Notch2 is a crucial regulator of self-renewal and tumorigenicity in human hepatocellular carcinoma cells

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Abstract. The Notch pathway plays an important role in both stem cell biology and cancer. Notch2 was reported to be upregulated in human hepatocellular carcinoma (HCC) tissues. However, the biological function of Notch2 in human HCC cells has not yet been documented. The aim of this study was to investigate its possible function on the progression of human HCC cells. The expression of Notch2 was detected in four human HCC cell lines by western blotting. Next, Notch2 was knocked down by small interference RNA (siRNA) in human HCC cells. The role of Notch2 in human HCC cells was investigated by cell proliferation assay, colony formation assay, chemoresistance and xenograft formation assay. In the present study, western blotting revealed that the expression of Notch2 was upregulated in human HCC cell lines. Genetic depletion of Notch2 in HCC cells not only resulted in significantly inhibited proliferation, cell cycle progression and colony formation ability but also increased its sensitivity to 5-fluorouracil (5-FU) compared with controls. In addition, upregulation of Notch2 was discovered in CD90 positive HCC cells, CD90 is a marker of hepatic stem cells. Most importantly, knockdown of Notch2 in HCC cells impaired the tumor formation *in vivo*. Taken together, our findings indicate that Notch2 may confer stemness properties in HCC; downregulation of Notch2 inhibited the proliferation and tumor formation of HCC cells and increase their sensitivity to 5-FU, suggesting Notch2 as a potential therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent carcinomas and the third leading cause of cancer-related deaths throughout the world (1). The best curative procedures for patients with HCC are hepatectomy and liver transplantation. However, curative therapies are not effective in a large number of patients due to diagnosis at advanced stage (2). Main risk factors for the development of HCC have been defined during the decades, nevertheless numerous aspects of the evolution of hepatocellular carcinogenesis, progress and metastasis are still not fully understood (3). Thus, HCC remains an aggressive cancer with a high mortality rate. In recent years, compelling evidence has emerged in support of the presence of cancer stem-like features that is responsible for chemoresistance and recurrence of HCC (4-7). Targeting cancer stem cell stemness determinants including self-renewal and drug-resistance has been proposed as a therapeutic goal. The Notch pathway is one of several key pathways linked to both stem cell biology and cancer (8). Four transmembrane Notch receptors (Notch1-4) exist in mammals. The receptors are mainly activated by ligands of the Delta and Jagged families, which are expressed on the surface of adjacent cells (9,10). In particular, dysregulated Notch2 activity has been reported to be associated with several human tumor types (11-14). Most importantly, Notch inhibition suppresses nasopharyngeal carcinoma by depleting cancer stem-like side population cells (15). Since aberrant Notch signaling has been implicated in cancer and cancer stem cell therapeutic strategies that effectively target Notch signaling could have a major impact on cancer patient survival.

In the liver, Notch2 are key regulators of liver development (16,17). The expression of Notch2 was found in HCC patients (18). Dill *et al* reported that activated Notch2 signaling in the liver leads to upregulation of pro-proliferative genes and proliferation of hepatocytes and biliary epithelial cells (BECs). Most importantly, using the diethylnitrosamine (DEN) HCC carcinogenesis model, they showed that constitutive Notch2 signaling accelerated DEN-induced HCC formation (19). However, the biological function of Notch2 in human HCC cells has not yet been documented. The objective of this study was to investigate the biological function of Notch2 in the progress of human HCC.

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Figure 1. The expression of Notch2 in HCC cell lines and downregulation of Notch2 by siRNA. Western blot analysis revealed that Notch2 was expressed in four HCC cell lines (A). The expression of N2ICD was significantly downregulated in HepG2 and SMMC-7721 cells by Notch2-siRNA (B).

Materials and methods

Cell culture. The human HCC cell lines HepG2, SMMC-7721, Bel-7402 and PLC were purchased from the Shanghai Cell Collection (Chinese Academy of Sciences). In brief, SMMC-7721 and BEL-7402 cell lines were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Biological Industries). HepG2 and PLC were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries).

Small interfering RNA transfection. Validated human siRNA for Notch2 (sense, 5'-GGAGGUCUCAGUGGAUUAUATT-3', antisense, 5'-UAUAUCCACUGAGACCUCCTT-3') and negative control siRNA (sense, 5'-UUCUUCGAACGUGUCACGUTT3', antisense, 5'-ACGUGACACGUUCGGAGAATT-3') were designed and purchased from Shanghai GenePharma Co., Ltd. One day before transfection, cells were plated on 6-well plates (2×10^5 cells/well) or 96-wells (5×10^3 cells/well), and then the cells were transiently transfected with either Notch2 or control siRNA (100 nM) using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western blot analysis. Protein lysates from the cells in culture were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were detected with primary antibodies recognizing Notch2 (1:1000, ab8926, Abcam) and β-tubulin (1: 2000, #2128, Cell Signaling Technology).

Cell proliferation analysis. Cell viability was measured using the Cell Counting Kit-8 (CCK-8, Dojindo). Briefly, transfected cells (5×10^3) on a 96-well plate with 5 replicate wells were allowed to incubate for different time interval from day 1 to 4. After washed in PBS, 100 μl medium containing 10% CCK-8 solution was added to each well and incubated for 2 h at 37°C. Samples are read directly in the wells using an absorbance of the 450 nm wavelength by an enzyme linked immunosorbent assay (ELISA) plate reader. The blank control absorbance (100 μl medium containing 10% CCK-8 alone) was subtracted from the experimental absorbance to adjust the background.

Cell cycle analysis. For cell cycle analysis, cells were collected 24 h after transfection, washed with PBS and fixed in 1 ml ice-cold 70% ethanol at 4°C overnight. Fixed cells were pelleted by centrifugation at 3000 rpm for 5 min. Then the cells were washed by PBS twice and stained with 1 ml staining solution containing 50 μg/ml propidium iodide (PI) and 0.5 μg/ml RNase A for 30 min and subjected to flow cytometric analysis.

Colony formation analysis. Standard colony formation assays were used to measure cell proliferation. At 24 h after transfection, transfected cells were seeded in 6-well tissue culture plates (50 cells per well). After an incubation period of 10 days, the medium was decanted and each well was washed twice with PBS. The cells were fixed in 100% methanol for 20 min and then stained with 1% crystal violet for 15 min, followed by detaining. Colonies (>20 cells/colony) were counted.

Mammosphere formation analysis. Cells were seeded on 6-well ultra low attachment plates (Corning, Tewksbury, MA, USA) at a density of 3,000 cells/500 μl in MammoCult Basal Medium and MammoCult Proliferation Supplement with 4 μg/ml heparin and 0.48 μg/ml hydrocortisone (Stem Cell Technologies, Vancouver, BC, Canada) and were incubated for 7 days at 37°C under 5% CO₂. Mammospheres were filtered by a 70-mm cell strainer and mammospheres >70 mm in diameter were collected and counted.

In vitro cytotoxicity assay. Twenty-four hours after transfection, transfected cells on a 96-well plate with 5 replicate wells were allowed to incubate for 48 h with the treatment of anticancer drug 5-fluorouracil (5-FU) with various concentrations (25, 50, 100, 200 and 400 μg/ml, respectively). After 48 h of incubation, cell viability was assessed utilizing the CCK-8 assay. The rate of cell growth inhibition (IR) was calculated according to the following equation: $IR = [1 - A_{450}(\text{drug}) / A_{450}(\text{control})] \times 100\%$, where $A_{450}(\text{drug})$ is the absorbance of the cells exposed to 5-FU and $A_{450}(\text{control})$ is the absorbance of the cells without 5-FU treatment.

In vivo xenograft study. All animal experimentation described in this study was performed in accordance with protocols

Figure 2. The effect of Notch2 on the proliferation and cell cycle progression of HCC cells *in vitro*. Depletion of Notch2 expression by Notch2-siRNA significantly inhibits the growth of HepG2 and SMMC-7721 cells (A). Downregulation of Notch2 induces G0/G1 cell cycle arrest of HCC cells. Left panel: PI staining demonstrated the induction of G0/G1 cell population but decrease in S and G2/M population. Right panel: representative histograms are displayed (B). **P<0.01.

approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University. Cells (3×10^6) were suspended in 100 μ l PBS and were injected subcutaneously into 5-week-old female nude mice 24 h after transfection. Tumor volumes were monitored every 3 days by caliper measurement of the length and width and calculated using the formula of $(\text{width}^2) \times \text{length} / 2$. Mice were sacrificed 21 days after the injection and the tumors were isolated and measured.

Immunofluorescence staining. Briefly, cells grown on coverslips were fixed in 4% fresh paraformaldehyde for 20 min and then permeabilized with 0.3% Triton X-100 in PBS for

10 min at room temperature. After the cells were blocked for 15 min with 10% normal goat serum, the coverslips were incubated overnight at 4°C with two primary antibodies, Notch2 (1:200, ab8926, Abcam) and CD90 (1:200, ab92574, Abcam). After being extensively washed with PBS, the cells were incubated with species-specific secondary antibodies (1:400, 711-545-152 or 715-095-150, Jackson Immuno Research) for 1 h at room temperature. Finally, the coverslips were washed, counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 0.1 mg/ml, Sigma) for 5 min and mounted on glass slides. Immunofluorescence images were photographed under a fluorescence microscope (Olympus).

A **B**

Figure 3. Silencing Notch2 represses self-renewal of HCC cells *in vitro*. Knockdown of Notch2 suppresses colony formation of HCC cells. Left panel: colony formation assays showed reduction of colony formation ability of HepG2 and SMMC-7721 cells after knockdown of Notch2 (A). Right panel: representative histograms are displayed. Silencing Notch2 suppresses mammosphere formation of HCC cells (B). Left panel: mammosphere formation assays showed reduction of mammosphere formation ability of HepG2 and SMMC-7721 cells after downregulation of Notch2. Right panel: representative histograms are displayed. **P<0.01.

Figure 4. Silencing Notch2 increases sensitivity to 5-FU of HCC cells *in vitro*. The effect of Notch2 knockdown on the chemo-sensitivity of HCC cells to 5-FU treatment. Cell viability of transfected cells treated with various concentration of 5-FU was assessed by CCK-8 (A and C). The inhibition rate of 5-FU in the Notch2-siRNA transfected HCC cells significantly increased compared to the control (B and D). *P<0.05, **P<0.01.

Statistical analysis. Each experiment was repeated 3-4 times. Statistical analysis was conducted with the 20.0 SPSS software package. The functional assays were compared using the paired student t-test. P-value <0.05 was considered statistically significant.

Results

The expression of Notch2 in four HCC cell lines. As showed in Fig. 1, western blot analysis revealed that Notch2 was expressed in four HCC cell lines and the expression of Notch2 was higher in HepG2 and SMMC-7721 cells compared to BEL-7402 and PLC cell lines (Fig. 1A). These two HCC cell lines were used in subsequent functional assays.

Transient knockdown of Notch2 by siRNA. To investigate the possible role of Notch2 on HepG2 and SMMC-7721 cells, we employed RNAi to deplete its expression in these cells, both of which were treated with NC-siRNA or Notch2-siRNA. After 24 and 48 h, the cells were examined by western blot analysis. As shown in Fig. 1B, the expression of Notch2 intracellular domain (N2ICD) was knocked down as determined by western blot analysis. This data indicated that Notch2-specific siRNA could effectively and obviously suppress the expression of Notch2 in HCC cells.

Transfection of Notch2-siRNA represses proliferation and cell cycle progression of HCC cells *in vitro*. CCK-8 cell prolifer-

ation assay revealed that the decrease in Notch2 expression caused by Notch2-siRNA significantly inhibited the proliferation of HCC cells (Fig. 2A). Additionally, cell cycle analysis by PI staining revealed significant increase in G0/G1 cell population but decrease in both S and G2/M population. The reduction of S+G2/G1 ratio after knockdown of Notch2 suggested that depletion of Notch2 would induce G0/G1 cell cycle arrest in HCC (Fig. 2B).

Transfection of Notch2-siRNA represses self-renewal and increases sensitivity to 5-FU of HCC cells *in vitro*. We next investigated the potential role of Notch2 on regulation of stem-like cell characteristics in HCC. Self-renewal and drug-resistance are two hallmarks of stemness behavior, so colony formation assay and chemoresistance were undertaken to assay the effect of Notch2 in HCC cells. Cell colony formation assay revealed that suppression of Notch2 could impede the self-renewal ability of HCC cells, which was reflected by fewer colonies and mammospheres in Notch2-depleted HCC cells (Fig. 3A and B). Moreover, knockdown of Notch2 sensitized HCC cells to anticancer drug 5-FU (Fig. 4A and C). Effect of Notch2-siRNA or 100 µg/ml 5-FU alone showed an inhibition rate on cell viability of HCC cells by 29.3 and 63.3%, respectively, however the synthetical outcome showed a clear distinct effect of 79.9% reduction on cell viability (Fig. 4B and D). Collectively, these data indicate that Notch2 maintains stem-like cell properties of HCC cells *in vitro*.

Figure 5. Silencing Notch2 suppresses tumorigenicity *in vivo*. Depletion of Notch2 suppresses HepG2 (A) and SMMC-7721 (B) tumor growth *in vivo*. Left panel: Notch2-siRNA could suppress tumor size on each left flank of the nude mice, whereas a larger volume tumor was observed on the right flanks injected with negative control-HCC cells. Right panel: the significant decrease in volume of xenograft by Notch2-siRNA. ***P*<0.01.

Transfection of Notch2-siRNA suppresses tumorigenicity in vivo. Since our *in vitro* studies suggested that Notch2 plays a regulatory role in proliferation and invasion of HCC cells, the biological significance of these results was further evaluated in an *in vivo* model of HCC. Both Notch2-siRNA tumor cells and control cells were implanted subcutaneously into nude mice and the resulting tumor formation was measured. Following downregulation of Notch2 expression, HCC cells showed significantly diminish *in vivo* tumor growth compared to control cells (Fig. 5A and B). These data corroborate our *in vitro* observations and support the notion that Notch2-siRNA significantly inhibits tumor growth of HCC.

Notch2 was highly expressed in CD90 positive HCC cells. To further determine the expression of Notch2 in HCC cells, expression of Notch2 and hepatic stem cell marker CD90 were examined by immunofluorescence staining. Of note,

Notch2 was markedly upregulated in CD90-positive HCC cells (Fig. 6). Based on this finding, we speculated that the extensive anticancer effects in HCC by silencing Notch2 might be through targeting cancer stem cells.

Discussion

Recent studies on Notch signaling pathway showed that it plays a crucial role in cell fate decision, tissue patterning and morphogenesis (20-23). The Notch pathway is one of several key pathways linked to both stem cell biology and cancer (8). Targeting Notch2 inhibits tumor growth and decreases tumor-initiating cells (24). In particular, dysregulated Notch2 activity has been associated with several human tumor types, including ovarian, breast, pancreas and colon cancers (11-14).

In the liver, Giovannini *et al* previously demonstrated that Notch1, Notch3 and Notch4 were over expressed in human

Figure 6. Notch2 is highly expressed in CD90⁺ HCC cells. Immunofluorescence double staining showed that Notch2 (red) was markedly upregulated in CD90⁺ HepG2 and SMMC-7721 cells (green) *in vitro* (white arrow).

HCC and Notch3 contributed to the doxorubicin resistance in HCC lines (25). In a recent study, Litten *et al* suggested that Notch2 expression and activation, independent of Jagged1 expression, might contribute to the pathogenesis of hepatoblastoma (26). Furthermore, Dill *et al* reported that constitutive Notch2 signaling accelerated DEN-induced HCC formation in the DEN HCC carcinogenesis model (19). These studies disclosed a key oncogenic role of Notch2 in HCC development. However, a previous report showed that Notch2 was downregulated in human HCC compared with adjacent non-tumor liver tissue (18). Of great interest, no existing data of functional assays elucidate the effect of Notch2 in human HCC cells.

In the present study, the expression of Notch2 was found in four human HCC cells. Functional studies disclosed that knockdown of Notch2 results in decreased cell proliferation, cell cycle progression, colony and mammosphere formation in HepG2 and SMMC-7721 cells, suggesting that Notch2 is essential for proliferation and self-renewal of HCC. Moreover, depletion of Notch2 leads to sensitized HCC cells to chemotherapeutic agent 5-FU, indicating that Notch2 plays an essential role in the drug-resistance of HCC cells. In addition, Notch2-knockdown inhibited the growth of HepG2 tumors in a xenograft model, which would in turn suggest that Notch2 plays an essential role in the progression of HCC.

One significant breakthrough in cancer research is the discovery of cancer stem cells (CSCs) (27), and it has received much attention. CSCs, characterized as a subset of tumor cells with stem cell properties, have been identified in mounting types of cancers helping to explain the cellular heterogeneity of tumor tissue, drug-resistance and tumor recurrence (28,29). In HCC, it is generally accepted that genes controlling self-renewal may sustain stem-like properties and chemoresistance ability. In line with this point of view, our present study deduced that Notch2 fortified stemness features can be seen in some types of HCC as depletion of Notch2 in HpeG2 and SMMC-7721 cell lines lead to an extensive reduction in growth and self-renewal ability both *in vitro* and *in vivo*.

Moreover, high expression of Notch2 was found in CD90-positive HCC cells. CD90 is the hepatic cancer stem cell maker in HCC (30). The results demonstrated that extensive anticancer effects by downregulation of Notch2 in HCC cells might be through targeting cancer stem cells.

In summary, Notch2 may confer stemness properties in HCC. Downregulation of Notch2 gene by RNAi can inhibit the proliferation and carcinogenesis of HCC cells and increase their sensitivity to 5-FU, which could provide a novel strategy for treatment of this fatal disease.

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