Notch1 activation contributes to tumor cell growth and proliferation in human hepatocellular carcinoma HepG2 and SMMC7721 cells

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Abstract. Notch signaling controls cellular differentiation and proliferation. Recent studies have shown that Notch signaling plays an important role in the carcinogenesis and progression of a growing number of malignant tumors. We investigated the effect of Notch1 activation on human hepatocellular carcinoma (HCC). In five human HCC cell lines, it was found that SMMC7721 had relatively high while HepG2 relatively low expression of Notch1 and the activity of Notch signaling. Notch1 activation by transfection of active intracellular region of Notch1 (ICN1) into HCC HepG2 cells enhanced cell growth and proliferation, including *in vitro* single cell colony formation, anchorage-independent proliferation, and *in vivo* tumorigenicity. Notch1 activation also promoted HepG2 cell cycle progression. Suppression of Notch1 activation by RNAi of Notch1 or by γ-secretase inhibitor (GSI) in HCC SMMC7721 cells decreased cell growth capability and blocked cell cycle progression. Moreover, it was found that suppression of Notch1 activation induced SMMC7721 cell apoptosis, as demonstrated by apoptosis assays. These findings indicate that Notch1 activation promotes human HCC cell growth and proliferation, which may contribute to the progression of this type of malignant carcinoma.

Introduction

Notch signaling pathway is important for many types of cell fate determinations and essential for proper embryonic development. Notch pathway comprises a family of transmembrane receptors and their ligands, negative and positive modifiers, and transcription factors. Notch receptors (Notch 1-4) and two groups of ligands, Jagged and Delta-like, have been identified in mammals (1). During maturation, Notch precursors are cleaved to produce an extracellular subunit (NEC) and a transmembrane subunit (NTM) (2). Binding of Notch ligands to receptors leads to two cleavages involving a metalloprotease and a presenilin-dependent protease and a release of the receptor intracellular region (ICN), which translocates to nucleus to activate transcription of a group of downstream genes and thus controls various biological events (3).

Since Notch signaling affects cell differentiation, proliferation and survival, recent studies have focused on its effects on malignant tumors and revealed its complex roles in cancer. In most malignant tumors, Notch plays an oncogenic role. Notch1 is identified as an oncogene responsible for acute T cell lymphoblastic leukemia (T-ALL) (4), and its oncogenic effects are also found in glioma, primary melanoma and pancreatic cancer (5-7). Notch2 is involved in the aberrant expression of CD23 in B-cell chronic lymphocytic leukemia and related to the failure of apoptosis (8). Notch3 contributes to the growth of human lung cancers by its effect on mitogen-activated protein kinase pathway (9). Notch4 is found to correlate with Ki67, and GSI treatment arrests the growth of breast cancer cells (10). However, in some circumstances, Notch is found to play a suppressive role. In mouse skin, Notch1 functions as a tumor suppressor (11). A recent report further demonstrates that inactivation of Notch1 and Notch2 simultaneously induces the development of a severe form of atopic dermatitis, which is accompanied by an increase in immature myeloid populations in the bone marrow and spleen, and the increase is revealed to be cell non-autonomous and caused by dramatic microenvironmental alterations (12). Moreover, in human breast cancer, Notch2 expression decreases as tumor grade increases and Notch2 signaling suppresses tumor growth (13).

In research on the liver, reports show that Notch signaling plays an important role in hepatoblast differentiation and all four Notch receptors are expressed in adult human liver (14,15). Most significant upregulation of Notch1, Notch2, Notch3, Delta1 and Jagged1 is observed in a hepatectomy (AAF/PHx) model (16). Notch1, ICN1 and Jagged1 proteins are all upregu-

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lated and Notch1/Jagged1 signaling is activated during rat liver regeneration (17). Notch3, Notch4, Jagged1, Delta1 and Hairy Enhancer of Split-1 (HES-1) are all expressed in HCC (18,19). And the levels of Notch1, Jagged1, and HES-1 expression are increased in HCC samples relative to the adjacent HCC-free liver tissue (20). Our previous study also shows that Notch1, Notch4 and Jagged1 are expressed higher in HCC than in adjacent nontumor tissue (21,22). All these studies suggest that Notch signaling might play a role in the development of HCC, however, investigations are lacking. Since Notch1 are demonstrated to be upregulated in HCC relative to adjacent nontumor liver in our previous experiment, we investigated the effects of Notch1 activation on human HCC HepG2 and SMMC7721 cell growth and proliferation in this study. We found that Notch1 activation contributed to the progression of HCC by enhancing cell growth activity and promoting cell cycle progression. Our data define a novel role for Notch1 in HCC progression and indicate that Notch1 might be a potential therapeutic target for the treatment of HCC.

Materials and methods

Cell lines and drugs. Five human HCC cell lines, SMMC7721, HepG2, HHCC, 9724, 9204 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and the Lab Animal Center of the Fourth Military Medical University (Xi'an, China). These cells were cultured at 37˚C in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Tulsa, OK, USA) supplemented with 10% fetal calf serum in 5% CO_2-humidified air. The plasmid of pcDNA3.1/ICN1-myc-His(-)C was a gift from Professor Tom Kadesch of University of Pennsylvania School of Medicine (23). ICN1 included 1760 to 2556 amino acid positions of full-length human Notch1 and was cloned into pcDNA3.1/myc-His(-)C at the *Xho*I and *Kpn*I sites. Generation of HepG2 cell line that stably expressed ICN1 was accomplished through transfection with pcDNA3.1/ICN1-myc-His(-)C using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Transgene expressed cells were selected and maintained with 600 μg/ml G418 (Gibco). Bulk cultured transfectants were picked and verified by measurement of myc-tagged ICN1 protein of exact molecular weight (MW), which were named HepG2-ICN1. Mock transfection was done by transfection of pcDNA3.1(-) vector (Invitrogen), which was named HepG2-pc. siRNAs for Notch1 were chemically synthesized (Invitrogen) and the target sequences were: si1, TATCGACGATTGTCCAGGA; si2, CAACA ATGAGTGTGAATCC; si3, TCCGAGGACTATGAGAGCT. The siRNAs were ligated with pSilencer 3.1-H1 neo vector (Ambion, Austin, TX, USA) respectively, and then pSilencer 3.1-Notch1/RNAi was constructed and confirmed by DNA sequencing. Control siRNA was processed as above and pSilencer 3.1-con/RNAi was constructed. Using Lipofectamine™ 2000 reagent, the pSilencer 3.1-Notch1/RNAi or pSilencer 3.1-con/RNAi was transfected, respectively, into SMMC7721 cells following the manufacturer's protocol. Transgene cells were selected and maintained with 800 μg/ml G418 (Gibco). Bulk cultured transfectants were picked and verified by measurement of Notch1 protein levels, named 7721-si. Mock transfectants were named 7721-con. For Notch signaling inhibition, HepG2 and SMMC7721 cells were treated with N-(N-(3,5-difluorophenacetyl-L-alanyl))-S-phenyl glycine t-butyl ester (DAPT) (Calbiochem, Darmstadt, Germany) at different final concentrations (2.5, 5 and 10 μM). 5 μM dimethyl sulfoxide (DMSO) was used as drug control.

Western blot analysis. Immunoblotting of cellular proteins from SMMC7721, HepG2, HHCC, 9724, 9204 cells was performed using anti-Notch1 goat polyclonal antibody (pAb) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA, 1:300). The anti-Notch1 pAb is raised against a peptide mapping at the C-terminus of Notch1 of human origin (sc-6014). Identification of 7721-si or HepG2-ICN1 was performed with the anti-Notch1 pAb or anti-myc monoclonal antibody (mAb) (1:300, Cell Signaling Technology, Boston, MA, USA). Nuclear protein extracts of SMMC7721 or HepG2 treated with 5 μM DAPT or 5 μM DMSO were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA). The level of ICN1 from the nuclear protein was detected with the anti-Notch1 pAb. The incubation of primary antibodies were followed by peroxidase coupled anti-goat or anti-mouse IgG. Proteins were then visualized by enhanced chemiluminescence (ECL). Western blot for β-actin or Histone H3 (1:200, BioLegend, San Diego, CA, USA) was used as internal sample. Autoradiograms were quantified by densitometry (software: Bio Image IQ). Relative protein levels were calculated by referring them to the amount of β-actin or Histone H3 protein. Mean values from three independent experiments were recorded as the results.

Luciferase assay. pGa981-6, a reporter gene plasmid which contained a hexamerized 50 bp Epstein-Barr virus nuclear antigen 2 response element (EBNA2RE) of the TP-1 promotor in front of the luciferase gene, was strictly dependent on recombination signal binding protein-Jκ (RBP-J) (24). pRL-TK (Promega Corporation, Madison, WI, USA) was co-transfected as an internal control for transfection efficiency. A negative control plasmid (neg-pGa981-6) was constructed by replacing EBNA2RE with an irrelevant DNA segment. For luciferase assay, pGa981-6 and pRL-TK were co-transfected into the above five HCC cell lines, and also the HepG2-ICN1, HepG2-pc, 7721-si and 7721-con cell lines, with Lipofectamine™ 2000 (Invitrogen). Neg-pGa981-6 replacing pGa981-6 was used as negative controls. After 48 h, co-transfected cells were lysed and luciferase assays were performed in Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA, USA). All assays were repeated three times.

MTT assay. The *in vitro* growth rates of HepG2-ICN1 and 7721-si cells were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, cells ($2x10^3$) were seeded into 96-well plates, respectively, and cultured in DMEM supplemented with 5% fetal calf serum. After 1, 2, 3, 4, 5, 6, 7 days, the culture medium was removed and 20 μl of 5 mg/ml MTT was added. After 4 h, the supernatant was discarded and 100 μl DMSO was added to each well. The mixture was shaken at room temperature for 10 min and measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). HepG2-pc and 7721-con cells were used as controls. The growth inhibitory rate was

calculated as: (control A value-treated A value)/control A value x 100%, where control A value and treated A value are the average absorbance of three parallel experiments from treated and control groups, respectively. DAPT-treated HepG2 and SMMC7721 cells were also performed as above. DAPT of different concentrations were added to the cells every day. DMSO was used as control. The cells in this experiment were also used in the following procedures.

Plate colony-forming assay. Cells mentioned above were seeded at a density of 500 cells/dish in 6-well plates and cultured in DMEM supplemented with 5% fetal calf serum. The culture medium was replaced by fresh medium or medium with drug (for DAPT treatment) everyday. After 2 weeks of incubation, cell colonies were washed and fixed with 100% methanol. Finally, cells were stained with Gimsa dye and colonies containing more than 50 cells were counted. Each experiment was done in triplicates.

Soft agar colony-forming assay. Anchorage-independent cell proliferation was determined by a soft agar assay, 24-well plates were coated with 0.5% of bottom agar solution, and $2x10^3$ cells per well were embedded into 0.3% top agar gel containing DMEM supplemented with 10% fetal calf serum, respectively. The cell suspensions were added over the precoated bottom agar gel in the 24-well plates. The dishes were examined with a vertical microscope for colony formation after a 2-week incubation period. For drug treatment, DAPT of different concentrations were added to the bottom agar and top agar solution and 5 μM DMSO was used as drug control. Colonies of >75 mm were counted. Each experiment was done in triplicates.

Tumorigenicity in nude mice. BALB/c (nu/nu) nude mice (4-6 week old; the Lab Animal Center of the Fourth Military Medical University) were injected subcutaneously into the right flank with $4x10^6$ HepG2-ICN1 or 7721-si cell suspension in DMEM. Five animals were used in each group. After 4 weeks, mice were euthanized, and the tumors were removed and weighted. HepG2-pc and 7721-con cells were inoculated as controls. This experiment was in accordance with approved ethical standards of the responsible committee of the University and with the Declaration of Helsinki (1975).

PI staining and flow cytometry (FCM). Cells ($1x10^6$) were seeded in 6-well plates and incubated overnight. Then cells were harvested, fixed and resuspended in propidium iodide (PI) solution. Samples were then analyzed for their DNA content by flow cytometer (FCM) (Coulter Co., Hialeah, FL, USA). For drug treatment, 5 μM DAPT was added to the culture solution and 5 μM DMSO was used as control (this drug concentration and control were employed in the following experiments). Each experiment was done in triplicates.

Annexin V binding assay. Cells undergoing early apoptosis were identified by binding of Annexin V to membrane phosphatidylserine and assayed using fluorescein isothiocyanate (FITC)-conjugated Annexin V (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were seeded in 6-well plates and incubated overnight. Then cells were harvested, added with PI (final concentration 1 μg/ml) and Annexin V (final concentration 1 μg/ml) successively, and then analyzed by flow cytometry. Each experiment was done in triplicates.

TUNEL assay. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining was performed using an *in situ* apoptosis detection kit (Dingguo Inc, Beijing, China). The positive cells were detected with nitroterazolium blue chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) reagent and counterstained with nuclear fast red dye, and then examined by light microscopy. The total number of apoptotic cells in 5 randomly selected fields was counted.

TEM assay. The above cells were fixed, dehydrated and embedded. Then they were sectioned and stained in 1% uranyl acetate and for 5 min with lead citrate. The cell ultrastructure was assessed by transmission electron microscopy (TEM) at 4,000-fold magnification.

Statistical analysis. Statistical analysis was performed using SPSS software (version 13.0, SPSS, Chicago, IL, USA). Two-sided Student's t-test was used. $P<0.05$ was considered as statistically significant.

Results

Identification of 7721-si and HepG2-ICN1 cells and detection of activity of Notch signaling. Firstly, we examined the expression of Notch1 in five human HCC cell lines and found that SMMC7721 had the relatively high while HepG2 the relatively low expression of this molecule, and the active ICN1 (the lower molecular band) was most abundant in SMMC7721 cells while undetectable in the other four cell lines (25). To further examine the activity of Notch signaling in the above cells, Notch signaling reporter plasmid pGa981-6 and the internal control plasmid pRL-TK were co-transfected into the above five cell lines. The results from luciferase assay showed that the activity of Notch signaling was relatively high in SMMC7721 while relatively low in HepG2 (Fig. 1A). Thus, these two cell lines were used as experimental targets in this study. After transfection with three pairs of siRNAs, it was found that Notch1 in SMMC7721 cells was significantly knocked down by the third siRNA ($P<0.05$), which showed the 7721-si cells was successfully achieved. Besides, after transfection with pcDNA3.1/ICN1-myc-His(-)C, the expression of myc-tagged ICN1 in transfected HepG2 cells was detected by western blot and HepG2-ICN1 cells were successfully constructed. We also examined the activity of Notch signaling in these transfectants and found that Notch signaling was significantly inhibited by RNAi of Notch1 and was activated by transfection with ICN1 (Fig. 1B). To compare the inhibition effects of DAPT on ICN1 between HepG2 and SMMC7721 cells, these two cell lines were treated with DAPT. The result showed that ICN1 in the nuclear protein of SMMC7721 treated with 5 μM DAPT was significantly reduced compared with treated with 5 μM DMSO ($P<0.05$), while no ICN1 was detected in HepG2 treated with DMSO or DAPT (Fig. 1C).

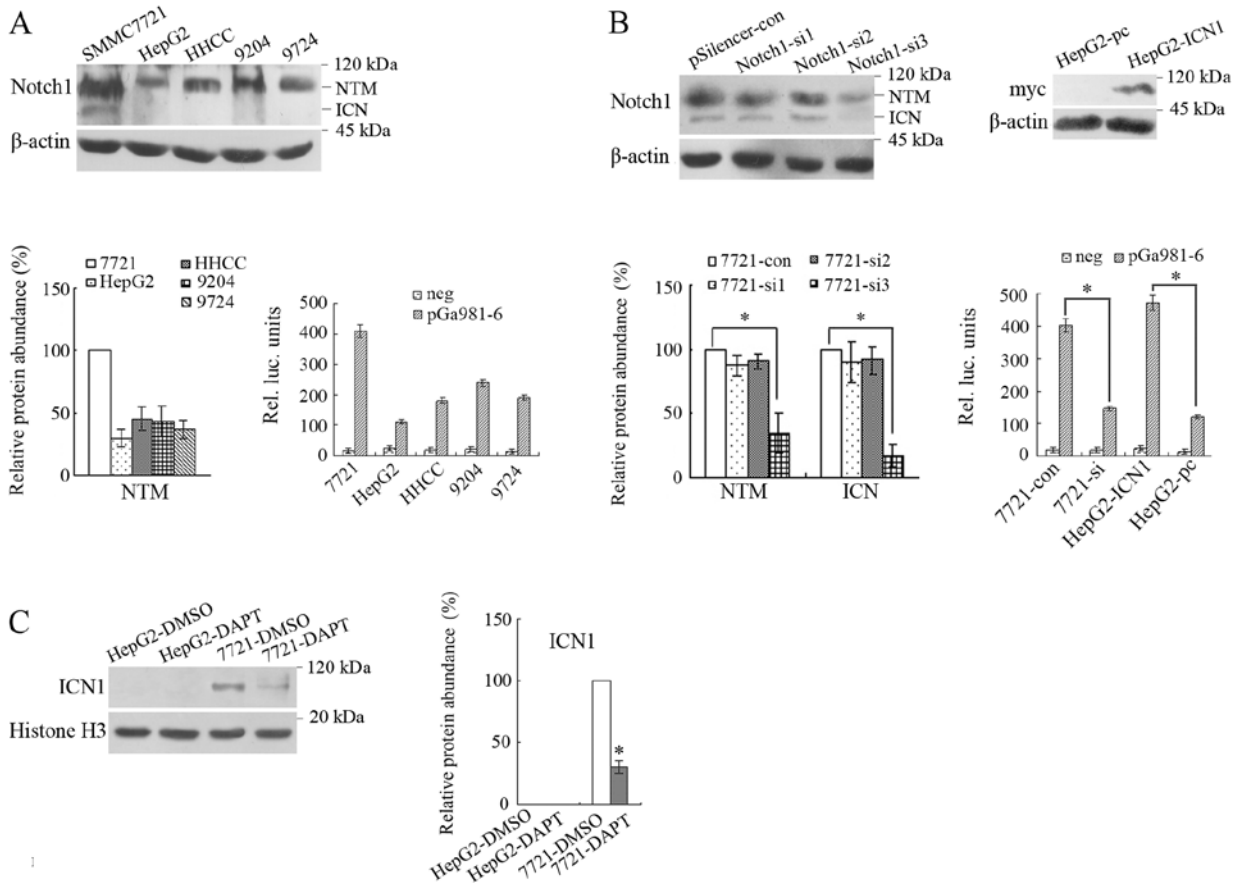

Figure 1. Construction of 7721-si and HepG2-ICN1 cell lines and detection of activity of Notch signaling. (A) Upper panel, the expression of Notch1 was examined by western blot in the five HCC cell lines. β-actin was an internal control. Lower left panel, autoradiograms were quantified by densitometry and relative protein levels were calculated by referring them to the amount of β-actin protein. Data were normalized by setting the ratios of the protein level of NTM1 against β-actin in SMMC7721 cells at 100%. Lower right panel, luciferase assay showed the activity of Notch signaling in the five HCC cell lines. (B) Upper left panel, three pairs of siRNAs were transfected into SMMC7721 cells and then Notch1 protein was examined with the anti-Notch1 pAb in the different tranfectants by western blot. Upper right panel, pcDNA3.1/ICN1-myc-His(-)C plasmid was transfected into HepG2 cells and the myc-tagged ICN1 of exact MW was examined with the anti-myc mAb by western blot. Lower left panel, data were normalized by setting the ratios of the protein levels of Notch1 NTM and ICN against β-actin in 7721-con cells at 100%. Lower right panel, luciferase assay shows the activity of Notch signaling in the tranfectants. *P<0.05. (C) Left panel, ICN1 in the nuclear protein of SMMC7721 and HepG2 treated with 5 μM DMSO or DAPT was examined with the anti-Notch1 pAb by western blot. Right panel, data were normalized by setting the ratios of the protein levels of ICN1 against Histone H3 in SMMC7721 cells treated with 5 μM DMSO at 100%. *P<0.05.

Notch1 activation promotes HCC cell growth and proliferation. Cell growth and proliferation *in vitro* was detected by MTT, plate colony-formation and soft agar assay. The MTT results showed that the growth of HepG2-ICN1 cells was significantly faster than that of HepG2-pc cells (since the 2nd day) (P<0.05), and the growth of 7721-si cells were significantly slower than that of 7721-con cells (since the 3rd day) (P<0.05). Since DAPT could significantly reduce the levels of ICN1 in SMMC7721 cells, cell growth in HepG2 and SMMC7721 treated with DAPT was also examined by the above assays. Compared with DMSO treatment, no significant cell inhibition was observed when DAPT was applied at final concentration of 2.5 μM, however, potent inhibitory effects of DAPT on SMMC7721 cells were observed at higher concentrations (5 and 10 μM) at the 1st day (P<0.05, P<0.05, Fig. 2A), and the degree of inhibition was positively correlated with the exposure time. It was also observed that the inhibitory rate of 10 μM was similar with 5 μM DAPT, which showed that 5 μM was a suitable final concentration. For HepG2 cells, DAPT at all tested concentrations did not significantly inhibit cell growth compared with DMSO, which might result from the low activity of Notch signaling in this cell line.

Cell growth was also detected by colony-formation and soft agar assay. As showed in Fig. 2B and C, the number of cell colonies of HepG2-ICN1 cells was much greater than that of HepG2-pc cells (P<0.05), and 7721-si cell colonies were significantly less than 7721-con cells (P<0.05). Compared with DMSO, 5 or 10 μM DAPT significantly reduced capacity of SMMC7721 cells for single cell colony formation or anchorage-independent cell proliferation (both P<0.05). Consistent with the MTT result, the inhibitory effect of 10 μM DAPT was not stronger than 5 μM DAPT. DAPT at all tested concentrations did not significantly inhibit cell growth of HepG2 cells compared with DMSO. The above results showed that Notch1 activation promoted the growth and proliferation of HCC cells, while suppression of Notch1 activation inhibited HCC cells growth.

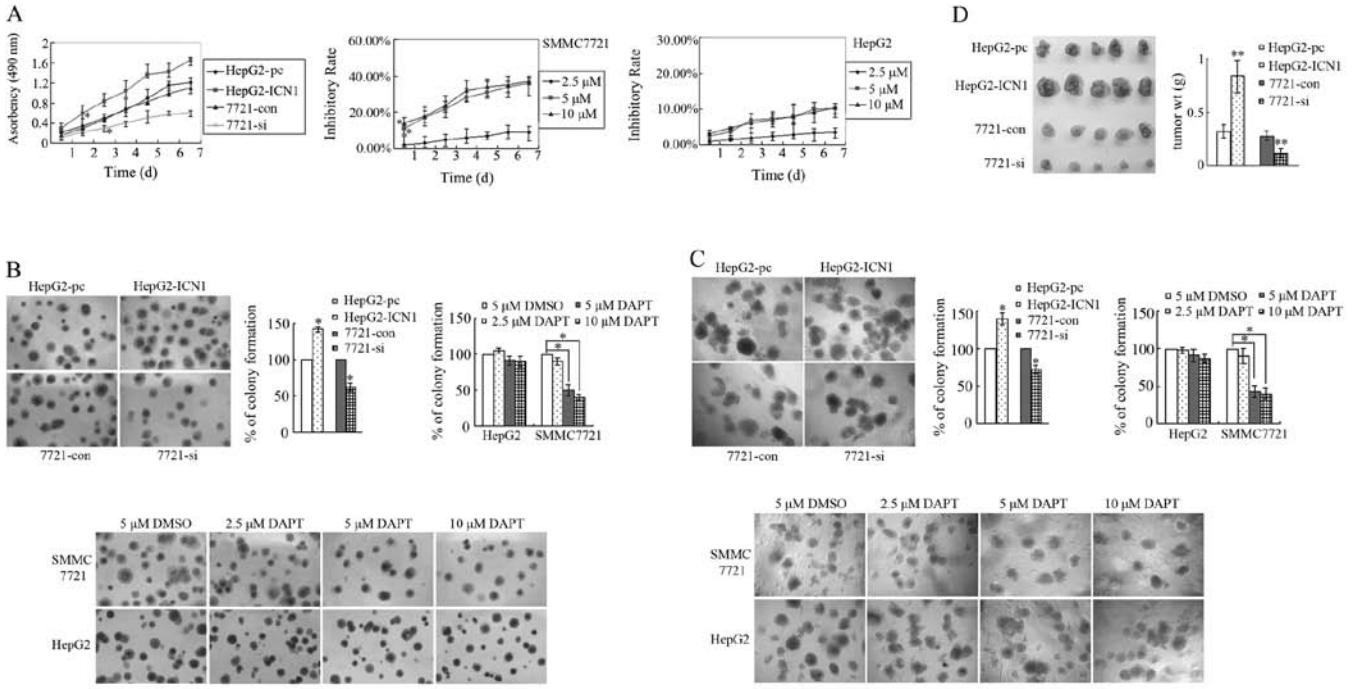

Figure 2. Notch1 activation promotes while suppression of Notch1 activation inhibits the growth and proliferation of HCC cells *in vitro* and *in vivo*. (A) Cell growth was detected by MTT assay in HepG2-ICN1, 7721-si, DAPT-treated HepG2 and DAPT-treated SMMC7721 cells. HepG2-pc, 7721-con, DMSO-treated HepG2 or DMSO-treated 7721 cells were used as controls. The cells of this experiment were also used in the following experiments. $^{*}P<0.05$. (B) Single cell colony formation was investigated by colony-formation assay in the above cell lines. (C) Soft agar assay was used to detect cell capacity of anchorage-independent proliferation in the above cell lines. Mean values from three independent experiments were taken as the results. Data were normalized by setting the number of cell colonies in the control cells at 100. $^{*}P<0.05$. (D) The HepG2-ICN1 and 7721-si cell growth *in vivo* was examined by tumorigenicity in nude mice. HepG2-pc and 7721-con cells were used as controls. Each experiment was done in triplicates. Data were expressed as mean ± SD. $^{**}P<0.01$.

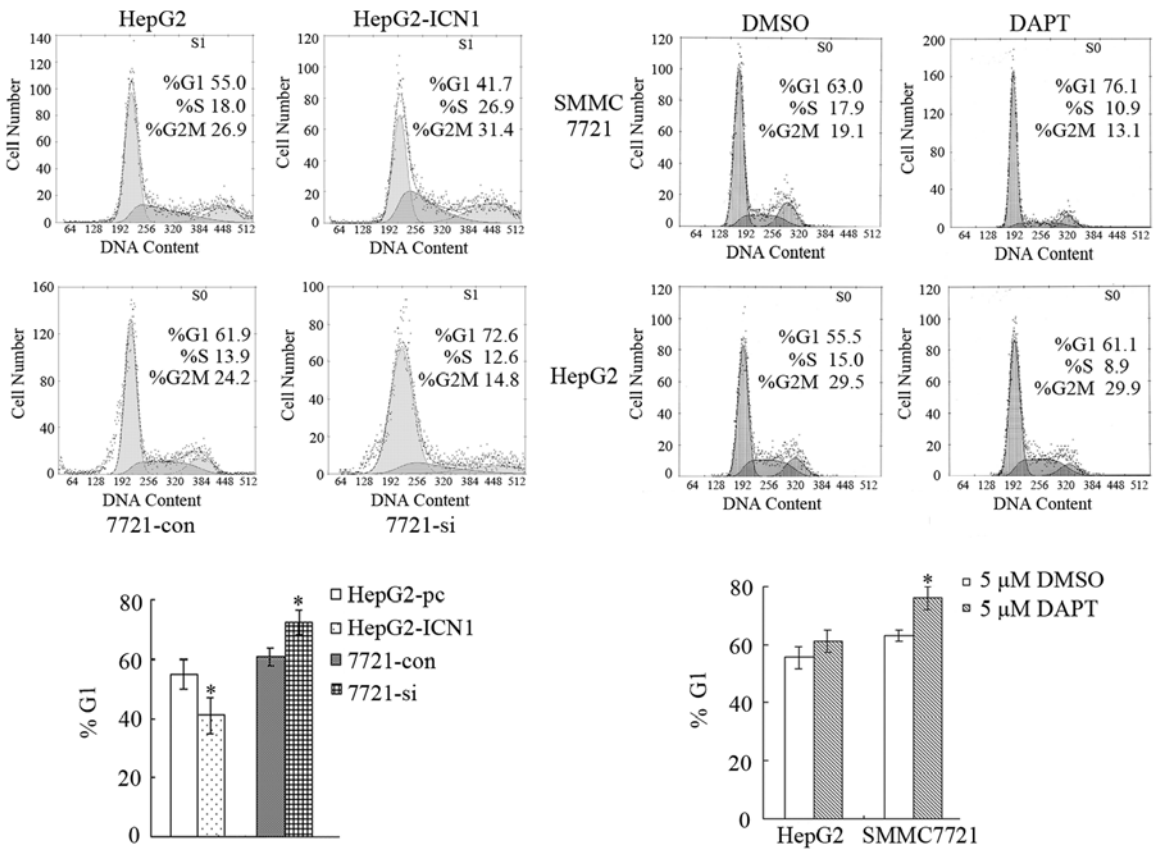

Figure 3. Notch1 activation promotes while suppression of Notch1 activation blocks HCC cell cycle progression. PI staining and FCM analysis were used to detect cell cycle progression in HepG2-ICN1, 7721-si, DAPT-treated HepG2 and DAPT-treated SMMC7721 cells. HepG2-pc, 7721-con, DMSO-treated HepG2 or DMSO-treated 7721 cells were used as controls. Each experiment was done in triplicates. $^{*}P<0.05$.

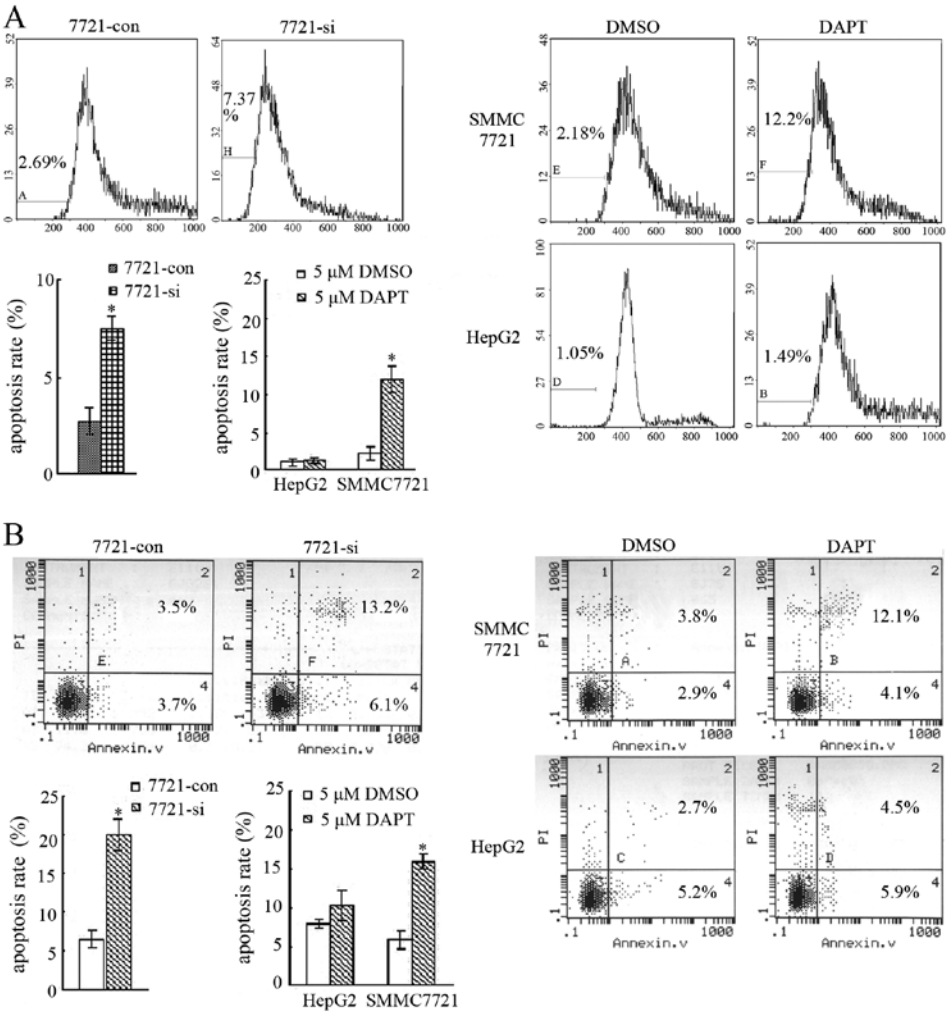

Figure 4. Suppression of Notch1 activation induces SMMC7721 cell apoptosis. (A) Apoptosis histograms from the above PI staining and FCM are presented. *P<0.05. (B) Apoptosis was assessed by Annexin V and PI double-staining assay. 7721-si, DAPT-treated HepG2 and DAPT-treated SMMC7721 cells were assayed using FITC-conjugated Annexin V and PI. 7721-con, DMSO-treated HepG2 or DMSO-treated 7721 cells were used as controls. Annexin V+/PI-, apoptosis of early stage; Annexin V+/PI+, late stage. *P<0.05.

Cell growth *in vivo* was examined by tumorigenicity in nude mice. All nude mice injected with HepG2-ICN1, HepG2-pc, 7721-si or 7721-con cells generated tumors in their right flank after four weeks. The tumors from nude mice injected with HepG2-ICN1 cells were heavier than those from HepG2-pc cells (P<0.01), and the mean weight from nude mice injected with 7721-si cells was less than that from 7721-con cells (P<0.01, Fig. 2D).

Notch1 activation promotes HCC cell cycle progression. The effects of Notch1 activation on cell cycle progression was examined by PI staining and flow cytometric analysis. DNA histograms of data from FCM analysis showed that the number of HepG2-pc cells in G1 phase was greater than that of HepG2-ICN1 cells (P<0.05), and the number of 7721-si cells in G1 phase was greater than that of 7721-con cells (P<0.05). Since 5 μM DAPT could reduce ICN1 and inhibit cell growth in SMMC7721 cells effectively, this drug condition was used in the following experiment. Compared with DMSO, the G1 phase cells in SMMC7721 treated with 5 μM DAPT increased significantly (P<0.05), while neither DAPT nor DMSO could influence HepG2 cell cycle distribution (Fig. 3). This result showed that the promotion of cell cycle might be a reason for the stimulation of cell growth in HepG2 and SMMC7721 cells by Notch1 activation.

Suppression of Notch1 activation induces HCC SMMC7721 cell apoptosis. Cell apoptosis was examined after Notch1 activation was suppressed in SMMC7721 cells. Firstly, apoptotic histograms from PI staining and FCM were analyzed. The result showed that apoptosis rate of 7721-si cells (7.37%) were much higher than that of 7721-con cells (2.69%) (P<0.05). The apoptotic rate of SMMC7721 increased significantly after treated with 5 μM DAPT (12.2%) compared with DMSO (2.18%) (P<0.05), whereas, DAPT did not affect the apoptosis of HepG2 cells significantly (1.05%, 1.49%) (Fig. 4A). Apoptosis was also assessed by Annexin V and PI double-staining assay. The percentage of apoptotic cells (Annexin V+/PI-, early stage; Annexin V+/PI+, late stage) of 7721-si was 19.3%, which was much higher than 7.2% of 7721-con cells (P<0.05). The percentage of apoptotic SMMC7721 cells treated with DMSO was 6.7%, and after treatment with 5 μM DAPT, the percentages of apoptotic cells increased to 16.2% (P<0.05). For HepG2 cells, drug treatment did not produce more apoptosis than control (Fig. 4B).

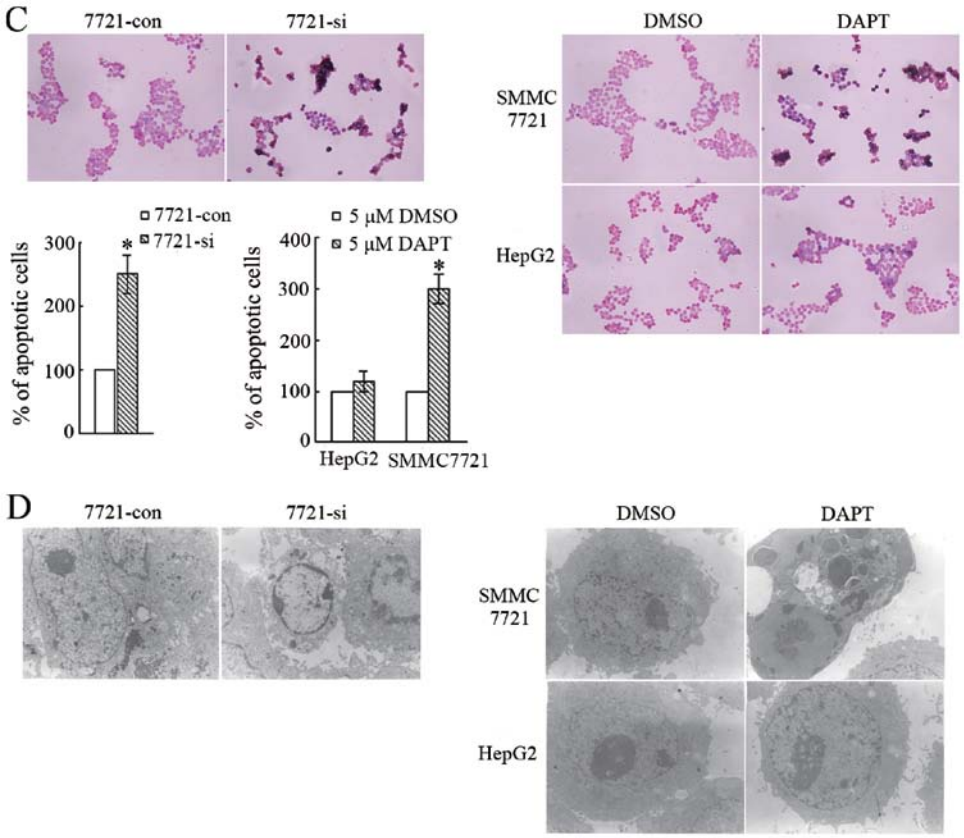

Figure 4. Continued. (C) TUNEL method was also used to detect apoptosis. NBT/BCIP staining in this TUNEL experiment stained amethyst apoptotic nucleus and red normal nucleus (magnification, x200). Counts of apoptotic cells in 5 randomly selected fields were determined and mean values from three independent experiments were taken as the results. Data were normalized by setting the number of apoptotic cells in the control cells at 100. The results are given in percentages. *P<0.05. (D) The ultrastructure of cell apoptosis was investigated by TEM. The above cells were treated according to the procedure of TEM section and examined at 60 kV on a Philips 100 M electron microscope at 4,000-fold magnification. Each experiment was done in triplicates.

Moreover, apoptotic cell morphological assessment was also carried on. TUNEL method, which labeled fragmented DNA *in situ*, was used to detect apoptosis. NBT/BCIP staining in our TUNEL experiment stained amethyst apoptotic nucleus and red normal nucleus. Besides, the ultrastructure of cell apoptosis was investigated by TEM. A characteristic pattern of apoptosis included chromosome condensation, nuclear fragmentation, cytoplasmic organelles closely packed, cell blebbing and emergence of apoptotic bodies. In contrast, normal cells exhibited membrane and nuclei with evenly distributed chromatin, as well as intact intracellular organelles. The result showed that 7721-si had more apoptosis than 7721-con cells ($P<0.05$). Treatment with 5 μM DAPT induced more cell apoptosis in SMMC7721 than DMSO of the same dose ($P<0.05$, Fig. 4C and D). For HepG2, either DAPT or DMSO could not lead to apparent cell apoptosis. These results demonstrated that suppression of Notch1 activation could lead to SMMC7721 cell apoptosis, which might also be a reason for the retarded growth of SMMC7721 cells by inhibition of Notch1 activation.

Discussion

HCC is one of the most common malignant tumors in the world. The molecular mechanisms of HCC have been studied for many years, and many molecules and signaling pathways are found to be involved in the development of HCC. Recent studies have revealed that Notch signaling is not only important in embryonic development and cell fate determination, but also plays crucial roles in the carcinogenesis, progression, invasion and neurovascular formation of many malignant tumors. Among Notch, Notch1 is most comprehensively investigated. For example, activated Notch1 signaling dramatically induces proliferation and inhibition of apoptosis in Hodgkin and anaplastic large cell lymphoma (26). Constitutive activation of the Notch1 pathway enhances primary melanoma cell growth and metastatic capability, which are mediated by β-catenin (27). Downregulation of Notch1 inhibits cell growth, contributes to apoptosis and suppresses invasion in pancreatic cancer cells (5,28). Up to now, there are several studies reporting the roles of Notch1 in HCC. Notch1 signaling could inhibit growth of human HCC through induction of cell cycle arrest and apoptosis (29), and Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human HCC cells (30), which indicates that Notch1 signaling might play a negative role in HCC. However, investigations from other teams come up with different results. A study shows that activation of Notch1 in the pre-neoplastic foci might be associated with the progression of HCC in the diethylnitrosamine-induced hepatocarcinogenesis model (31). Downregulation of Notch1 signaling by a Notch1 inhibitor curcumin, inhibits tumor growth in human HCC (32). Besides, a research team newly reports that abnormal Notch1

expression is strongly associated with HCC metastatic disease and knockdown of Notch1 reverses HCC tumor metastasis in a mouse model (33).

We examined the expression of Notch1 in five human HCC cell lines and found that SMMC7721 had the relatively high, while HepG2 the relatively low expression, and the active ICN1 was most abundant in SMMC7721 cells while undetectable in the other four cell lines. Since ICN1 is rapidly degraded after it activates transcription in the nucleus, it is reasonably undetectable even in cells that require it for survival, due to its short half-life. Luciferase assay further showed that the activity of Notch signaling was relatively high in SMMC7721 while relatively low in HepG2 cells. Ectopic expression of ICN1 activated Notch signaling in HepG2, while Notch1 knockdown by RNAi led to inhibition of Notch signaling in SMMC7721. Although it is deemed that Notch1 is vital for the survival of mammalian cells, and without Notch, cells will probably die, the protein expression of Notch1 and activity of Notch signaling still existed in 7721-si cells of our experiments, which might support the survival of this transfected cell line. Moreover, our data showed that Notch1 activation by ectopic expression of ICN1 promoted the growth and proliferation of HepG2 cells, while suppression of Notch1 activation by RNAi of Notch1 inhibited SMMC7721 cell growth. Since our previous study demonstrates that DAPT reduces the expression of ICN1 and HES-1 in SMMC7721 cells (34), we also compared the inhibition effects of DAPT on ICN1 between HepG2 and SMMC7721 cells in this study. We found that DAPT could reduce the level of ICN1 in SMMC7721 cells, while no ICN1 was detected in HepG2 at all. Further, it was demonstrated that DAPT inhibited tumor cell growth in SMMC7721 while not HepG2 cells. Thus, our results indicate an oncogenic effect of Notch1 on HCC, which is consistent with those recent reports. Our data indicate Notch1 as a critical player in HCC development and progression, while not except other Notch molecules involved, since other Notch receptors and ligands are also expressed in HCC (22,35).

The influence of Notch activation on cell growth is related to its effects on cell cycle and apoptosis, which has been proved in a series of malignant tumors. For example, in T-ALL cells, inhibition of the Notch pathway activity leading to derepression of retinoblastoma protein Rb and subsequent exit from the cell cycle (36). In pancreatic cancer, Notch1 downregulation leads to increasing cell population in the G0-G1 phase (5). There are also studies showing the relationship of Notch and apoptosis. It is reported that GSI induces breast cancer cell apoptosis (37). GSI treatment also induces apoptosis of myeloma cells via specific inhibition of Notch signaling, and enhances sensitivity to chemotherapy (38). Since Notch1 activation was found to promote HCC cell growth and proliferation in our investigation, we also examined its effect on cell cycle and apoptosis. Indeed, we found that Notch1 activation increased HepG2 cell number in the S and G2M phase, while inhibition of Notch1 activation arrested the cell cycle of SMMC7721 in the G1 phase. Besides, it was demonstrated that inhibition of Notch1 activation induced SMMC7721 cell apoptosis. The suppression of cell growth, arrest of cell cycle and induction of cell apoptosis by inhibition of Notch1 activation in our case is consistent with the roles of Notch1 in the reported other cancers, and the underlying related molecular mechanisms remain for further study.

To date, Notch has been considered as a new approach to overcome cancers since RNAi of Notch1 or GSIs was found to inhibit malignant transformation and development in many tumors (39,40). For instance, after phase I clinical trial of MK-0752 in patients with T-ALL and other leukemias in 2006 (41), phase I trial of this novel Notch inhibitor for children with refractory central nervous system malignancies is also currently completed (42). The inhibition of cell growth and induction of cell apoptosis in SMMC7721 in our investigation also indicate that the siRNA of Notch1 and specific GSIs might be served as new drugs for HCC therapy. Considering the discrepant expression of Notch1 and activity of Notch signaling in different HCC cell lines, we suppose that this kind of novel therapy should be individualized, which also needs thorough and careful research.

In conclusion, our study demonstrates that Notch1 activation contributes to tumor cell growth and proliferation whereas suppression of Notch1 activation inhibits cell growth, induces arrest of cell cycle and cell apoptosis in human HCC cells. Our findings refer to Notch1 as a new molecular mechanism in the development of HCC and support a potential oncogenic role of this molecule in HCC. Further studies clarifying the composition of Notch1 signaling and its downstream molecules mediating multiple steps in HCC progression will contribute to make clear how Notch1 activation regulates HCC and will also be helpful for providing more support for Notch1 used as a valuable approach for HCC therapy.

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