ORIGINAL ARTICLE

The Down-Regulation of Notch1 Inhibits the Invasion and Migration of Hepatocellular Carcinoma Cells by Inactivating the Cyclooxygenase-2/Snail/E-cadherin Pathway In Vitro

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Abstract

Background The Notch signaling pathway plays an important role in cancer, but the mechanism by which Notch1 participates in invasion and migration of hepatocellular carcinoma (HCC) cells is unclear.

Aims Our purpose is to confirm the anti-invasion and anti-migration effects of the down-regulation of Notch1 in HCC cells.

Methods The invasion and migration capacities of HCC cells were detected with Transwell cell culture chambers. The expressions of Notch1, Notch1 intracellular domain (N1ICD), E-cadherin, Snail, and cyclooxygenase-2 (COX-2) were analyzed by RT-PCR and/or western blotting. Notch1 and Snail were down-regulated by RNA interference, and COX-2 was inhibited by NS-398. Cell apoptosis was analyzed by MTT and flow cytometry.

Results In HCC cells, Snail, Notch1, and COX-2 were up-regulated, and E-cadherin was down-regulated in mRNA and/or protein levels. The down-regulation of Snail or Notch1 or the inhibition of COX-2, respectively, can increase the mRNA and protein expressions of E-cadherin and decrease the invasion and migration capabilities of HCC cell. Down-regulated Notch1 or inhibited COX-2 can reduce the mRNA and protein expressions of Snail. The down-regulation of Notch1 can also reduce the protein expression of COX-2. However, exogenous PGE2 can reverse the role of down-regulated Notch1. The results of MTT and flow cytometry showed that down-regulated Notch1 did not affect HCC cell viability.

Conclusions Down-regulated Notch1 may be an effective approach to inactivating Snail/E-cadherin by regulating COX-2, which results in inhibiting the invasion and migration of HCC cells. The inhibitory effects of down-regulated Notch1 on cell invasion and migration were independent of apoptosis.

Keywords Notch1 signaling pathway · E-cadherin · Snail · Cyclooxygenase-2 · Invasion · Migration

Introduction

HCC is one of the most common malignancies worldwide [1]. Despite the development of various therapies, the prognosis for HCC patients is still poor. The major reason for this poor prognosis is that HCC often causes intrahepatic and distant metastases after resection or transplantation [2]. Thus, the discovery and development of new agents to block metastasis are the primary research objectives for addressing HCC.

A number of different steps in the complex metastatic process are associated with alterations in the adhesive properties of the tumor cells. The disruption of cell–cell adhesion contributes to the metastasis of tumor cells [3, 4]. This disruption can be achieved by decreasing cadherin or catenin family members or by activating certain signaling pathways [3]. A considerable number of previous studies have shown that the reduction of E-cadherin is relevant to tumor invasion, metastasis, and unfavorable prognosis [5–7]. Indeed, E-cadherin expression seems to be beneficial for intraepithelial expansion and invasiveness in a variety of solid tumors, as well as for the intrahepatic metastasis of

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HCC [8–11]. Snail, a zinc-finger transcription factor, has been shown to contribute to the repression of the transcription of the E-cadherin gene by binding to the E-boxes of the CDH1 promoter [12]. The up-regulation of Snail is also correlated with metastasis and poor prognosis, whereas the silencing of Snail is critical for reducing tumor growth and invasiveness [13, 14].

As an important signaling pathway, Notch is not only involved in cell development and fate determination but also plays an important role in cancer [15, 16]. The Notch signaling pathway includes Notch ligands, negative and positive modifiers, and Notch target transcription factors. Notch1, one of the Notch signaling pathway receptors, mRNA, and protein are significantly higher in HCC than in adjacent non-tumor liver tissue [17]. In the MHCC97L cell line, which is one of the HCC cell lines, abnormal Notch1 expression has been shown to be strongly associated with HCC metastatic disease, which may be mediated through the Notch1/Snail1/E-cadherin pathway [18]. A previous study has shown that Notch directly up-regulates Snail expression via the recruitment of the Notch intracellular domain to the Snail promoter and elevates the hypoxia-induced up-regulation of lysyloxidase, which stabilizes the Snail-1 protein in some tumor cells [19]. However, Lim et al. [20] have demonstrated that the Notch1 intracellular domain (N1ICD) can oppose Snail-dependent HCC cell invasion by binding and inducing proteolytic the degradation of Snail. Thus, the mechanism by which Notch1 participates in the invasion and migration of HCC cells through the regulation of the Snail/E-cadherin is complex and depends on the tissue and cell type.

We sought to address whether Notch1 is involved in the control of the invasion and migration of human HCC cells. We also delineated the Notch1/COX-2/Snail/E-cadherin pathway as an additional potential mechanism involved in the invasion and migration of HCC cells.

Materials and Methods

Cell Culture and Reagents

The human liver non-tumor cell line (HL-7702, obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences) and HCC cell lines (HepG2, HuH-7, and SMMC-7721, obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, and MHCC97H, obtained from the Liver Cancer Institute of Fudan University) were cultivated in DMEM medium supplemented with 10 % fetal calf serum (Sigma Chemical, St. Louis, MO, USA). The liver non-tumor cells and HCC cells were seeded into 6-well cell culture plates at a density of 1×10^5 cells/well. All experiments were carried out using a confluent monolayer of HCC cell cultures. To

attain a normoxic condition, the cultures were maintained at 37 °C in a humidified incubator containing 20 % O_2, 5 % CO_2, and 75 % N_2. The primary antibodies for Notch1 (120 kDa), E-cadherin (120 kDa), Snail (29 kDa), COX-2 (72 kDa), and GAPDH (37 kDa) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody for the N1ICD (110 kDa) was purchased from Abcam (Cambridge, UK). All secondary antibodies were obtained from Pierce (Rockford, IL, USA). Notch1 small interfering RNA (siRNA), Snail siRNA, and the siRNA control were obtained from Santa Cruz Biotechnology. LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA, USA). To inhibit endogenous COX-2 activity, NS-398 (Sigma-Aldrich) in DMSO was used at 50 μmol/l [21]. Prostaglandin E2 (PGE2; Sigma-Aldrich) in ethanol was used at 10 μg/ml. Dexamethasone (Sigma-Aldrich) was used at 100 nmol/l. All other chemicals and solutions were purchased from Sigma-Aldrich unless otherwise indicated.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cells using the Trizol reagent, according to the manufacturer's instructions (Invitrogen). The reverse transcription of total cellular RNA was performed using the one-step RT-PCR kit (MBI Fermentas, Lithuania) in accordance with the manufacturer's instructions. The polymerase chain reaction (PCR) primers used were as follows: 5′-CCGTCATCTCCGACTT CATCT-3′ (forward) and 5′-GTGTCTCCTCCCTGTTGTT CTG-3′ (reverse) for Notch1 (468 bp), 5′-TCCCATCAGC TGCCCAGAAA-3′ (forward) and 5′-ATTGTCCTTGTGT CCTCAGT-3′ (reverse) for E-cadherin (502 bp), 5′-TTC T TCTGCGCTACTGCTGCG-3′ (forward) and 5′-AGAAGG AGAGGTATGGACGGG-3′ for Snail (883 bp), and 5′-AC CACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCAC CACCCTGTTGCTGTA-3′ (reverse) for GAPDH (452 bp). The conditions of PCR were as follows: after initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at each appropriate temperature as described for 30 s and extension at 72 °C for 45 s. PCR products were separated by electrophoresis on 1 % agarose gel and visualized with ethidium bromide staining. Gene expression was presented as the relative yield of the PCR product from target sequences compared to that of the GAPDH gene. The mean values from three independent experiments were taken as the results.

Small Interfering RNA Transfection

According to the protocols for LipofectAMINE 2000, the HepG2 and MHCC97H cells were transfected with Notch1

siRNA, Snail siRNA, and the siRNA control. The cells transfected with siRNA were seeded into 6-well cell culture plates at a density of 1×10^5 cells/well. The cells were allowed to grow for 24 h and were then harvested for further analysis.

Protein Extraction and Western Blotting

The cells were lysed in lysis buffer (50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5 % NP40, 0.5 % Triton X-100, 2.5 mmol/l sodium orthovanadate, 10 μl/ml protease inhibitor cocktail, and 1 mmol/l PMSF) by incubating for 20 min at 4 °C. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA, USA). Total proteins were fractionated using SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5 % nonfat dried milk or bovine serum albumin in 1× TBS buffer containing 0.1 % Tween 20 and subsequently incubated with the appropriate primary antibodies. Horseradish-peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody, and the protein bands were detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The quantification of western blots was performed using laser densitometry, and the relative protein expression was then normalized to the GAPDH levels in each sample. The results are presented as the means of three independent experiments with error bars representing SDs. For probing different proteins in the same membranes, membranes were incubated for 30 min at 50 °C in a buffer containing 2 % SDS, 62.5 mmol/l Tris (pH 6.7), and 100 mmol/l 2-mercaptoethanol to remove the first primary antibody, washed, and incubated with another desired primary antibody.

MTT Assay

The differently treated cells were seeded into 96-well cell culture plates at a density of 1×10^4 cells/well and were grown for up to 48 h. Cell viability was assessed with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma Chemicals) in accordance with the manufacturer's protocols. Each experiment included six replications and was repeated three times. The data were summarized as means ± SDs.

Flow Cytometry for the Analysis of Cell Apoptosis

To determine the number of apoptotic cells, Annexin V assays were performed using an apoptosis detection kit (Annexin V-FITC/propidium iodide (PI) Staining Kit; Immunotech, Marseille, France). Briefly, 1.5×10^5 differently treated cells were plated in 24-well cell culture plates. After 48 h, the cells were harvested, washed in cold PBS, incubated for 15 min with fluorescein-conjugated Annexin V and PI, and analyzed using flow cytometry. PI-negative and Annexin V-positive cells were considered to be early apoptotic, whereas cells that were both PI- and Annexin V-negative were considered non-apoptotic.

Invasion and Migration Assays

The cell migration was analyzed with non-Matrigel-coated Transwell cell culture chambers (8-μm pore size) (Millipore, Billerica, MA, USA). The cell invasion was analyzed with Matrigel-coated Transwell cell culture chambers (8-μm pore size) (Millipore). Briefly, differently treated cells (5×10^4 cells/well) were serum starved for 24 h and plated in the upper insert of a 24-well chamber in a serum-free medium. A medium containing 10 % serum as a chemoattractant was added to the well. The cells were incubated for 24 h. Cells on the upper side of the filters were mechanically removed by scrubbing with a cotton swab, after which the membrane was fixed with 4 % formaldehyde for 10 min at room temperature and stained with 0.5 % crystal violet for 10 min. Finally, invasive or migrated cells were counted at ×200 magnification from 10 different fields of each filter. For treatment with NS-398 or PGE2, the cells were pretreated for 2–4 h, and the treatment continued during the invasion or migration experiment.

Statistical Analysis

Each experiment was repeated at least three times. All data were summarized and presented as means ± SDs. The differences among means were statistically analyzed using a t test. All statistical analyses were performed using SPSS 13.0 software (Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

Results

Snail/E-cadherin participated in the invasion and migration of HCC cells.

As illustrated in Fig. 1a, the HCC cell showed higher levels of penetration through Transwell cell culture chambers versus the liver non-tumor cells. These results demonstrated that the HCC cells had higher invasion and migration capabilities than the liver non-tumor cells. In HCC cells, the HepG2 cell line had the lowest invasion capability, and the MHCC97H cell line had the highest invasion capability. To confirm the universality of the experimental results, we selected the HepG2 and MHCC97H cell lines, two different
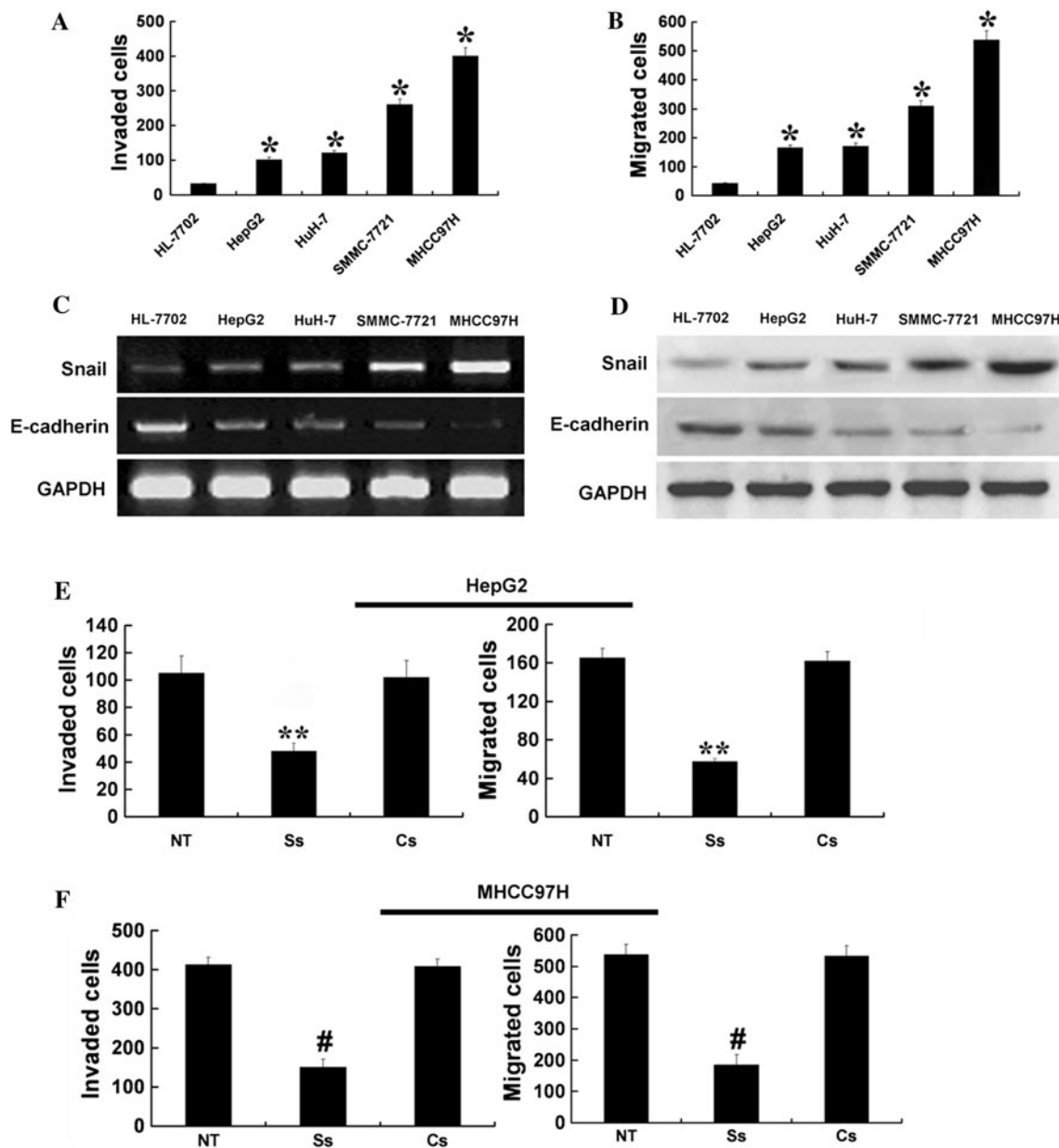
Fig. 1 Snail/E-cadherin participated in HCC cell invasion and migration of HCC cells. **a**, **b** The invasion and migration capacities of different cell lines were measured by Transwell cell culture chambers. **c**, **d** In different cell lines, the mRNA and protein expressions of Snail and E-cadherin were measured by RT-PCR and western blotting. **e**, **f** Analyses of the invasion and migration capacities in different treatments of HepG2 and MHCC97H cells.

The mRNA and protein expressions of Snail and E-cadherin were normalized to GAPDH (Snail or E-cadherin/GAPDH). Non-transfected and control siRNA-transfected cells were used as controls. *NT* non-transfection, *Ss* Snail siRNA-transfection, *Cs* control siRNA-transfection. The data represent means ± SDs; *$P < 0.05$ compared with HL-7702 cells, **$P < 0.05$ compared with Cs in HepG2 cells, #$P < 0.05$ compared with Cs in MHCC97H cells

HCC cell lines with different invasion capabilities, for the subsequent experiments.

Next, we examined the mRNA and protein expressions of Snail and E-cadherin in liver non-tumor cells and HCC cells. With RT-PCR and western blotting, E-cadherin was down-regulated and Snail was up-regulated in mRNA and protein levels compared with liver non-tumor cells (Fig. 1c, d). There was an inverse correlation between Snail and E-cadherin. These results also showed that up-regulated Snail had a positive correlation with invasion and migration capability, but E-cadherin demonstrated the opposite effect. The HepG2 and MHCC97H cells were transfected with human Snail siRNA or control siRNA. As shown in Fig. 1e, f, Snail siRNA-transfected cells exhibited a low level of penetration through the membrane compared with control cells. These results indicated that
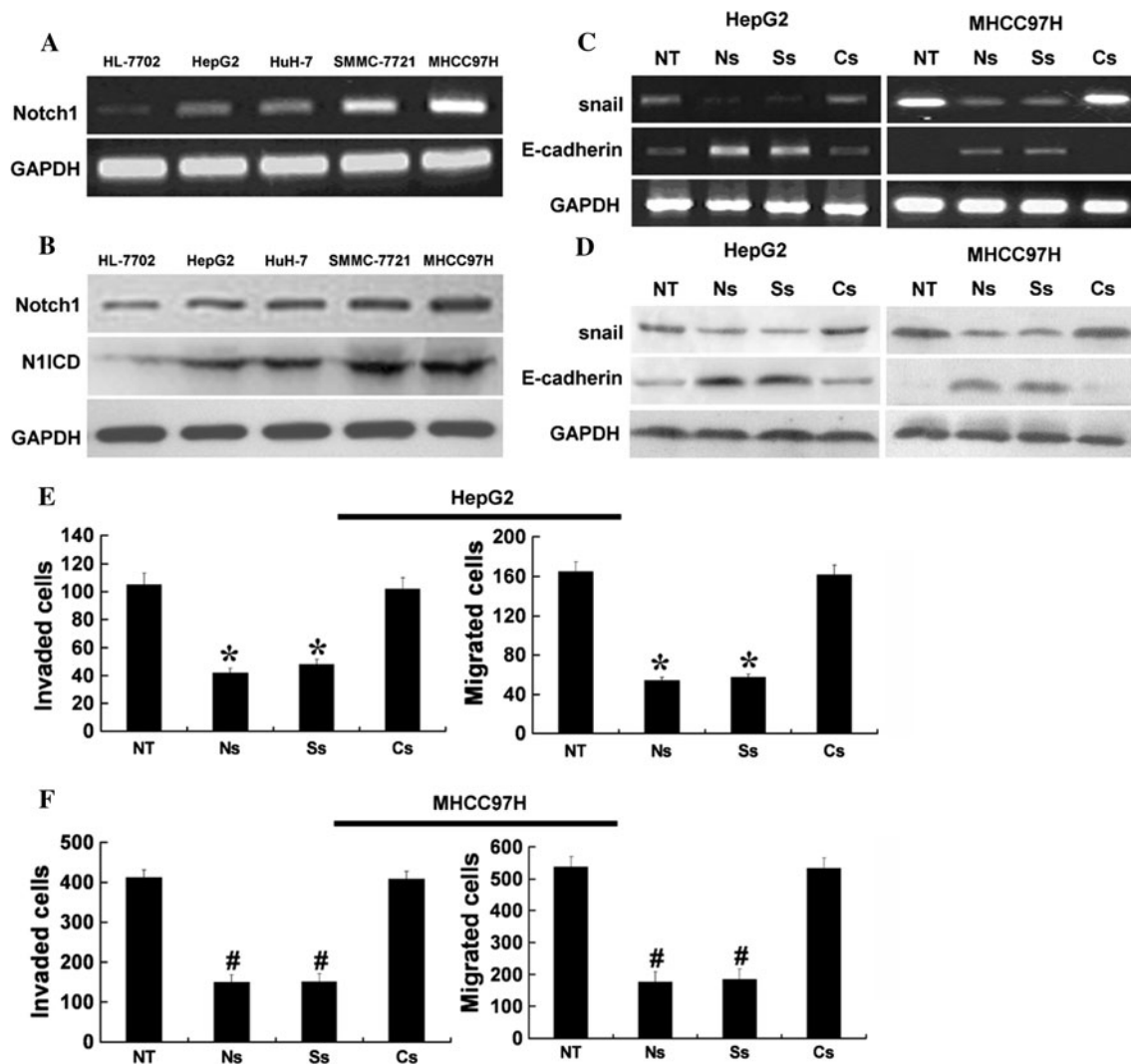
Fig. 2 The down-regulation of Notch1 can reduce HCC cell invasion and migration by regulating Snail/E-cadherin. **a**, **b** In different cell lines, RT-PCR was used to measure the mRNA expressions of Notch1, and western blotting was used to measure the protein expressions of Notch1 and N1ICD. **c**, **d** The mRNA and protein expressions of Snail and E-cadherin in different treatments of HepG2 and MHCC97H cells were measured by RT-PCR and western blotting. **e**, **f** Analyses of the invasion and migration capacities in different treatments of HepG2 and MHCC97H cells. Non-transfected and control siRNA-transfected cells were used as controls. *NT* non-transfection, *Ns* Notch-1 siRNA-transfection, *Ss* Snail siRNA-transfection, *Cs* control siRNA-transfection. The data represent means ± SDs; *$P < 0.05$ compared with Cs in HepG2 cells, #$P < 0.05$ compared with Cs in MHCC97H cells

Snail/E-cadherin participates in the invasion and migration of HCC cells.

The down-regulation of Notch1 can reduce the invasion and migration of HCC cells by regulating Snail/E-cadherin.

To address whether Notch1 participates in the invasion and migration of HCC, we first examined the mRNA and protein expressions of Notch1 and the protein expression of N1ICD in liver non-tumor cells and HCC cells. RT-PCR analysis showed that the HCC cells exhibited higher expressions of Notch1 in mRNA levels compared with the liver non-tumor cells (Fig. 2a). The protein expressions of Notch1 and N1ICD also exhibited similar tendencies toward increased levels (Fig. 2b). The mRNA and protein

expressions of Notch1 were also the lowest in HepG2 cells and the highest in MHCC97H cells. These results indicated that up-regulated Notch1 may have a positive correlation with invasion and migration capability.

To further examine whether the Notch1 was involved in the invasion and migration of HCC cells by regulating Snail/E-cadherin, the HepG2 and MHCC97H cells were transfected with human Notch1 siRNA, Snail siRNA, and control siRNA. We detected the expressions of Snail and E-cadherin in mRNA and the protein levels in different treatments of cells. As shown in Fig. 2c, d, Notch1 siRNA or Snail siRNA was able to down-regulate the expression of the Snail or up-regulate the expression of the E-cadherin

with respect to mRNA and protein levels. However, no differences were detected between the Notch1 siRNA-transfected and Snail siRNA-transfected HCC cells with regard to mRNA and protein expressions of Snail or E-cadherin. As illustrated in Fig. 2e, f, Notch1 siRNA-transfected cells or Snail siRNA-transfected cells exhibited a low level of penetration through the membrane, compared with control cells. No difference was observed between the Notch1 siRNA-transfected and Snail siRNA-transfected cells with regard to the decreased levels of invasion and migration capacity. The cells transfected with Notch1 siRNA exhibited low expressions of Notch1 or N1ICD in mRNA and protein levels, as confirmed by RT-PCR and western blotting (Fig. 3a, b). To confirm further that the inhibitory effects of down-regulated Notch1 on cell invasion and migration were independent of apoptosis, we used an MTT assay and flow cytometry to detect Notch1 siRNA-transfected cells. As the results of MTT assay and flow cytometry show, down-regulated Notch1 did not affect cell viability (Fig. 3c–e).

The down-regulation of Notch1 was able to inactivate Snail/E-cadherin by regulating COX-2, which resulted in the inhibition of HCC cell invasion and migration.

To explore the potential mechanisms by which Notch1 regulates Snail/E-cadherin, we focused on COX-2. Western blot analysis demonstrated that HCC cells exhibited a higher protein expression of COX-2 compared with the liver non-tumor cells (Fig. 4a). As the invasion and migration capacities of HCC cells increased, the protein expression of COX-2 also increased (Fig. 4a). These results illustrated that the up-regulated protein expression of COX-2 may also have a positive correlation with the invasion and migration capability of HCC cells. To address whether COX-2 was able to regulate Snail/E-cadherin, HepG2 and MHCC97H cells were treated with the COX-2 inhibitor NS-398 to block COX-2 activity. As shown in Fig. 5a, b, 50 µmol/l NS-398 does not affect the viability of either the HepG2 or MHCC97H cells. As shown in Fig. 4b, c, 50 µmol/l NS-398 was able to up-regulate the expression of E-cadherin and down-regulate the expression of Snail with regard to mRNA and protein levels. In the subsequent experiment, we investigated the protein expression of COX-2 in Notch1 siRNA-transfected cells. As shown in Fig. 4d, the down-regulation of Notch1 is able to decrease the protein expression of COX-2. As shown in Fig. 5c, d, 10 µg/ml exogenous PGE2 does not affect the viability of either HepG2 or MHCC97H cells. We treated Notch1 siRNA-transfected
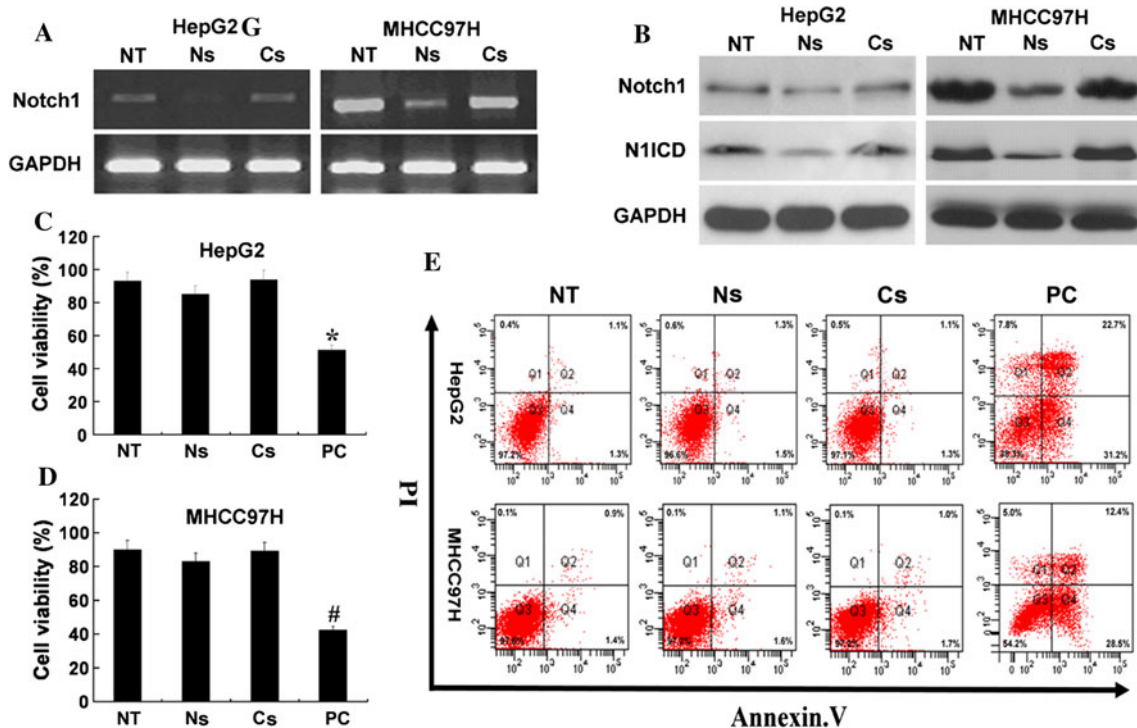

Fig. 3 siRNA can down-regulate the expression of Notch1 and had no effect on HCC cell viability. **a**, **b** In different treatments of HepG2 and MHCC97H cells, RT-PCR was used to measure the mRNA expressions of Notch1, and western blotting was used to measure the protein expressions of Notch1 and N1ICD. **c–e** In different treatments of HepG2 and MHCC97H cells, cell viability was measured by MTT and flow cytometry. *NT* non-transfection, *Ns* Notch-1 siRNA-transfection, *Cs* control siRNA-transfection, *PC* positive control (cells treated with 100 nmol/l dexamethasone as a positive control). *$P < 0.05$ compared with Cs in HepG2 cells, #$P < 0.05$ compared with Cs in MHCC97H cells
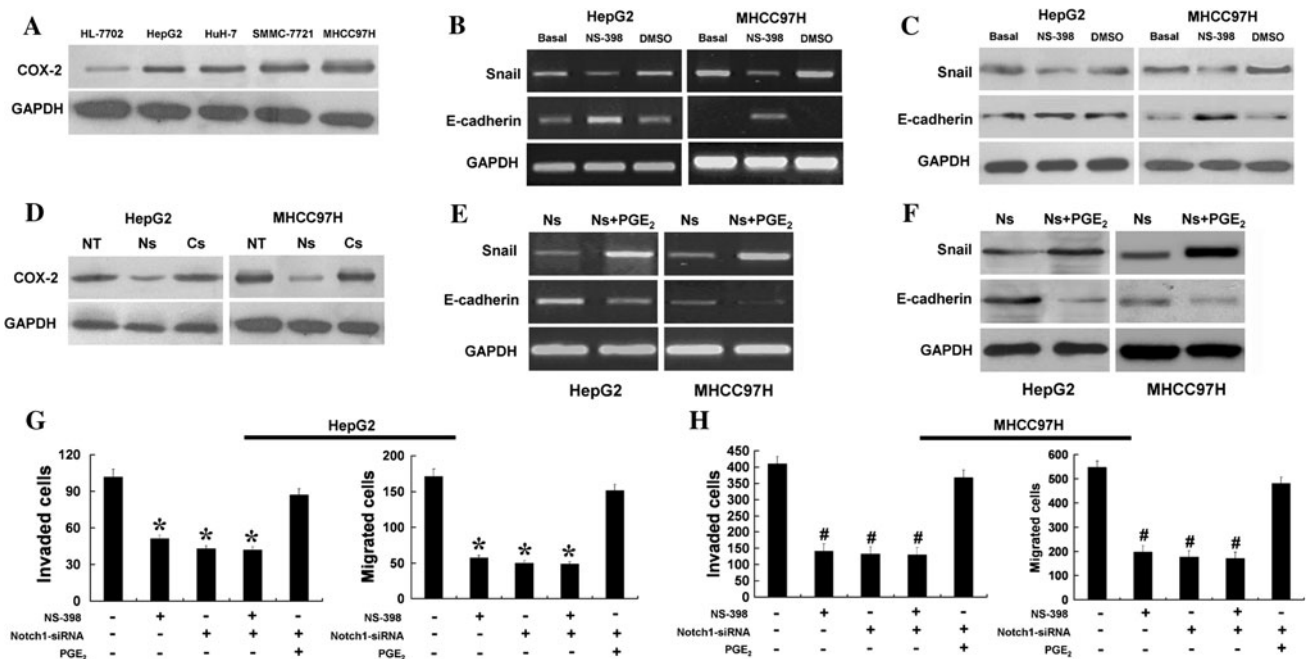
Fig. 4 The down-regulation of Notch1 can inactivate Snail/E-cadherin by regulating COX-2/PEG2, resulting in the inhibition of invasion and migration. **a** In different cell lines, the protein expressions of COX-2 were measured by western blotting. **b, c** In different treatments of HepG2 and MHCC97H cells, the mRNA and protein expressions of Snail and E-cadherin were measured by RT-PCR and western blotting. HepG2 and MHCC97H cells were treated with 50 μmol/l NS-398. Basal cells (non-treated cell) and cells treated DMSO was used as controls. **d** In different treatments of HepG2 and MHCC97H cells, the protein expressions of COX-2 were measured by western blotting. Non-transfected and control siRNA-transfected cells were used as controls. **e, f** In different treatments of HepG2 and cells with 10 μg/ml exogenous PGE2. The results showed that PGE2 can increase the expression of Snail and decrease the expression of E-cadherin with regard to mRNA and protein levels (Fig. 4e, f). To examine further the relationship between COX-2 and Notch1 in the control of HCC cell invasion and migration, we treated HCC cells with NS-398 or Notch1 siRNA to block COX-2 activity or Notch1, respectively. Treatment with NS-398 or Notch1 siRNA alone reduced the invasion and migration capacities of HepG2 and MHCC97H cells. However, treatment with NS-398 in combination with Notch1 siRNA did not block these biological functions of HCC cells to a greater extent than treatment with NS-398 or Notch1 siRNA alone. The suppressed capacity of invasion and migration by down-regulated Notch1 were reversed after treatment with exogenous PGE2 in the HCC cells (Fig. 4g, h).

MHCC97H cells, the mRNA and protein expressions of Snail and E-cadherin were measured by RT-PCR and western blotting. HepG2 and MHCC97H cells were treated with Notch1-siRNA and 10 μg/ml PEG2. Cells transfected with Notch1-siRNA were used as a control. **g, h** In different treatments of HepG2 and MHCC97H cells, invasion and migration capacities were measured with Transwell cell culture chambers. HepG2 and MHCC97H cells were treated with Notch1-siRNA and/or 50 μmol/l NS-398 and/or 10 μg/ml PEG2. *NT* non-transfection, *Ns* Notch-1 siRNA-transfection, *Cs* control siRNA-transfection. The data represent means ± SDs; *$P < 0.05$ compared with non-treated HepG2 cells, #$P < 0.05$ compared with non-treated MHCC97H cells

regulating Snail/E-cadherin through COX-2. These results supplemented the invasion and migration mechanisms and further confirm the importance and complexity of Notch1 in HCC. To the best of our knowledge, this report described the first correlation of the Notch1/COX-2/Snail/E-cadherin pathway with HCC cell invasion and migration.

Cell–cell adhesion, which is achieved by cell adhesion molecules, is critical to the establishment and maintenance of normal tissue architecture and organ development [22]. Alterations affecting cell adhesion molecules are considered to play a critical role in the invasive process. Among these molecules, E-cadherin, a member of the cadherin family, is involved in homotypic, calcium-dependent cell–cell adhesion in epithelial tissues [23]. Physiologically, E-cadherin regulates a variety of morphogenetic events, including cell migration, the separation and formation of boundaries between cell layers, and the differentiation of each cell layer into functionally distinct structures. The pathological loss or reduced expression of E-cadherin results in de-differentiation, invasiveness, and lymph node or distant metastasis in a variety of human neoplasms, including HCC [24–27]. The loss of E-cadherin expression
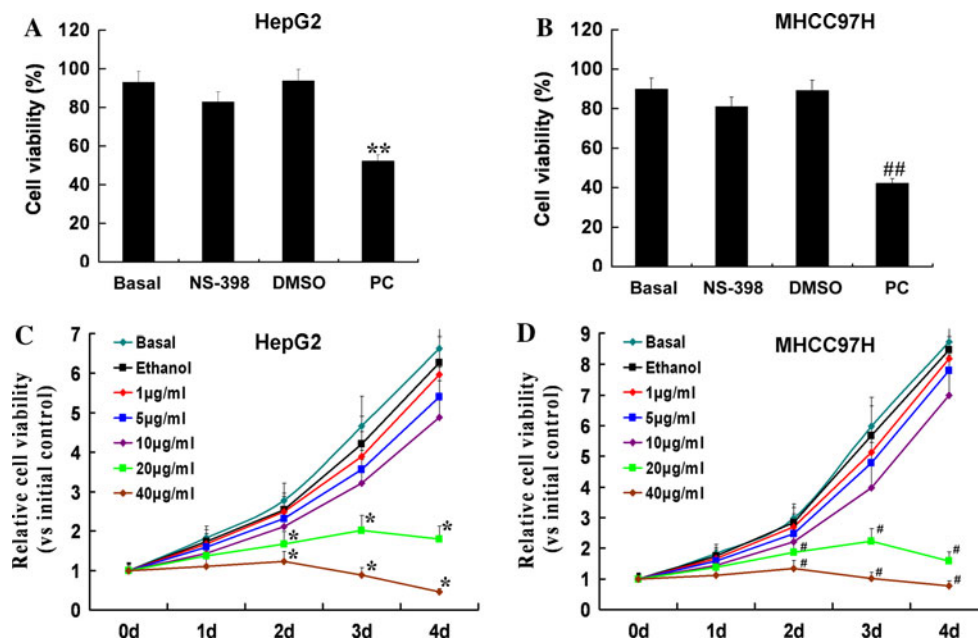
Discussion

The results of the current experiments showed that Notch1 contributes to HCC cell invasion and migration by

Fig. 5 The effects of NS-398 and PEG2 on the growth and viability of HepG2 and MHCC97H cells. **a**, **b** The HepG2 and MHCC97H cells were treated with 50 μmol/l NS-398. The viability of the HepG2 and MHCC97H cells was measured by MTT assay. Basal cells (non-treated cell) and cells treated DMSO or ethanol were used as negative controls. *PC* positive control (cells treated with 100 nmol/l dexamethasone as a positive control). **c**, **d** The HepG2 and MHCC97H cells were treated with different doses of PEG2 (1, 5, 10, 20, and 40 μg/ml) for 4 days. The cells not treated (basal cells) or treated with ethanol were used as controls. The viability of HepG2 and MHCC97H cells was measured by MTT assay. The data represent means ± SDs; $*P < 0.05$ compared to HepG2 cells treated with ethanol, $^{\#}P < 0.05$ compared to MHCC97H cells treated with ethanol, $**P < 0.05$ compared to HepG2 cells treated with DMSO, $^{\#\#}P < 0.05$ compared to MHCC97H cells treated with DMSO

and the disassembly of E-cadherin adhesion plaques on the cell surface enable tumor cells to disengage from the primary mass and to move through conduits of dissemination [28]. Snail, a zinc-finger transcription factor, has been shown to contribute to the repression of the transcription of the E-cadherin gene by binding to the E-boxes of the CDH1 promoter [12]. Extensive studies have shown the involvement of Snail in the development and metastasis of cancer [29, 30]. Thus, Snail/E-cadherin is a key component of cancer metastasis. Strong inverse correlations have been shown to exist between Snail and E-cadherin expression in a panel of epithelial and dedifferentiated cells derived from carcinomas of various etiologies, including: oral squamous carcinoma; breast, pancreas, colon, and bladder cancer; and melanomas, fibroblasts, and HCC [31–34]. Our results indicated similar results in HCC cells. The down-regulation of Snail can reduce the expression of E-cadherin and decrease the capacities of invasion and migration in HCC cells. Thus, Snail/E-cadherin is involved in HCC cell invasion and migration. Determining the regulatory mechanism of Snail/E-cadherin is important for the prevention of cancer metastasis.

As has been well established, the Notch signaling pathway is involved in the carcinogenesis, progress, invasion, and neovascular formation of many malignant tumors [35–37]. However, knowledge of the role of Notch1 in HCC cell invasion and migration is limited. Previous studies have suggested that the Notch signaling pathway increases Snail expression in endothelial cells to promote mesenchymal transformation [38, 39]. The Notch signaling pathway is required to convert the hypoxic stimulus into changes in Snail/E-cadherin, increased motility, and the invasiveness of cervical, colon, glioma, and ovarian cancer cells [19]. In contrast, Lim et al. [20] demonstrated that the Notch1 intracellular domain (N1ICD) can induce the proteolytic degradation of Snail, thereby resulting in a decrease in the invasion of Snail-dependent HCC cells. The results of the present study indicate that, in HCC, activated Notch1 can promote the invasion and migration capabilities of HCC cells. To explore the potential mechanism involved in this process, we found that down-regulated Notch1 can regulate the Snail/E-cadherin, which was involved in cancer invasion and migration. Our results were consistent with the results found by Wang et al. [18]. The means by which the Notch1 signaling pathway mediates Snail/E-cadherin in tumor cells is complex and depends on the tissue and cell type. In addition, the role of Notch1 involved in HCC cell invasion and migration is perplexing. However, the specific mechanism involved in Notch1-regulated Snail/E-cadherin should be further studied. Toward this aim, we focused on COX-2.

Tumor COX-2 and its metabolite, prostaglandin E2 (PGE2), play important roles in regulating diverse cellular functions under physiological and pathological conditions [40, 41]. COX-2 is also over-expressed in a variety of malignancies, including colon, gastric, esophageal, prostate, pancreatic, breast, and lung carcinomas [40–43]. Elevated COX-2 expression is often associated with invasion and metastasis in lung and breast cancer [44, 45]. COX-2/PEG2-dependent pathways contribute to the modulation of E-cadherin expression through PGE2 exposure, leading to enhanced Snail binding at the chromatin level [46]. This relationship may be the mechanism by which COX-2 is involved in the metastasis of tumors. Notch1 can also regulate COX-2 expression in gastric cancer through N1IC bound to a COX-2 promoter [21]. Thus, we speculated that up-regulated Notch1 can activate COX-2, resulting in up-regulating PGE2, and up-regulated PGE2 can enhance Snail, resulting in down-regulating E-cadherin, while the Notch1/COX-2/Snail/E-cadherin pathway may participate in HCC cell invasion and migration.

Our findings suggested that COX-2 may have relevance in the metastatic potential of HCC cells. In agreement with the results of previous studies [46, 47], we found that the inhibitions of COX-2 can down-regulate the expression of Snail and up-regulate the expression of E-cadherin with regard to mRNA and protein levels. The inhibition of COX-2 reduced the invasion and migration capabilities of HCC cells. These data indicated that COX-2 participates in HCC cell invasion and migration. It was interesting that in down-regulated Notch1 HCC cells, the expression of COX-2 dramatically decreased, and PEG2 was able to reverse the role of Notch1 in regulating Snail/E-cadherin. In contrast, the down-regulation Notch1 and/or COX-2 play the same role in inhibiting HCC cell invasion and migration. After treatment with PGE2, the invasion and migration capabilities of HCC cells with down-regulated Notch1 increased. These results can be explained by the fact that down-regulated Notch1 caused COX-2 to decrease. Down-regulated COX-2 can also reduce the expression of PGE2, which further decreases the expression of Snail, resulting in the up-regulation of E-cadherin. Exogenous PGE2 can also increase the expression of Snail [46], causing the effect of down-regulated Notch1 to be neutralized. These results further confirmed our previous speculation.

The results of the present study indicated the potential mechanisms by which the Notch1/COX-2/Snail/E-cadherin pathway was involved in HCC cells invasion and migration. The data presented in this study provide experimental evidence that supported the anti-invasion and anti-migration effects of down-regulated Notch1 on HCC cells. We speculate that targeting Notch1 for specific cell types may be useful in the near future for devising novel preventive and therapeutic strategies for HCC.

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