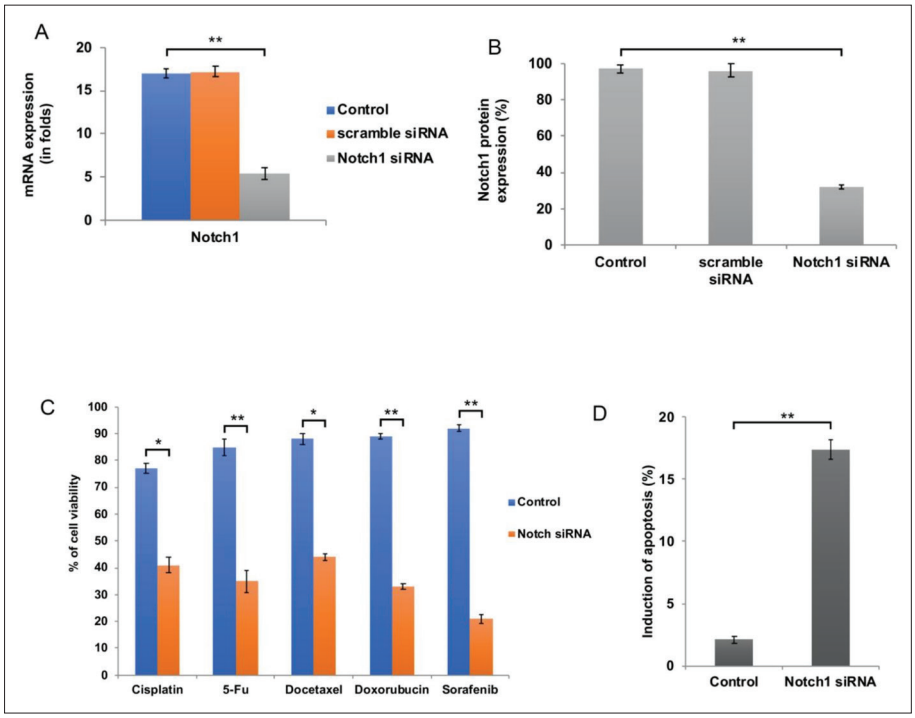

Fig. 2. NOTCH1 downregulation induced apoptosis by improving chemosensitivity. RT-PCR (**A**) and Western blot analysis (**B**) confirming NOTCH1 depletion in NOTCH1 siRNA-transfected cells. Chemoresistance assay (**C**) and flow cytometry based on the propidium iodide staining assay (**D**) showing significantly reduced cell viability and induction of apoptosis after siRNA-*NOTCH1* transfection; * P<0.05; ** P<0.01, respectively.

transfected cells (Fig. 2D). These findings clearly show that NOTCH1 inhibition led to the suppression of Hes1 oncogene and thus efficiently prevented tumor growth by inducing apoptosis in HCC cells.

Sorting of CD44⁺ TICs from hepatocellular carcinoma cell lines

CD44 plays a key role in cancer cell migration and metastasis and is therefore used as a potential biomarker for the isolation of TICs from different solid tumors [8]. Herein, we used two HCC cell lines, HepG2 and SNU449, for the sorting of CD44⁺ TICs. By FACs analysis we identified a small population of TICs (HepG2: >7%; SNU449: >6%) from both cell lines, whose fluorescence signal intensities for CD44 was maximum according to dot plot analysis of the FACs profile (Fig. 3A). The quantification graph from Western blot analysis revealed that the expression of CD44 protein and NOTCH1 protein was significantly enhanced in CD44^{+ve} TICs when compared to non-TICs (Fig. 3B). From these findings, the existence of CD44⁺ TICs in HCC cell lines is clear and that they displayed activation and overexpression of NOTCH1.

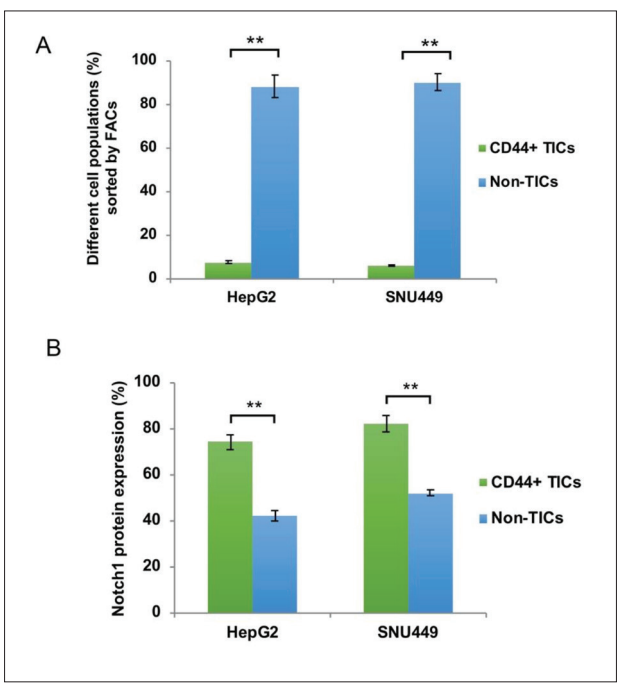

Fig. 3. Sorting of CD44 positive tumor initiating cells (TICs) from HepG2 and SNU449 cell lines. **A** – Quantitative graph from dot plot analysis of the FACs profile showing the percentage of the subpopulation of CD44^{+ve} overexpressed TICs in HCC cell lines. **B** – Quantification graph obtained from Western blots showing enhanced NOTCH1 protein expression in CD44^{+ve} TICs; ** P<0.01.

NOTCH1 overexpression is associated with chemotherapy and apoptosis resistance in human liver cancer cell lines

We investigated the effect of *NOTCH1* depletion in sorted CD44$^+$ TICs from HepG2 and SNU449 cell lines for apoptosis induction after treatment with chemotherapeutic drugs. As an initial step, we transfected *NOTCH1* siRNA to HepG2/SNU449 CD44$^+$ TICs and subjected them to RT-PCR analysis to confirm the *NOTCH1* downregulation (Fig. 4A). Consistent with the HCC RNA depletion results (Fig. 2), *NOTCH1* depletion in CD44$^+$ TICs was accompanied by significantly improved sensitivity to chemotherapeutic drugs. Again, flow cytometry-based PI staining further confirmed the induction of apoptosis after *NOTCH1* siRNA transfection (Fig. 4B). In non-transfected cells, the viability of the cells ranged from 97-99%. However, after *NOTCH1* siRNA transfection, the CD44$^+$ TICs showed early and late apoptotic features with significantly reduced cell viability (HepG2: 23%; SNU449: 28%).

We also found evidence for the release of cytochrome C and activation of caspase 9 in CD44$^+$ TICs upon siRNA transfection. The quantification graph from Western blot analysis showed that protein signal intensities for cytochrome C and caspase 9 were significantly higher after *NOTCH1* silencing of TICs (Fig. 4C and D). Together, these results suggest that overexpression of NOTCH1 in CD44$^+$ TICs facilitated liver cancer progression by resisting DNA targeting drugs and by apoptosis inhibition.

Inhibition of NOTCH1 induces apoptosis via downregulation of PI3K-Akt signaling

Several studies have reported that cytochrome C-dependent intrinsic apoptosis induction is mediated by the attenuation of the PI3K-Akt signaling pathway in different types of cancers, and that increased chemosensitivity of HCC is acquired via the downregulation of Akt signaling [17,18]. Therefore, in order to understand the molecular mechanism that underlies apoptosis induction in *NOTCH1*-depleted CD44$^+$ TICs, we

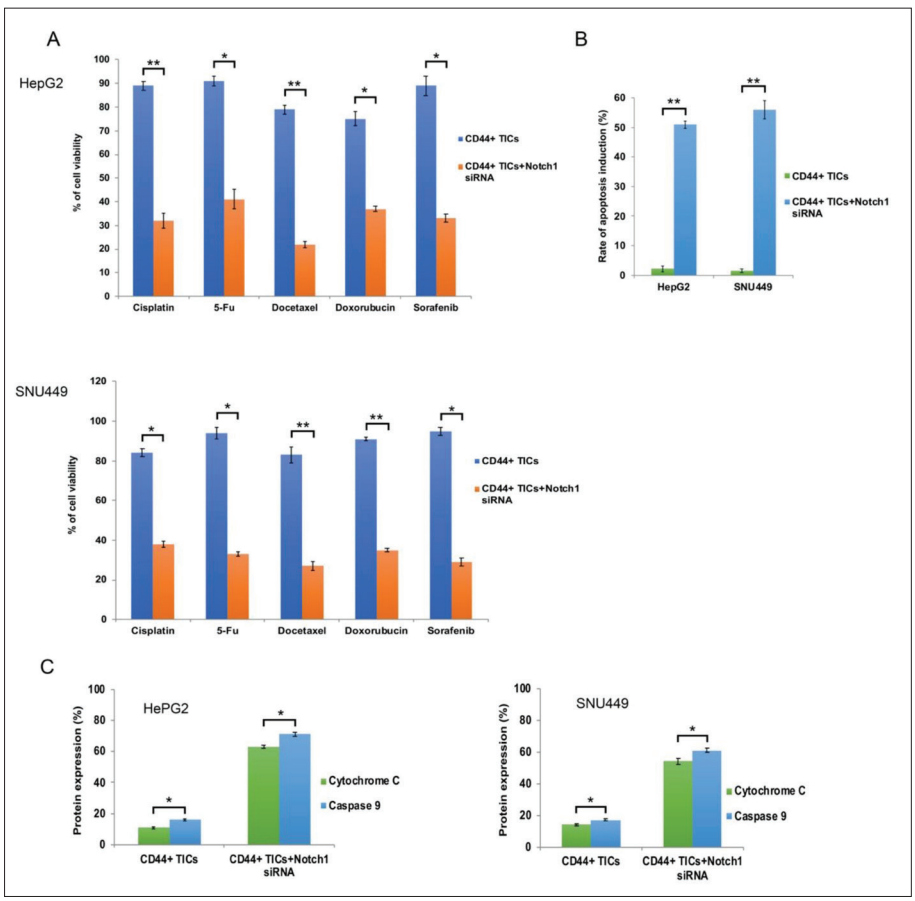

Fig.4. NOTCH1 depletion efficiently induced apoptotic cell death in CD44^{+ve} TICs. Chemoresistance assay (**A**) and flow cytometry-based PI staining assay (**B**) showing significantly reduced cell viability and induction of apoptosis after siRNA *NOTCH1* transfection in CD44^{+ve} TICs of HepG2 and SNU449. **C**, **D** – Quantification graph obtained from Western blots displaying enhanced expression of cytochrome C and caspase 9, respectively, in siRNA *NOTCH1* transfected CD44^{+ve} TICs; * P<0.05; ** P<0.01, respectively.

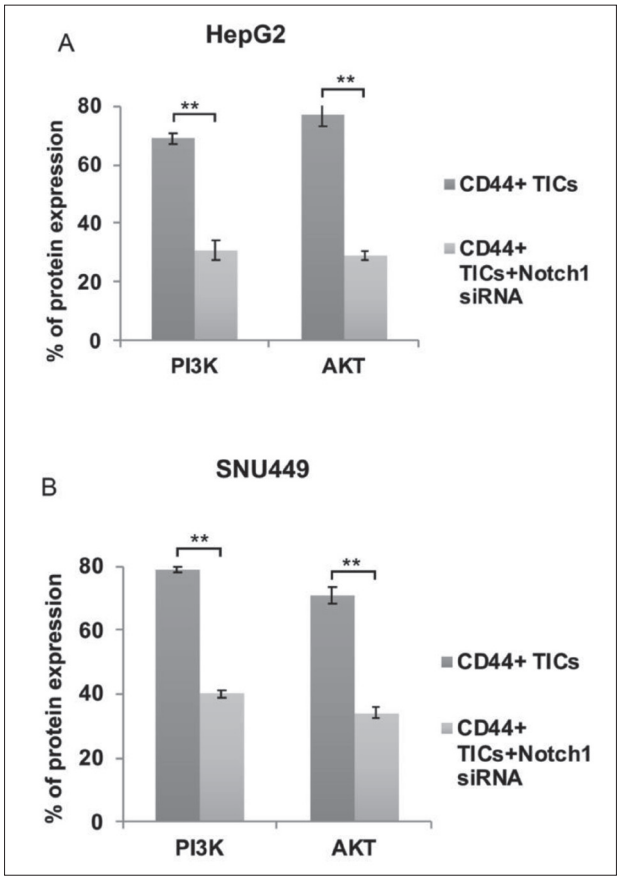

Fig. 5. Quantification graph obtained from Western blots showing inactivation of PI3K and Akt proteins in siRNA *NOTCH1*-transfected CD44^{+ve} TICs of HepG2 (**A**) and SNU449 (**B**) cell lines; * P<0.05; ** P<0.01, respectively.

evaluated the expression level of Akt by Western blot analysis. Fig. 5 shows a decrease in the relative levels of both PI3K and Akt in *NOTCH1*-silenced CD44^{+} TICs. Thus, the data indicated that overexpression of NOTCH1 in HCC CD44^{+} TICs might activate PI3K-Akt signaling for apoptosis attenuation, which facilitates liver cancer progression.

DISCUSSION

NOTCH1 is a multifunctional protein that regulates a wide array of functions such as cellular proliferation, differentiation and survival [11-14]. NOTCH1 receptors and ligands are transmembrane proteins with large extracellular domains whose activation releases the NOTCH1 intracellular domain (NICD), translocating it to the nucleus where it activates transcription of one of the target genes of NOTCH1, called the "hairy and enhancer of split-1 (HES1)". The downstream activation of Hes1 leads to the modulation of cellular processes such as cell growth, survival and migration [12,19]. To date, several studies have demonstrated that aberrant NOTCH1 signaling and overexpression of NOTCH1 and its receptors are associated with human malignancies such as breast, prostrate, cervical and pancreatic cancers [20,21]. In the present study, we observed that malignant behavior was associated with NOTCH1 in hepatocellular carcinoma, after analyzing the NOTCH1 expression pattern by IHC in HCC specimens. We identified an intensive NOTCH1-positive staining and upregulated NOTCH1 expression in HCC tissues when compared to corresponding control tissues. Further, when *NOTCH1* expression was downregulated by siRNA, HCC cells showed significant induction of apoptosis after efficient uptake of DNA-targeting drugs. Consistent with this finding, NOTCH1 downregulation by curcumin leads to significant growth inhibition of HCC [12], and downregulated NOTCH1 HCC cells possess decreased invasion capability due to reduced E-cadherin expression [22].

Based on these observations, our research objective was to elucidate the role of NOTCH1 in TIC-mediated apoptosis resistance of HCC. We first sorted the small population of TICs, based on enhanced expression of CD44, from human HCC cell lines HepG2 and SNU449. CD44, a transmembrane glycoprotein that is a widely accepted biomarker for isolating TIC- elevated CD44 expression facilitates tumorigenesis by interacting with extracellular matrix components and thus leads to cell adhesion, migration and angiogenesis [22]. Henceforth, we assumed that the presence of a small percentage of CD44^{+} TICs is the root cause for rapid tumor growth, metastasis and tumor recurrence. Moreover, HCC tissues and HCC CD44^{+} TICs displayed aberrant activation of NOTCH1 signaling. Thus, we speculated that elevated NOTCH1 might be involved in apoptosis and chemotherapy resistance. Following *NOTCH1*-specific siRNA transfection, HepG2 and SNU449 cells were evaluated for the induction of apoptosis. As expected, siRNA-NOTCH1 significantly reduced tumor progression and induced apoptosis as compared to non-transfected TICs. The siRNA-*NOTCH1* cells became more sensitive to chemotherapy treatment and underwent apoptotic cell death, and hence cell viability declined. This suggests

that NOTCH1 plays a key role in apoptosis inhibition and in promoting cell survival of HCC. However, lack of *in vivo* experiments and xenograft models are the limitations of this study's attempt to unravel the underlying mechanism of tumorigenesis.

It has been shown that activated NOTCH1 is involved in the liver cancer development in human HCC and mice models [23]. Enhanced expression of NOTCH1 in HepG2 was found to be involved in xenograft growth in nude mice with accelerated vasculogenic mimicry in cancer samples. Therefore, pharmacological inactivation of NOTCH1 is an attractive and clinically therapeutic target for HCC because it was demonstrated that the combination of NOTCH1 siRNA with interleukin-24 (IL-24) effectively induces apoptotic cell death in HCC and prevents HepG2 cell migration [24]. To ascertain the mechanism that underlies NOTCH1-mediated apoptotic cell death, we examined the levels of cytochrome C and caspase 9. The increased expression of these protein in siRNA-*NOTCH1* cells demonstrated that after transfection the mitochondrial-dependent intrinsic apoptotic pathway was induced in both cell lines, as revealed by the release of cytochrome C and the cleavage and activation of caspase 9 [16,17]. Recently, it was demonstrated that NOTCH1 induces apoptosis in HCC through the regulation of the JNK signaling pathway [12]. Herein we found that after transfection, PI3K-Akt signaling was inhibited and apoptosis was induced. These data suggest that activation of NOTCH1 contributed to apoptosis inhibition and tumor progression of HCC via the activation of Akt signaling. However, an opposite role of NOTCH1 was reported in cholangiocarcinoma-like tumors where its inhibition facilitates tumor development [25]. A possible explanation could be that NOTCH1 function depends on the biological microenvironment in a cell-specific and gene dosage-dependent manner [26,27].

In summary, our study demonstrated that activation of NOTCH1 in HCC and TIC HCC cell lines promotes tumor progression by apoptosis and treatment resistance. Hence, NOTCH1 inhibitors and anticancer therapy specifically targeting the Notch signaling pathway might provide better treatment outcome.

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Author contributions: Yang Rui performed most experiments and was assisted by Zhan Gang who performed the RNAi and Western blot experiments. Zhou Jun and Jin Weidong performed the primary cancer tissues experiments (immunohistostaining, apoptotic assays and life span assays). Jiang Hui was involved in designing the research and in the results and discussion. Yang Rui and Jiang Hui wrote the manuscript. Jiang Hui wrote the results part with suggestions from Jiang Hui who conceived and designed the research. The final version of the manuscript was approved by all the authors.

Conflict of interest disclosure: All authors declare no conflict of interest.

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