Epigenetic knockdown of Notch1 inhibits hepatitis B virus X protein-induced hepatocarcinogenesis of L02/HBx cells

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Abstract. The present study aimed to investigate the effects of Notch1 on the development of hepatitis B virus X protein (HBx)-induced hepatocarcinogenesis. The L02/HBx cells were transfected with a short hairpin RNA (shRNA) specially targeting Notch1 (Notch1-shRNA). The mRNA and protein expression levels of Notch1 signaling pathway-related molecules (Notch1, Hes1 and NICD) were detected after knockdown of Notch1. The effects of Notch1 knockdown on the proliferation was analyzed by Cell Counting Kit-8 assay, and cell cycle and apoptosis of L02/HBx cells *in vitro* were investigated by flow cytometry. The *in vivo* tumor xenograft model was established by subcutaneously injection of mice with Notch1-shRNA or sh-NC transfected cells. The effects of Notch1 knockdown on tumor progression *in vivo* were then explored by H&E staining and immunohistochemistry. The results showed that knockdown of Notch1 inhibited the activation of the Notch1 signaling pathway. In addition, decreased viability and colony formation ability of L02/HBx cells were detected along with downregulated protein expression levels of Ki-67 and PCNA (proliferating cell nuclear antigen). In addition, knockdown of Notch1 led to L02/HBx cell cycle arrest at G_0/G_1 phase by decreasing the expression of cyclin D1, CDK4, E2F1 and increasing the expression of p21 and retinoblastoma gene (Rb). Moreover, knockdown of Notch1 promoted the apoptosis of L02/HBx cells by activation of caspase-3 and caspase-9. *In vivo* experiments demonstrated that knockdown of Notch1 inhibited the tumorigenicity of L02/HBx cells. Our findings revealed that inhibition of the Notch1 signaling pathway may inhibit the development of HBx-induced hepatocellular carcinoma. Notch1 may serve as a promising therapeutic target for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor with high incidence and mortality (1,2). Surgical resection and liver transplantation are the most effective treatments for HCC, but the survival rate of HCC patients remains at only 30-40% (3). The diagnostic methods for HCC include biopsy, computed tomography (CT), and magnetic resonance imaging (MRI) (4,5); however, most patients are diagnosed at advanced stages without curative approaches (6). Moreover, the current molecular pathogenesis of HCC is poorly understood (7). Therefore, elucidation of key mechanism underlying HCC is still essential for the development of effective therapeutic strategies for HCC therapy.

Hepatitis B virus X protein (HBx) plays a key role in the replication of viral genomes and the development of chronic hepatitis B (CHB) to HCC (8,9). HBx has been found to be involved in HCC development via regulating the Notch signaling pathway (10-12). The Notch signaling pathway has been described to promote cell survival, and angiogenesis in a variety of human cancers and may serve as a promising therapeutic strategy for cancer therapy (13-15). Accumulating evidence has confirmed the role of Notch1 in regulating cell invasion and metastasis in prostate (16), breast (17) and colorectal cancer (18). In addition, Notch1 was found to participate in the pathogenesis of liver cancer by various functions and pathways. Sun *et al* (19) confirmed that Notch1 could regulate the Wnt signaling pathway, and further affect the proliferation of hepatocellular carcinoma cells. Importantly, constitutively active Notch1 alone failed to transform immortalized L02 cells *in vivo*, yet it synergized with the Ras pathway to promote hepatic cell transformation (20). However, there is a discrepancy concerning the role of Notch1 in cancer development due to the high context dependency of the Notch cascade (21). In addition to the limited and inconsistent data regarding

Notch1 involvement in HCC considering anti-tumoral effects following its inhibition (22,23), there is still an urgent need for exploring the effects of Notch1 on the tumorigenesis and progression of HCC.

In the present study, we knocked down the expression of Notch1, and then detected the regulatory relationship between Notch1 and the Notch signaling pathway. We then investigated the effect of Notch1 knockdown on the proliferation, cell cycle and apoptosis of L02/HBx cells *in vitro* and the tumor formation ability of L02/HBx cells *in vivo*.

Materials and methods

Cell culture. The L02/HBx cells were obtained from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in Gibco™ Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) and 250 μg/ml G418 in a 37˚C incubator with 5% CO_2.

Cell transfection. The short hairpin RNAs specially targeting Notch1 (Notch1-shRNAs) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of Notch1-shRNAs were as follows: shRNA1, 5'-CCGGGATGC CAAATGCCTGCCAGAACTCGAGTTCTGGCAGGCATT TGGCATCTTTTT-3'; shRNA2, 5'-CCGGCAAAGACATGA CCAGTGGCTACTCGAGTAGCCACTGGTCATGTCTTTG TTTTT-3'; shRNA3, 5'-CCGGCTTTGTTTCAGGTTCAG TATTCTCGAGAATACTGAACCTGAAACAAAGTTTTT-3'; and shNC, 5'-CCGGGCCGAACCAATACAACCCTCTCT CGAGAGAGGGTTGTATTGGTTCGGCTTTTT-3'. For the *in vitro* group, L02/HBx cells were divided into 5 groups by transfection with Notch1-shRNAs, Notch2-shRNAs, Notch3-shRNAs, sh-NC or without any treatment using Invitrogen™ Oligofectamine (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The shRNA, which induced the lowest relative mRNA of Notch1, was used for subsequent experiments.

Quantitative reverse-transcription PCR (qRT-PCR). When the cell reached 80-90% confluence, cell were harvested and total RNA was extract by TRIzol RNA kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The concentration and purity of total RNA were assessed by a ultraviolet spectrophotometer. Reverse transcription into cDNA was then conducted by a reverse cDNA transcription Kit (Takara Bio Inc., Otsu, Japan). Quantitative SYBR ExScript qRT-PCR Kit (Takara Bio Inc.) was used to detect the expression of target genes and the primers used in this study are showed in Table I. The reactions were performed at 95˚C for 10 min followed by 40 cycles of 95˚C, 15 sec and 60˚C for 1 min. Finally, the expression levels of target genes were normalized to GAPDH and then calculated using the $2^{-\Delta\Delta Cq}$ method (24).

Western blot analysis. Total protein was extracted using radioimmunoprecipitation assay (RIPA) (Sangon Biotech, Shanghai, China). The protein samples were run by 10% SDS-PAGE gel electrophoresis, transferred to the polyvinylidene fluoride (PVDF) membranes for 2 h, blocked using skimmed milk powder antigen for 30 min, and incubated with primary rat antibodies against Notch1 (cat. no. ab44986), Hes1 (cat. no. ab108937), NICD (cat. no. ab8925), Ki-67 (cat. no. ab15580), proliferating cell nuclear antigen (PCNA) (cat. no. ab18197), Bcl-2 (cat. no. ab182858), cyclin D1 (cat. no. ab16663), CDK4 (cat. no. ab199728), E2F1 (cat. no. ab4070), p21 (cat. no. ab109199), retinoblastoma gene (Rb) (cat. no. ab181616), cyclin E1 (cat. no. ab133266), caspase-3 (cat. no. ab13585), caspase-9 (cat. no. ab32539), caspase-8 (cat. no. ab25901) and actin (cat. no. ab8226) (Abcam, Cambridge, MA, USA; 1:1,000 dilution) overnight at 4˚C, respectively. Actin was used as the internal control. Then the samples were incubated with secondary rabbit anti-rat antibody (cat. no. sc-516132; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5,000 dilution) at room temperature for 2 h, followed by luminescence development using an enhanced chemiluminescence (ECL) analysis system (Santa Cruz Biotechnology).

CCK-8 assay. Cells were seeded in 96-well plates at a density of $5x10^4$ cells/well. After transfection for 24, 48, 72 and 96 h, the cells in the corresponding well were replaced with primary culture medium (no red phenol), and WST-8 solution was added to each well at 37˚C for 4 h. The absorbance of each well at 450 nm was detected by a microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA).

Colony formation assay. Cells were digested by 0.25% trypsin and filtered using a 40-μm nylon mesh. Then, cells were seeded in 24-well plates and maintained in a 37˚C incubator with 5% CO_2 for 14 days. Thereafter, the cells were fixed with 4% methanol for 15 min, followed by incubation with Giemasa (Kaiji Biotechnology, Shanghai, China) for 30 min. The colonies containing >30 cells were counted under a light microscope (Olympus IX83; Olympus Corp., Tokyo, Japan).

Cell cycle assay. Cells were digested by 0.25% trypsin. Cells ($1x10^6$ cells/ml) were collected and then fixed with 70% volume ethanol overnight at 4˚C. After discarding the supernatant by centrifugation (in 5 min x 1,500 g), 50 μl RNase A was added to the cell precipitation for 30 min at 37˚C and then 200 μl propidium iodide (PI) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added for staining. Cell cycle was then analyzed by flow cytometry (Sysmex Partec, Munster, Germany).

Cell apoptosis. Cells were digested by 0.25% trypsin. Cells ($1x10^6$ cells/ml) were collected and then suspended in 1X binding buffer. Thereafter, the cells were stained with Annexin V-PE and 7-amino-actinomycin (7-AAD) at room temperature for 15 min. Cell apoptosis was then detected by flow cytometry within 1 h.

In vivo tumor xenograft test. A total of 12 male nude mice (4-6 weeks old, 18-20 g in weight; Cyagen, Shanghai, China) were housed for one week and then divided into 2 groups: Notch1-shRNA and sh-NC groups (n=6 each group). The mice were housed in temperature-controlled cages with a 12-h

Table I. Primer sequences for the amplification of target genes.

Gene	Sequences	PCR product (bp)
Notch1	5'-CCGCAGTTGTGCTCCTGAA-3' 5'-ACCTTGGCGGTCTCGTAGCT-3'	109
Hes1	5'-GCTAAGGTGTTTGGAGGCT-3' 5'-CCGCTGTTGCTGGTGTA-3'	122
NICD	5'-GATGTCACCAGGTCTTACTAC-3' 5'-GTATATCTTCAGCAGAAATGG-3'	132
Cyclin D1	5'-GGCTGAAGTCACCTCTTGGTTACAG-3' 5'-TAGCGTATCGTAGGAGTGGGACA-3'	177
CDK4	5'-TGGTGTCGGTGCCTATGG-3' 5'-GAACTGTGCTGATGGGAAGGG-3'	128
E2F1	5'-CCAGGAAAAGGTGTGAAATC-3' 5'-AAGCGCTTGGTGGTCAGATT-3'	74
P21	5'-TGATTAGCAGCGGAACAAGGAG-3' 5'-GGAGAAACGGGAACCAGGACA-3'	254
Rb	5'-TCAAGGGTCATTATGGGTTAGGC-3' 5'-CTTTAGGTGTAGGGGAGGGGAG-3'	115
Cyclin E1	5'-CCTGGATGTTGACTGCCTTGA-3' 5'-CTATGTCGCACCACTGATACCCT-3'	116
Caspase-3	5'-GGTTCATCCAGTCGCTTTG-3' 5'-ATTCTGTTGCCACCTTTCG-3'	99
Caspase-9	5'-GCGAACTAACAGGCAAGCA-3' 5'-CATCACCAAATCCTCCAGAAC-3'	149
Caspase-8	5'-AGAGTCTGTGCCCAAATCAAC-3' 5'-GCTGCTTCTCTCTTTGCTGAA-3'	78
Actin	5'-GTTGCGTTACACCCTTTCTTG-3' 5'-GACTGCTGTCACCTTCACCGT-3'	157

light/dark cycle and given free access to water and normal chows. The maximum allowable tumor size was diameter of 2.0 cm and volume of 4.2 cm^3. Then Notch1-shRNA and sh-NC transfected cells at logarithmic growth phase were digested and suspended in DMEM respectively, at a cell density of $2x10^6$. Next, 0.2 ml cells were injected subcutaneously into the neck of each nude mouse, respectively. The nude mice were sacrificed by ether anesthesia after 25 days. The tumors were removed, and the tumor volume was calculated according to the formula: $V = (LxW^2)/2$ (V, volume, L, length and W, width). Then, the tissues were fixed in 10% formalin for haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) straining. Non-retrospective ethical approval was obtained for the animal experiments conducted in the study and tumor burden did not exceed the recommended dimensions. The animal experiments in this study were approved by the Animal Care and Research Committee of Chengdu Military General Hospital. All experiments were performed in compliance with relevant laws and guidelines. In addition, all experiments were conducted following the institutional guidelines of Chengdu Military General Hospital (Chengdu, Sichuan, China).

H&E staining. To assess the pathological changes, the fixed samples of the lungs in 10% formaldehyde were embedded in paraffin. Tissue sections cut from paraffin-embedded samples were then stained with H&E to detect inflammatory cell infiltration in the lung tissue.

IHC staining. Paraffin sections (3-5 μm in thickness) on slides with suitable tissue adhesive were processed for deparaffinization and hydration. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxidase for 15-20 min. Antigen retrieval was conducted with a microwave oven heating for 30 min with citrate buffer (0.01 M, pH 6.0). After blocked with 5% bovine serum albumin for 1 h to reduce nonspecific reactions, the sections were incubated with the appropriate monoclonal antibodies, including Notch1 (cat. no. ab44986), Hes1 (cat. no. ab108937), NICD (cat. no. ab8925), Ki-67 (cat. no. ab15580), cyclin D1 (cat. no. ab16663), retinoblastoma gene (Rb) (cat. no. ab181616), caspase-3 (cat. no. ab13585) and actin (cat. no. ab8226) (Abcam, Cambridge, MA, USA; 1:1,000 dilution) overnight at 4°C, followed by incubation with the secondary rabbit anti-rat antibody (cat. no. sc-516132; Santa Cruz Biotechnology) (1:5,000 dilution). The reaction was visualized using DAB (3,3'-diaminobenzidine), and nuclei were counterstained with hematoxylin.

Statistical analysis. Data were derived from at least three independent experiments. The data were analyzed using SPSS Software v10.0 (IBM Corp., Armonk, NY, USA). Data are presented as mean ± standard deviation and experiments were

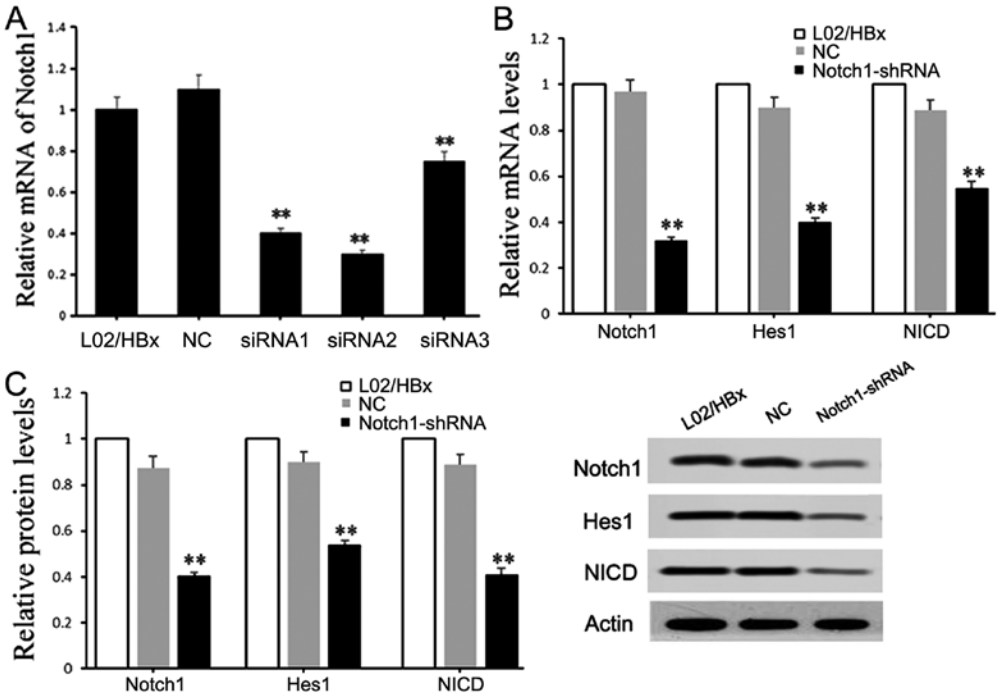

Figure 1. Knockdown of Notch1 inhibits activation of the Notch1 signaling pathway. L02/HBx cells were transfected with Notch1-shRNAs, sh-NC or without any treatment. (A) The mRNA expression of Notch1 was detected by RT-PCR. (B) The mRNA expression of Notch1 signaling pathway-related molecules was detected by RT-PCR, including Notch1, Hes1 and NICD in L02/HBx, sh-NC and Notch1-shRNA groups. (C) The protein levels of Notch1 signaling pathway-related molecules were measured by western blot analysis, including Notch1, Hes1 and NICD in L02/HBx, sh-NC and Notch1-shRNA groups. Data are represented as the mean ± SD of at least three independent experiments. **P<0.01 vs. LO2/HBX group.

repeated at least three times. Group statistical comparisons were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A value of P<0.05 indicated a statistically significant difference.

Results

Knockdown of Notch1 inhibits the activation of the Notch1 signaling pathway. Three pairs of chemically synthesized shRNAs (shRNA1, 2, 3) targeting Notch1 and negative controls (sh-NC group) were transfected into L02/HBx cells, respectively. The results of qRT-PCR showed that compared with the blank control, the expression of Notch1 was obviously inhibited by all siRNAs (P<0.01), and the inhibition efficiencies were 64.2% (shRNA1), 72.9% (shRNA2) and 22.4% (shRNA3), respectively (Fig. 1A). The L02/HBx cells were transfected with shRNA2 for subsequent experiments. Moreover, the effects of recombinant plasmid Notch1-shRNA on Notch1 signaling pathway were detected. The results showed that the mRNA and protein expression levels of Notch1 signaling pathway-related molecules, including Notch1, Hes1 and NICD, were significantly decreased in the Notch1-shRNA transfected cells compared with those in the blank control (P<0.01) (Fig. 1B and C), indicating that Notch1-shRNA could inhibit the activation of the Notch1 signaling pathway.

Knockdown of Notch1 impairs the proliferation of L02/HBx cells. The effect of Notch1 on the proliferation of L02/HBx cells was detected by CCK-8 and colony formation assays. Compared with the blank control, the cell viability of the Notch1-shRNA group was significantly decreased after 48 h of transfection (P<0.05) (Fig. 2A). Consistent with the results of the CCK-8 assay, the number of clones formed by Notch1-shRNA transfected cells was significantly reduced in comparison with the blank control or sh-NC groups (P<0.05) (Fig. 2B and C). Furthermore, the protein expression levels of Ki-67, PCNA and Bcl-2 were detected. The results showed that the protein expression levels of Ki-67 and PCNA were significantly downregulated in the Notch1-shRNA transfected cells relative to those in blank control (P<0.01) (Fig. 2D).

Knockdown of Notch1 leads to L02/HBx cell cycle arrest at the G_0/G_1 phase. The effect of Notch1 on L02/HBx cell cycle was detected by flow cytometry. The result showed that the proportion of cells at the G_0/G_1 phase in the Notch1-shRNA group was increased significantly compared with the blank control group (P<0.01), and the proportion of cells at S phase was decreased significantly (P<0.01); no obvious change was detected in the proportion of cells at G2/M (Fig. 3A and B). These data indicated that inhibition of Notch1 significantly arrested the cell cycle at the G_0/G_1 phase. Moreover, we determined the expression changes in cell cycle-related molecules. As shown in Fig. 3C and D, the mRNA and protein expression levels of Cyclin D1, CDK4 and E2F1 were significantly decreased in the Notch1-shRNA group (P<0.01), while the mRNA and protein levels of P21 and Rb were significantly increased compared with the blank control group, (P<0.01). These results indicate that knockdown of Notch1 led to L02/HBx cell cycle arrest at G_0/G_1 phase.

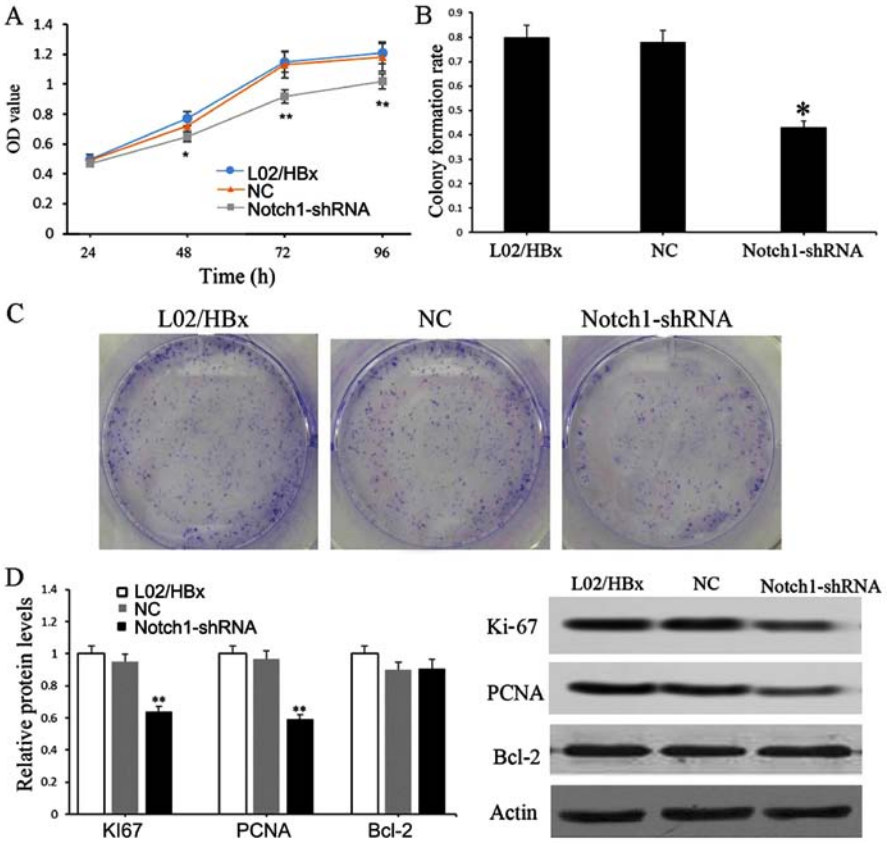

Figure 2. Notch1 knockdown inhibits the proliferation of L02/HBx cells *in vitro*. L02/HBx cells were transfected with Notch1-shRNAs, sh-NC or without any treatment. (A) The effect of Notch1-shRNA on cell viability was detected by CKK-8 assay. (B and C) The effect of Notch1-shRNA on the colony formation ability of L02/HBx cells was detected by colony formation assay. (D) The effect of Notch1-shRNA on the protein expression of Ki-67, PCNA and Bcl-2 as determined by western blot analysis. Data are represented as the mean ± SD of at least three independent experiments. *P<0.05 and **P<0.01 vs. LO2/HBX group.

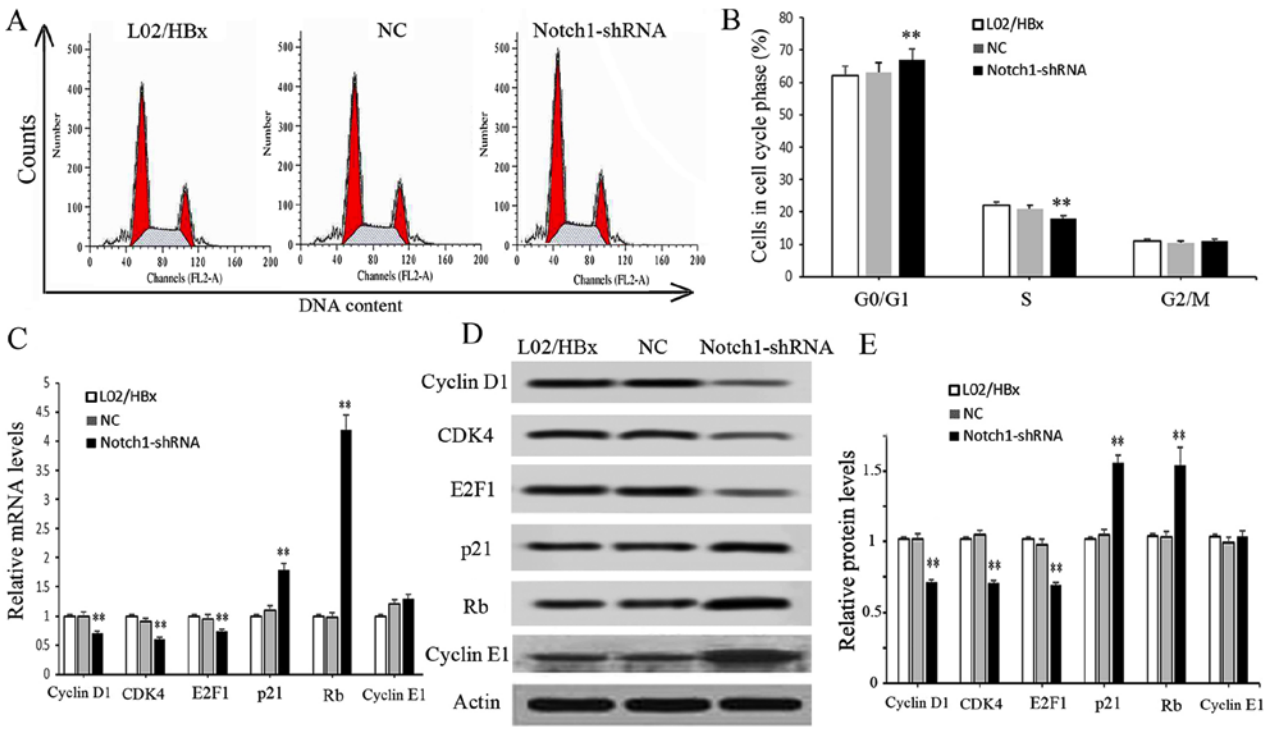

Figure 3. Notch1 knockdown arrests L02/HBx cell cycle *in vitro*. L02/HBx cells were transfected with Notch1-shRNAs, sh-NC or without any treatment. (A and B) The cell cycle phase was detected by flow cytometry. (C) The mRNA levels of cell cycle-related molecules were detected by RT-PCR, including cyclin D1, CDK4, E2F1, P21 and Rb. (D) The protein levels of cell cycle-related molecules including cyclin D1, CDK4, E2F1, p21, Rb, cyclin E1 and actin, were detected by western blot analysis. (E) Relative protein levels of cell cycle-related molecules were analyzed by bar graph. Data are represented as the mean ± SD of at least three independent experiments. **P<0.01 vs. LO2/HBX group.

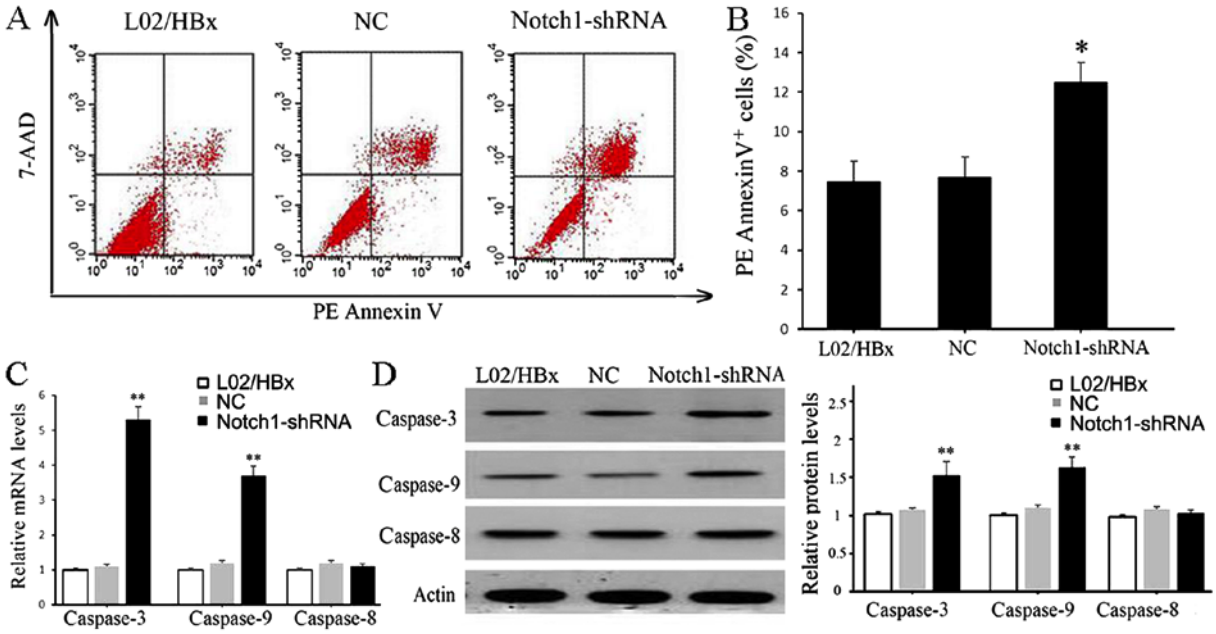

Figure 4. Notch1 knockdown promotes the apoptosis of L02/HBx cells *in vitro*. L02/HBx cells were transfected with Notch1-shRNAs, sh-NC or without any treatment. (A and B) Cell apoptosis was detected by flow cytometry after Annexin V-PE and 7-AAD double staining. (C) The mRNA expression of caspase-3, caspase-9 and caspase-8 was detected by RT-PCR. (D) The protein expression of caspase-3, caspase-9 and caspase-8 was detected by western blot analysis. Data are represented as the mean ± SD of at least three independent experiments. *P<0.05 and **P<0.01 vs. LO2/HBX group.

Knockdown of Notch1 promoted apoptosis of L02/HBx cells. The effect of Notch1 on L02/HBx cell apoptosis was also assessed by flow cytometry. As shown in Fig. 4A and B, the percentage of apoptotic cells in the Notch1-shRNA transfected group was significantly increased compared with the blank control or sh-NC groups (P<0.05). Furthermore, the mRNA and protein expression levels of caspase-3 and caspase-9 were significantly increased in the Notch1-shRNA group compared with the blank control (P<0.01), but the expression of caspase-8 exhibited no significant difference (Fig. 4C and D).

Knockdown of Notch1 inhibits the tumorigenicity of L02/HBx cells in vivo. The *in vivo* tumor xenograft model was established to further explore the effects of Notch1. The results showed that the tumor volume was significantly inhibited in the Notch1-shRNA group compared with that noted in the L02/HBx group (P<0.05) (Fig. 5A and B). A statistically difference was detected at 10 days after inoculation (Fig. 5A). In addition, the results of H&E staining revealed that the tumor tissues were hepatocellular carcinoma, characterized by a cord like arrangement, with round nuclei, large nuclear shelves and frequent nuclear division (Fig. 5C). The results of IHC staining showed that the expression levels of Notch1, Ki-67 and cyclin D1 in the Notch1-shRNA group were significantly decreased compared with these levels in the L02/HBx group, while the expression levels of Rb and caspase-3 were significantly increased (P<0.05) (Fig. 5D and E). Moreover, the results of western blot analysis showed that the expression levels of Notch1 pathway-related proteins, including Notch1, Hes1 and NICD were all significantly decreased in the Notch1-shRNA tumor group in comparison with the L02/HBx group (P<0.01) (Fig. 5F). There was no significant difference in the above indices between the NC and blank groups.

Discussion

HCC is the leading cause of cancer-related death around the world with limited effective treatments. In addition, the key mechanism underlying HCC is largely unknown. HBx functions by interfering with cellular functions, causing aberration in cellular behavior and transformation, while Notch signaling is involved in cellular differentiation, cell survival and cell death processes in various types of cells (12). Notably, Notch1 has been found to regulate cell invasion and metastasis in prostate (16), breast (17) and colorectal cancer (18). In 2017, Niloofar *et al* (25) used bioinformatic methods to predict targets for Notch1 and HBX genes in chronic hepatitis B-induced HCC. However, reports concerning the specific role of Notch1 in the tumorigenesis and progression of HCC are limited. The present study firstly demonstrated a regulatory relationship between Notch1 and the Notch signaling pathway, and investigated the inhibitory effect of Notch1 knockdown on the proliferation, cell cycle and apoptosis of L02/HBx cells *in vitro* and the tumor formation ability of L02/HBx cells *in vivo*.

One important finding of this study was that the viability and colony formation ability of L02/HBx cells were inhibited and the protein expression levels of Ki-67 and PCNA were markedly downregulated after knockdown of Notch1. Ki-67 is considered a key marker to assess cell proliferation in breast cancer (26). Li *et al* confirmed that Ki-67 expression is associated with tumor cell proliferation and growth, and may be a promising target in the diagnosis and treatment of cancer (27). In addition, PCNA is also regarded as a marker of cell proliferation in cancer (28). Upregulation of PCNA can mediate the role of long non-coding RNA CCHE1 in the proliferation of cervical cancer (29). Given the key role of Ki-67 and PCNA in proliferation, we speculated that knockdown of Notch1 may

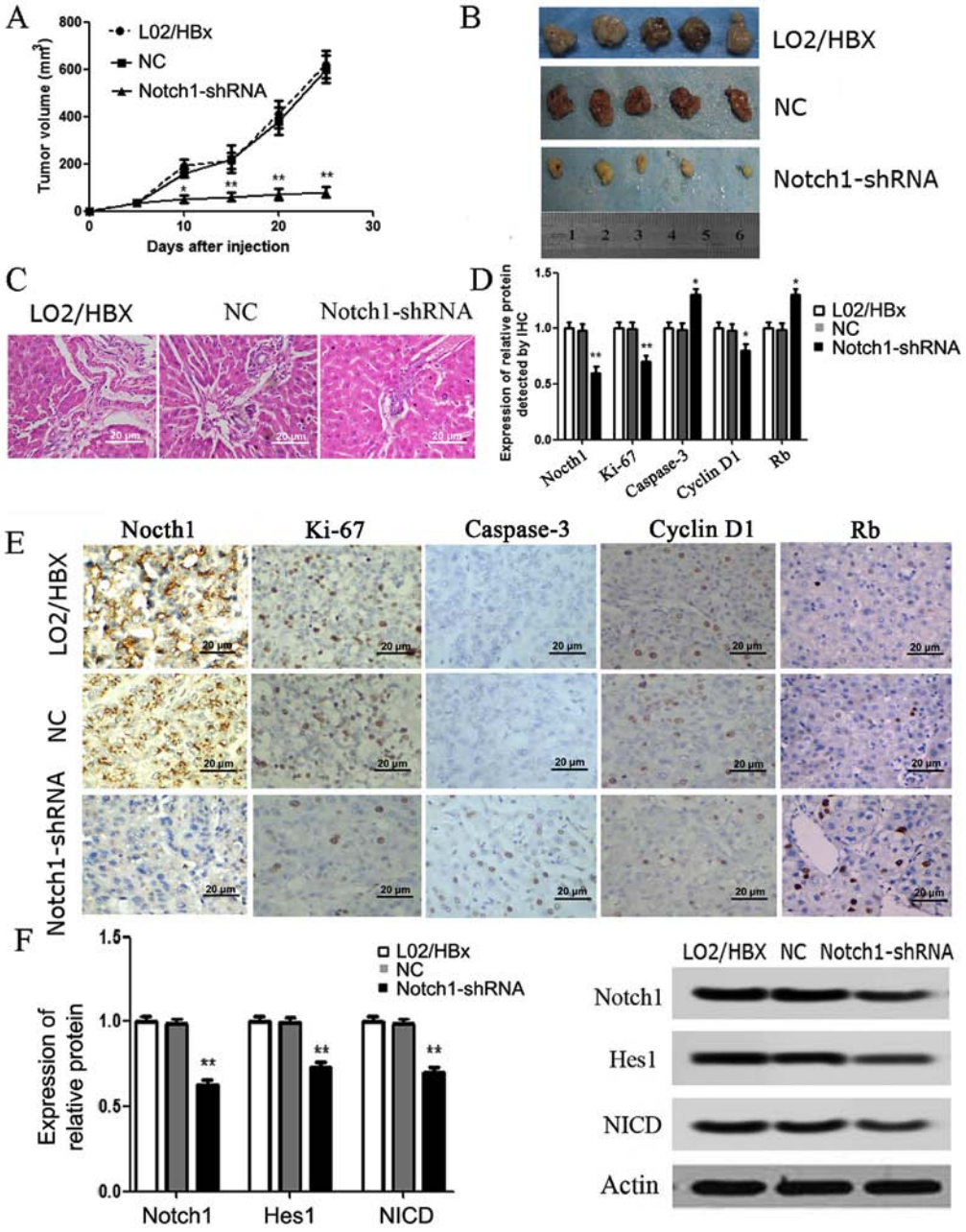

Figure 5. Notch1 knockdown inhibits the tumorigenicity of L02/HBx cells *in vivo*. The Notch1-shRNA and sh-NC transfected cells were injected subcutaneously into the neck of each nude mouse, and the nude mice were divided into sh-NC, LO2/HBX and Notch1-shRNA groups. (A) Tumor volume of cell tumorigenicity in mice of the sh-NC and Notch1-shRNA group. (B) Tumor size *in vivo* of sh-NC and Notch1-shRNA groups after 25 days. (C) The pathological changes of tumor xenograft detected by H&E staining. (D and E) The protein expression of Notch1, Ki-67, cyclin D1, Rb and caspase-3 in sh-NC and Notch1-shRNA was detected by immunohistochemistry. (F) The protein expression of the Notch1 signaling pathway-related molecules (Notch1, Hes1 and NICD) was detected by western blot analysis. Data are represented as the mean ± SD of at least three independent experiments. *P<0.05 and **P<0.01 vs. the LO2/HBX group.

inhibit the proliferation of L02/HBx cells by suppressing the expression levels of Ki-67 and PCNA.

Cell apoptosis is a key step in cancer development. Caspase-9 is the major molecule of the endogenous pathway, and caspase-8 is the major molecule of the exogenous pathway. These two pathways eventually cause caspase-3 activation. Some studies have found that HBx does not directly induce apoptosis, but increases the sensitivity of these cells to apoptotic factors (30-33). Overall, it seems that the effects of HBx on cell apoptosis are related to the cell type and duration of expression of caspases (34). In addition, knockdown of Notch-1 is found to induce apoptosis of prostate cancer cells (35). Thus, we aimed to ascertain whether the Notch1 pathway could regulate the apoptosis of L02/HBx cells, and to assess the expression of caspase-8, caspase-9 and caspase-3 apoptotic proteins. Our results showed that the apoptosis of L02/HBx cells was increased after Notch1 knockdown, together with increased expression of caspase-3 and capase-9. The expression of caspase-8 did not exhibit significant changes after Notch1 knockdown. Therefore, we speculate that Notch1 may cause dysregulation of cell apoptosis via regulating caspase-9 and caspase-3, an endogenous apoptosis pathway.

A growing body of evidence suggests that downregulation of cell cycle progression is one of the significant reasons for the early stage of liver cancer (36). p21 is a major target gene for p53-induced cell cycle arrest through DNA damage (37,38), and DNA damage repair and activation of p53 can both activate p21, thereby regulating the expression of pRb (39). In addition, Rb protein was found to play an important role in the initiation and maintenance phase of cell cycle arrest (39). Kunter *et al* confirmed that inhibition of PI3K/Akt signaling could arrest HCC cells at the G_0/G_1 phase by regulating Rb/E2F1 (40). Chen *et al* indicated that silencing of cyclin D1 could arrest HCC cells in the G_0/G_1 phase (41). In addition, CDK4 was found to be enzymatically activated by D-type cyclins to inactivate retinoblastoma, and thus arrest G1/S transition (42). In addition, a previous study showed that the activation of the Notch1 signaling pathway led to tumor cell growth and proliferation in HepG2 and SMMC7721 cells (43). Qi *et al* demonstrated that Notch1 signaling could induce cell cycle arrest and apoptosis, thus inhibiting the growth of human HCC (44). In this study, we investigated the effect of Notch1 inhibition on the cell cycle and the expression of critical cycle regulators. The results showed that inhibition of Notch1 led to l02/HBx cell cycle arrest at the G_0/G_1 phase. Moreover, the expression levels of cyclin D1, E2F1 and CDK4 were decreased and the expression levels of p21 and Rb were increased. Therefore, we hypothesized that Notch1 may cause cell cycle disturbances in L02/HBx cells via the cyclin D1/CDK4 pathway. The Notch pathway is a major member of the network that is involved in the pathogenesis of HBx-induced HCC. Activated Notch1 was observed in HBx-induced L02 cell malignant transformation, while inhibition of the Notch1 pathway decreased cell proliferation (19). In addition, Villanueva *et al* suggested that Notch signaling is activated in human HCC samples and promotes formation of liver tumors in mice. These reports indicate that Notch signaling inhibition contributes to the suppression of HBx-induced HCC (45). Consistent with this research, knockdown of Notch1 in this study was found to inhibit tumor growth and induce cell cycle arrest in L02/HBx cells *in vitro* and *in vivo*. These findings confirmed that knockdown of Notch1 inhibited the tumorigenicity of L02/HBx cells.

In conclusion, our findings revealed that knockdown of Notch1 can inhibit the activation of the Notch1 signaling pathway, and thus suppress the development of HBx-induced HCC. Knockdown of Notch1 may inhibit the proliferation of L02/HBx cells by regulating the expression levels of Ki-67 and PCNA, and induce cell cycle arrest at the G_0/G_1 phase by decreasing the expression of cyclin D1, CDK4, E2F1 and increasing the expression of p21 and retinoblastoma gene (Rb). Moreover, knockdown of Notch1 may promote apoptosis of L02/HBx cells by activation of caspase-3 and caspase-9. *In vivo* experiments also confirmed that knockdown of Notch1 inhibited the tumorigenicity of L02/HBx cells. Thus, Notch1 may serve as a promising therapeutic target for HCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YP interpreted the main data regarding the cell transfection and cell function analysis. DZ and YY were involved in the *in vivo* tumor xenograft test. CK and ZJ were responsible for immunohistochemistry. ZZ and XW interpreted the western blot analysis and conducted the statistical analysis. JX was responsible for the design and drafting of the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal experiments in this study were approved by the Animal Care and Research Committee of Chengdu Military General Hospital. All experiments were performed in compliance with relevant laws and guidelines. In addition, all experiments were conducted following the institutional guidelines of Chengdu Military General Hospital (Chengdu, Sichuan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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