

# Associations between high levels of Notch1 expression and high invasion and poor overall survival in hepatocellular carcinoma

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**Abstract** Although Notch1 expression has been associated with progression or prognosis in various tumors, the role of Notch1 in hepatocellular carcinoma (HCC) remains unknown. This study sought to investigate the clinicopathological and prognostic relevance of Notch1 expression in HCC as well as the underlying mechanisms responsible. HCC tissues were stained with an anti-Notch1 antibody. The invasion capacities of cells were measured using Transwell cell culture chambers. Reverse transcription PCR and/or western blot were used to evaluate the expression levels of Notch1, matrix metalloproteinase (MMP)-2, and MMP-9. Notch1 expression was downregulated by RNA interference. The activity of MMP-2/MMP-9 was quantified by enzyme-linked immunosorbent assay, and cellular apoptosis was analyzed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Notch1 expression was mainly localized within the cytoplasm and at the cell membrane. High Notch1 expression correlated with tumor size, tumor grade, metastasis, venous invasion, and American Joint Committee on Cancer TNM stage ( $P<0.05$ ), and patients with high levels of Notch1 expression were at a significantly increased risk for shortened survival time ( $P<0.05$ ). In vitro, the downregulation of Notch1 expression decreased the invasion capacity of HCC cells via the regulation of MMP-2 and MMP-9. The results of the MTT assay showed that downregulation of

Notch1 did not affect HCC cell viability. Notch1 may represent a novel candidate marker for patient prognosis as well as a molecular target for HCC therapy.

**Keywords** Hepatocellular carcinoma · Notch1 · Invasion · Metastasis · Overall survival · Clinicopathological correlation

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide and is currently the second most lethal tumor in China [1]. Despite improvements in tumor detection and clinical treatment strategies, the overall outcome for patients with HCC remains very poor. The main cause of death in HCC patients is the high rate of recurrence or metastasis following treatment [2]. Tumor markers for HCC, such as the level of alpha-fetoprotein (AFP), have been reported as additional indicators of tumor progression associated with patient survival [3]. However, few markers, besides TNM stage or AFP level, have been validated as independent prognostic factors. Moreover, molecules involved in tumor metastasis may serve as markers for the early detection of recurrence or metastasis as well as prognostic indicators for surgical intervention. Therefore, it is necessary to further investigate novel indicators for the evaluation of tumor progression and the prediction of patient outcome.

As an evolutionally conserved signaling pathway, the Notch signaling pathway has been implicated in cell fate determination, tissue patterning, and morphogenesis, and cell differentiation, proliferation, and death [4, 5]. Because the Notch signaling pathway plays important roles in cellular processes such as proliferation and apoptosis, alterations in this pathway have been associated with tumorigenesis [6]. The

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Notch signaling network is frequently deregulated in human malignancies, and the upregulated expression of Notch receptors and /or their ligands has been reported in various human tumors including HCC [7–10]. In recent studies, long-term exposure to constitutive Notch signaling in the liver induces HCC in mice with high penetrance, and that Notch pathway activation occurs in roughly one third of human HCCs [11]. Notch1 has been shown to play a paradoxical role, as it can act as either a tumor suppressor or oncogene depending on the tissue type, and this signaling pathway was shown to have a tumor-suppressive effect on murine skin tumors and in human non-small cell lung cancer [12, 13]. In contrast to its tumor-suppressive role, Notch1 has also been shown to be upregulated in prostate cancer, small cell lung cancer, pancreatic cancer, and HCC and has been shown to be involved in tumor cell invasion in pancreatic cancer, lingual squamous cell carcinoma, and breast cancer, which suggests that Notch1 may act as an oncogene in many tumors [10, 14–18]. Together, these findings indicate that Notch1 has a variable role in tumor development. Many studies have demonstrated complex mechanisms for Notch1 in the invasion and metastasis of tumors including HCC, and one such mechanism is the Notch1-Snail/E-cadherin pathway. One previous study found that Notch can induce the invasion and metastasis of tumor cells via the upregulation and stabilization of Snail protein expression and the downregulation of E-cadherin protein expression [19]. In the MHCC97L cell line (a HCC cell line), abnormal Notch1 expression was shown to be strongly associated with HCC metastatic disease, which may be mediated through the Notch1-Snail1-E-cadherin pathway [20]. However, Lim et al. demonstrated that the Notch1 intracellular domain can oppose Snail-dependent HCC cell invasion by binding and inducing the proteolytic degradation of Snail [21]. Furthermore, in the presence of wild-type p53, Notch1/Snail activation was shown to increase the invasiveness of HCC cells, whereas in the absence of wild-type p53, Notch1 decreased the invasiveness of HCC cells [22]. 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. However, the Notch1-matrix metalloproteinase (MMP) pathway may also serve as an important mechanism in HCC development. Our previous studies demonstrated that in HCC, a Notch signaling pathway inhibitor could suppress the invasion of HCC cells via downregulation of MMP-2 and MMP-9 [23]. In pancreatic cancer and lingual squamous cell carcinoma, downregulated Notch1 was shown to inhibit invasion via the inactivation of MMP-2 and MMP-9 [16, 17]. It remains unknown whether Notch1 participates in the invasion and metastasis of HCC cells via the regulation of MMP-2 and MMP-9. From the above-mentioned results, it is clear that Notch1 plays an important and complex role in tumor cell invasion and metastasis, which directly affect patient

prognosis. In some tumors, such as breast cancer and colorectal cancer, high Notch1 expression has been related to poor overall survival rate [24, 25]. However, the relationship between Notch1 expression and survival in HCC patients has not been explored.

The present study used immunohistochemistry to investigate Notch1 protein expression and was the first to examine the potential relationship between Notch1 protein expression and patient prognosis in HCC. Furthermore, this study explored the role of Notch1 in the invasion and metastasis of HCC via its effect on the regulation of MMP-2 and MMP-9 *in vitro*.

## Materials and methods

### Patients and tissue specimens

Tissue specimens from HCC and adjacent non-cancerous hepatic tissues (at least 1.5 cm away from the tumor) were collected from 120 patients who underwent surgical treatment for primary HCC at the Department of Hepatobiliary Surgery at Xijing Hospital (Xi'an, China) between 2004 and 2007. Specimens were obtained from patients who had not received preoperative treatments such as chemotherapy, ethanol injection, or transarterial chemoembolization. The study included 74 male and 46 female patients with a median age of 48.5 years (range, 29–80 years). The median size of the tumors was 6.7 cm (range, 2.0–16.2 cm). This study was approved by the Ethics Committee of the Fourth Military Medical University and conformed to the ethical guidelines of the 2004 Declaration of Helsinki. Written informed consent was obtained from each patient or from his/her legal guardians. Before the study was initiated, histopathological examinations were performed to confirm that there were enough cancer cells in the tumor samples, and that no cancer cells had contaminated the non-cancerous hepatic tissues. All specimens were fixed in 10 % formalin and embedded in paraffin, and 4- $\mu$ m serial sections were examined by immunohistochemistry. Clinical parameters such as gender, age, tumor location, tumor size, tumor grade, metastasis, satellite lesions, tumor number, American Joint Committee on Cancer (AJCC) TNM stage, and AFP were collected. For the 38 cases diagnosed with metastasis, these included venous invasion ( $n=26$ ), bile duct tumor thrombi ( $n=11$ ), and lymph node metastasis ( $n=6$ ) which were verified by pathological analysis. The enrolled patients were followed for 5 years to perform survival calculations.

### Cell culture and reagents

The human HCC cell lines (HepG2, SMMC-7721, and MHCC97H) were cultivated in DMEM medium

supplemented with 10 % fetal calf serum (Sigma Chemical Co., St. Louis, MO). The HCC cells were seeded into six-well cell culture plates at a density of  $1 \times 10^5$  cells/well. Primary antibodies against Notch1, MMP-2, MMP-9, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All secondary antibodies were obtained from Pierce (Rockford, IL, USA). An SP immunostaining kit was purchased from ZYMED (ZSGB; Beijing, China). Notch1 small interfering RNA (siRNA) and siRNA controls were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals and solutions were purchased from Sigma-Aldrich unless otherwise indicated.

#### Immunohistochemistry and evaluation of staining

Immunohistochemistry was performed using the avidin-biotin-peroxidase method for all tissues. All sections were deparaffinized in xylene and dehydrated through a graded alcohol series prior to the blockade of endogenous peroxidase activity using 0.5 %  $H_2O_2$  in methanol for 10 min. Nonspecific binding was blocked by incubating the sections with 10 % normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Without washing, the sections were incubated with an anti-Notch1 antibody (1:50) in PBS at 4 °C overnight in a humidified chamber. Biotinylated IgG (1:200, Sigma) was then added, and the sections were incubated for 2 h at room temperature. Detection was performed using a streptavidin-peroxidase

complex. The brown color indicative of peroxidase activity was obtained by incubating with 0.1 % 3,3-diaminobenzidine (Sigma) in PBS with 0.03 %  $H_2O_2$  for 10 min at room temperature. The tissue specimens were scored independently by two pathologists, who were blinded to the clinicopathological results and patient outcome, using a previously described immunoreactivity scoring system [25]. Based on the score, we divided all HCC specimens into two subgroups: the low expression group (score of 0–4) and high expression group (score of 5–12).

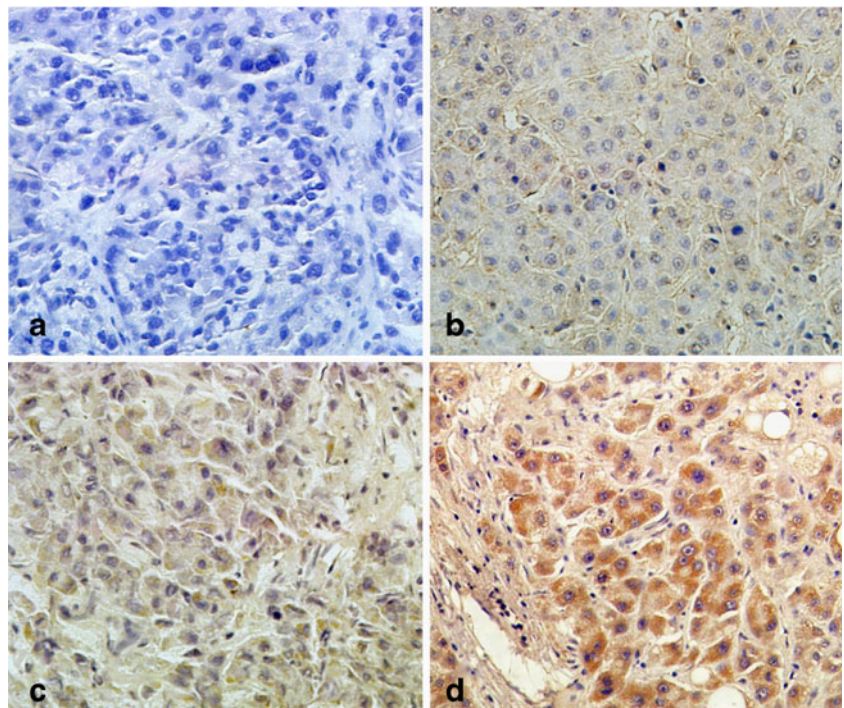
#### Small interfering RNA transfection

According to the protocol supplied with the Lipofectamine 2000, HepG2, SMMC-7721, and MHCC97H cells were transfected with either Notch1 or control siRNA. siRNA-transfected cells were seeded into six-well cell culture plates at a density of  $1 \times 10^5$  cells/well. The cells were allowed to grow for an additional 24 h and were then harvested for further analysis.

#### Real-time reverse transcription PCR

Total RNA was extracted and reverse transcribed. The primers used for the PCR reaction were as follows: Notch1, forward primer (5'-CACCCATGACCACTACCCAGTT-3') and reverse primer (5'-CCTCGGACCAATCAGAGATGTT-3'); and GAPDH, forward primer (5'-AAATCCCATCACC ATCTTCC-3') and reverse primer (5'-TCACACCCA TGACGAACA-3'). The primer sequences were verified by

**Fig. 1** Different levels of Notch1 expression in HCC tissues. **a** Negative, **b** weak, **c** moderate, and **d** strong



running a virtual PCR, and the primer concentrations were optimized to avoid primer-dimer formation. Additionally, dissociation curves were evaluated to avoid nonspecific amplification. Real-time PCR amplifications were performed using an Mx4000 Multiplex QPCR System (Stratagene, La Jolla, CA) with 2× SYBR Green PCR Master Mix (Applied Biosystems). Data were analyzed according to the comparative  $C_t$  method and were normalized to GAPDH expression in each sample.

#### 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 nitrocellulose membranes. The membranes were blocked with 5 % nonfat dried milk or bovine serum albumin in 1× TBS buffer containing 0.1 % Tween 20 and then 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

**Table 1** Association of Notch1 expression with clinicopathologic factors of the HCC patients

Tumor characteristic	n	Notch1		P value	$\chi^2$
		High (5–12 score, %)	Low (0–4 score, %)		
All cases	120	64 (53.3)	56 (46.7)		
Gender				0.841	0.040
Male	74	40 (54.1)	34 (45.9)		
Female	46	24 (52.2)	22 (47.8)		
Age (years)				0.379	0.773
≤50	63	36 (57.1)	27 (42.9)		
>50	57	28 (49.1)	29 (50.9)		
Tumor location				0.354	0.860
Left	61	30 (49.2)	31 (50.8)		
Right	59	34 (57.6)	25 (42.4)		
Tumor size (cm)				<0.001	14.255
≤5	51	17 (33.3)	34 (66.7)		
>5	69	47 (68.1)	22 (31.9)		
Tumor grade (differentiation)				0.006	7.574
Well	41	29 (70.7)	12 (29.3)		
Moderately or poorly	79	35 (44.3)	44 (55.7)		
Metastasis				0.002	9.254
Yes	38	28 (73.7)	10 (26.3)		
No	82	36 (43.9)	46 (56.1)		
Venous invasion				0.006	7.421
Positive	26	20 (76.9)	6 (23.1)		
Negative	94	44 (46.8)	50 (53.2)		
Satellite lesions				0.448	0.575
Positive	34	20 (58.8)	14 (41.2)		
Negative	86	44 (51.2)	42 (48.8)		
Tumor number				0.325	0.967
Solitary	87	44 (50.6)	43 (49.4)		
Multiple	33	20 (60.6)	13 (39.4)		
AJCC TNM stage				<0.001	29.233
I and II	32	4 (12.5)	28 (87.5)		
III and IV	88	60 (68.2)	28 (31.8)		
AFP (ng/ml)				0.957	0.003
≤400	34	18 (52.9)	16 (47.1)		
>400	86	46 (53.5)	40 (46.5)		

chemiluminescence detection system (Amersham Pharmacia Biotech). Quantification of the western blots was performed using laser densitometry, and relative protein expression was then normalized to GAPDH levels.

#### MTT assay

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

#### Invasion assays

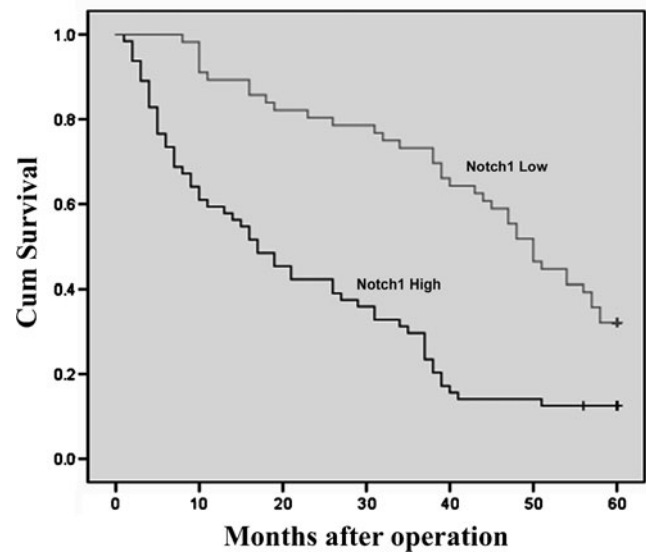
Cell invasion was analyzed using Matrigel-coated Transwell cell culture chambers (8  $\mu$ m pore size) (Millipore, Billerica, MA, USA). Briefly, treated cells ( $5 \times 10^4$  cells/well) were serum starved for 24 h and plated in the upper insert of a 24-well chamber in a serum-free medium. Medium containing 10 % serum as a chemoattractant was then added to the wells, and the cells were incubated for 24 h. Cells on the upper side of the filters were mechanically removed using 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 cells were counted at  $\times 200$  magnification from ten different fields in each filter.

#### Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) technique (Amersham, Buckinghamshire, UK) was used to quantify the activity of MMP-2 and MMP-9. The samples were thawed on ice, and all reagents were equilibrated to room temperature. All assays were carried out according to the manufacturer's instructions.

#### Statistical analysis

Statistical analysis was performed using SPSS 15.0 software (Chicago, IL, USA). Each experiment was repeated at least three times, and all data were summarized and presented as the means  $\pm$  SDs. The differences between means were statistically analyzed using a *t* test. The  $\chi^2$  test for proportions was used to analyze the relationship between Notch1 expression and various clinicopathologic factors. Survival curves were



**Fig. 2** Kaplan–Meier statistical analysis of postoperative survival curves according to Notch1 expression

calculated using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazard analysis was used for univariate and multivariate analyses to explore the effect of clinicopathological factors

**Table 2** Univariate and multivariate analyses for overall survival of 120 patients

Tumor characteristic	Relative risk (95 % CI)	<i>P</i> value
Univariate		
Gender	0.771 (0.512–1.163)	0.215
Age (years)	1.451 (0.964–2.185)	0.074
Tumor location	0.984 (0.656–1.476)	0.938
Tumor size	2.078 (1.340–3.222)	0.001
Tumor grade (differentiation)	0.840 (0.543–1.298)	0.432
Metastasis	15.769 (8.722–28.510)	<0.001
Venous invasion	18.413 (9.694–34.973)	<0.001
Satellite lesions	1.464 (0.938–2.283)	0.093
Tumor number	2.198 (1.419–3.405)	<0.001
AJCC TNM stage	6.230 (3.410–11.383)	<0.001
AFP (ng/ml)	0.847 (0.541–1.325)	0.466
Notch1	2.691 (1.765–4.103)	<0.001
Multivariate		
Tumor size	1.675 (0.839–3.344)	0.143
Metastasis	7.449 (3.365–16.488)	<0.001
Venous invasion	7.898 (3.360–18.566)	<0.001
Tumor number	3.567 (2.181–5.834)	<0.001
AJCC TNM stage	2.351 (0.908–6.086)	0.078
Notch1	2.086 (1.262–3.448)	0.004

95 % CI 95 % confidence interval

and the Notch1 expression on survival. *P* values of  $<0.05$  were considered statistically significant.

## Results

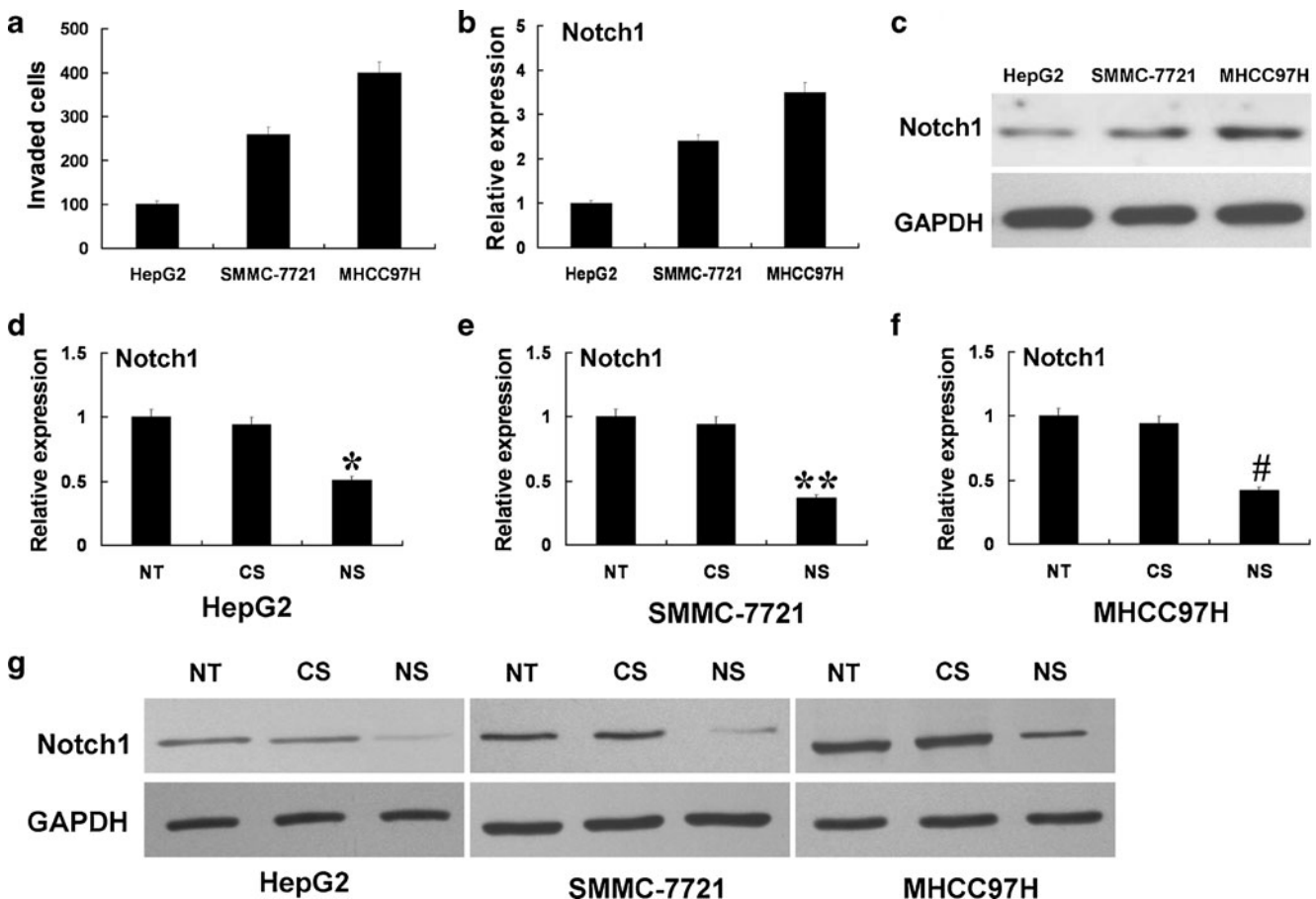
### Notch1 immunohistochemistry

Notch1 expression was mainly localized within the cytoplasm and at the cell membrane. There was no significant Notch1 expression in adjacent non-cancerous hepatic tissues, with only weak staining for Notch1 at the cell membrane and in the cytoplasm. As shown in Fig. 1, the expression of Notch1 differed between HCC tissues. Notch1 staining was negative in 17 samples of HCC, whereas weak positive staining was detected in 39 samples of

HCC; moderate positive staining was detected in 27 samples of HCC, and strong positive staining was detected in 37 samples of HCC.

### Relationship between Notch1 expression and clinicopathological characteristics

The pathological factors examined for 120 cases of HCC included gender, age, tumor location, tumor size, tumor grade, metastasis, tumor number, AJCC TNM stage, and AFP. In cases diagnosed with metastasis, we also analyzed vascular invasion. For this analysis, we divided the 120 patients into two subgroups: a high Notch1 expression group ( $n=64$ ) and a low Notch1 expression group ( $n=56$ ). The relationship between Notch1 expression and the clinicopathological factors is summarized in Table 1. The results



**Fig. 3** siRNA effectively inhibited the expression of Notch1 mRNA and protein in HCC cells. **a** Using Transwell cell culture chambers, we detected the invasion capabilities of different HCC cell lines. **b, c** RT-PCR and western blot were performed to assess the expression levels of Notch1 in different HCC cell lines. **d–g** RT-PCR and western blot were performed to assess the expression of Notch1 in three Notch1 siRNA-transfected HCC cell lines. The

expression of Notch1 was normalized to that of GAPDH (Notch1/GAPDH). The data represent the mean  $\pm$  SD, \* $P<0.05$  compared to control siRNA-transfected HepG2 cells; \*\* $P<0.05$  compared to control siRNA-transfected SMMC-7721 cells; # $P<0.05$  compared to control siRNA-transfected MHCC97H cells. *NT* non-transfection, *NS* Notch-1 siRNA transfection, *CS* control siRNA transfection

demonstrated that high Notch1 expression was strongly correlated with tumor size ( $P < 0.001$ ), tumor grade ( $P = 0.006$ ), metastasis ( $P = 0.002$ ), venous invasion ( $P = 0.006$ ), and AJCC TNM stage ( $P < 0.001$ ). However, there were no significant associations between Notch1 expression and the other pathological factors examined ( $P > 0.05$ ). These results indicate that Notch1 may be involved in the differentiation, invasion, and metastasis of HCC.

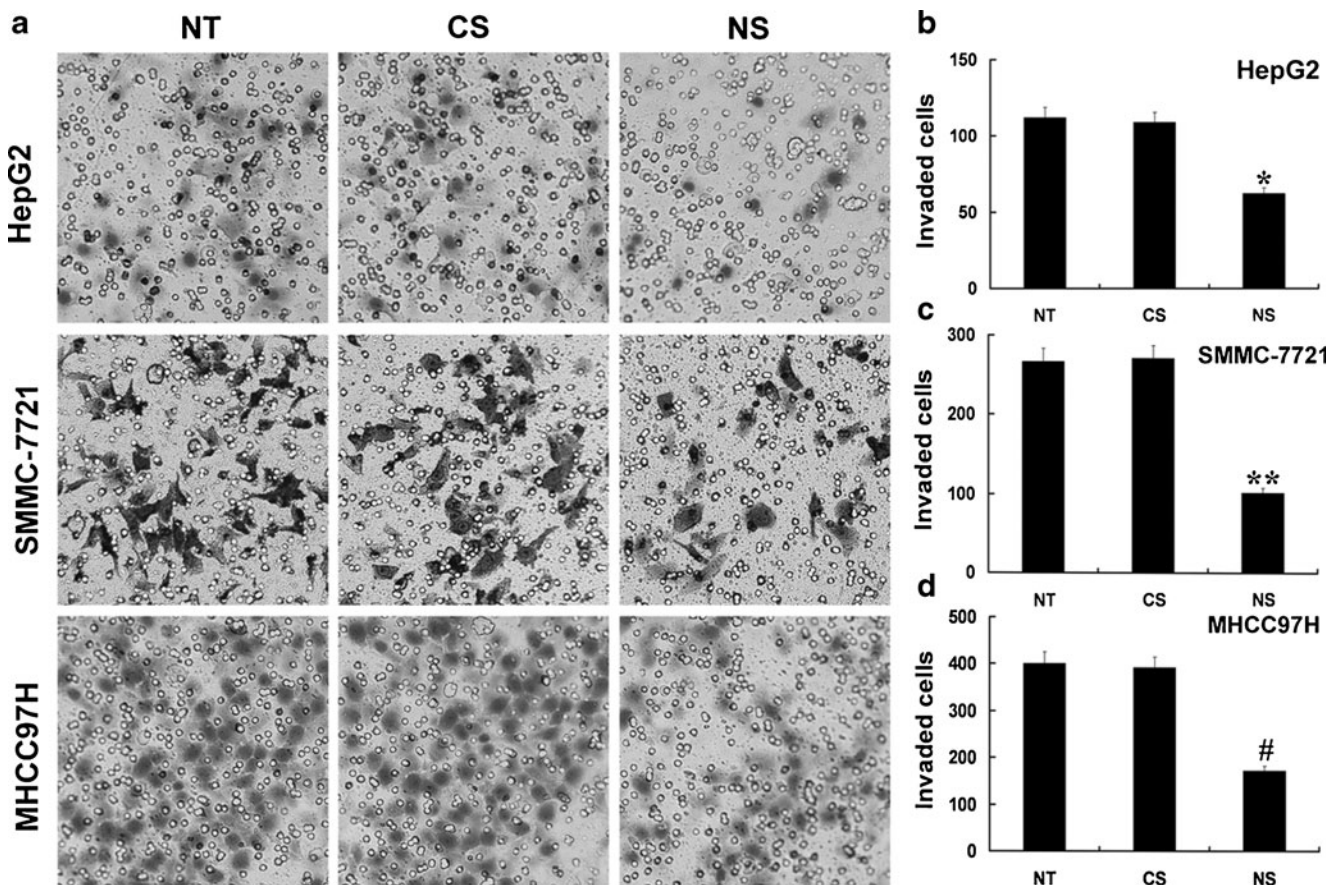
#### Correlation between Notch1 expression and prognosis of HCC patients

Because the level of Notch1 expression correlated with tumor size, tumor grade, metastasis, venous invasion, and AJCC TNM stage, we further speculated that the level of Notch1 expression may affect the prognosis of HCC patients. Kaplan–Meier postoperative survival curves were used to evaluate the overall survival rates of patients with HCC in comparison to their levels of Notch1 expression. The log-rank test showed that survival time was significantly different between low and high Notch1 expression groups

( $P < 0.001$ ). The low Notch1 expression group demonstrated increased survival, whereas the high Notch1 expression group demonstrated reduced survival (Fig. 2). The cumulative 5-year survival rate was 32.1 % in the low Notch1 expression group, whereas this rate was only 12.5 % in the high Notch1 expression group.

A univariate Cox regression analysis also found that tumor size, metastasis, venous invasion, tumor number, AJCC TNM stage, and Notch1 protein expression were significantly associated with overall survival (Table 2). Furthermore, to evaluate the potential of high Notch1 expression to serve as an independent predictor for overall survival among HCC patients, multivariate Cox regression analyses was performed. The results indicated that only metastasis, venous invasion, tumor number, and Notch1 expression could predict overall survival among HCC patients (Table 2).

Downregulated expression of Notch1 by siRNA reduced the invasiveness of HCC cells. Because high expression of Notch1 was strongly correlated with metastasis ( $P = 0.002$ ) and venous invasion ( $P = 0.006$ ), we next sought to



**Fig. 4** Inhibition of Notch1 by siRNA decreased the in vitro invasion capabilities of HepG2, SMMC-7721, and MHCC97H cells in Transwell assays, compared to control siRNA treatment. The data represent the mean  $\pm$  SD. \* $P < 0.05$  compared to control siRNA-transfected

HepG2 cells; \*\* $P < 0.05$  compared to control siRNA-transfected SMMC-7721 cells; # $P < 0.05$  compared to control siRNA-transfected MHCC97H cells. NT non-transfection, NS Notch1 siRNA transfection, CS control siRNA transfection

determine whether Notch1 was involved in invasion and metastasis in HCC. We first examined the expression levels of Notch1 in different HCC cells with different invasion capabilities. As shown in Fig. 3a, the invasion capacity of HepG2 cells was the lowest, whereas the invasion capacity of MHCC97H cells was the greatest. Reverse transcription (RT)-PCR and western blot analysis showed that the

expression levels of Notch1 mRNA and protein exhibited similar increased tendencies related to invasion capability (Fig. 3b, c) in HCC cells. In HCC cells, siRNA was used to effectively downregulate the expression of Notch1 mRNA and protein (Fig. 3d–g). Using Transwell cell culture chambers, we measured the invasiveness of Notch1 siRNA-transfected cells using three HCC lines. As illustrated in

**Fig. 5** In HepG2, SMMC-7721, and MHCC97H cells, the inhibition of Notch1 by siRNA decreased the protein expression and proteolytic activity of MMP-2 and MMP-9. **a** The protein expression levels of MMP-2 and MMP-9 were measured by western blot. **b–d** The proteolytic activity of MMP-2 and MMP-9 was measured by ELISA. The data represent the mean  $\pm$  SD. \* $P$ <0.05 compared to control siRNA-transfected HepG2 cells; \*\* $P$ <0.05 compared to control siRNA-transfected SMMC-7721 cells; # $P$ <0.05 compared to control siRNA-transfected MHCC97H cells. *NT* non-transfection, *NS* Notch1 siRNA transfection, *CS* control siRNA transfection

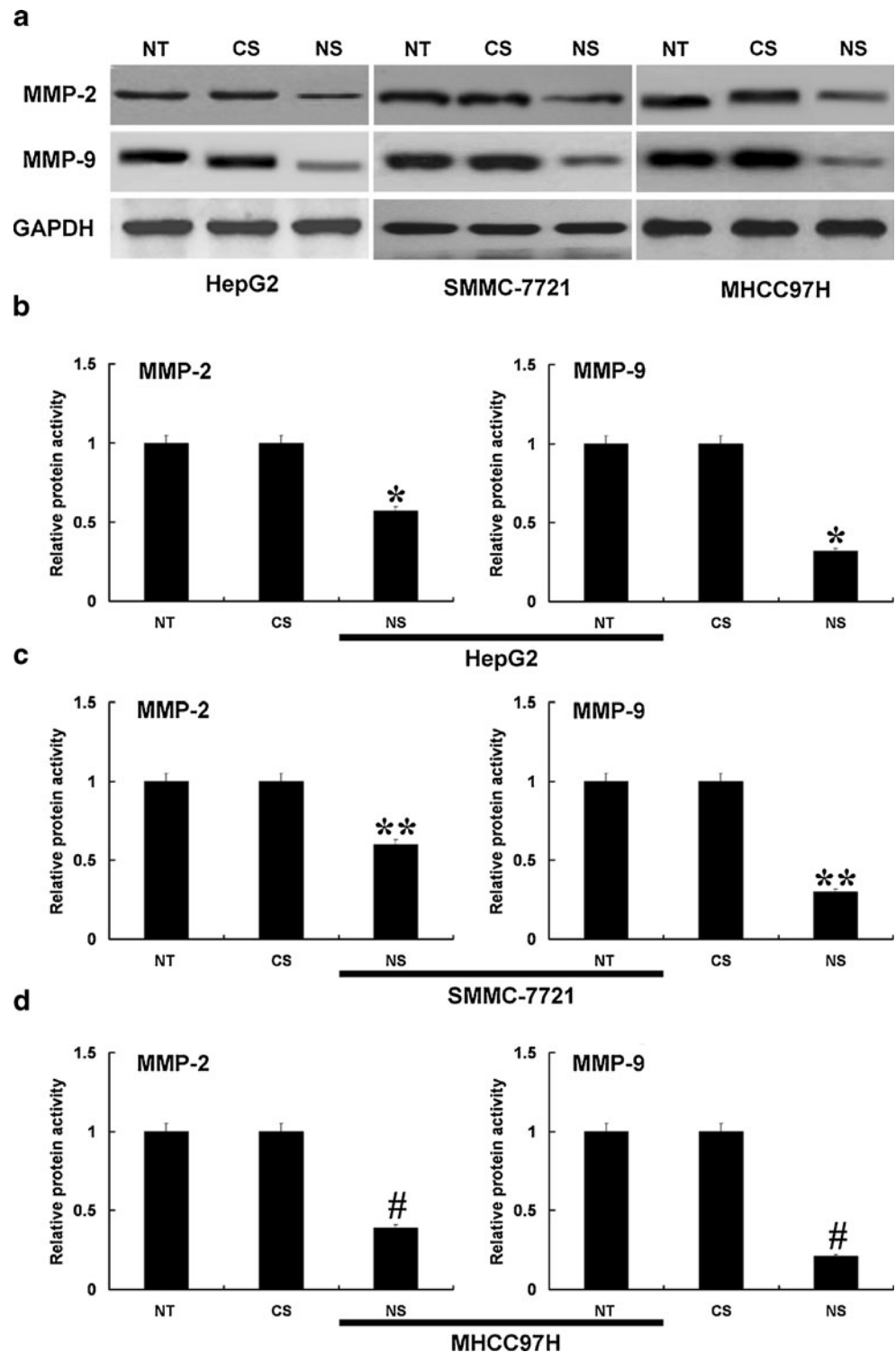




Fig. 4, the number of Notch1 siRNA-transfected HepG2 cells that migrated through the Transwell was significantly less than the number of control siRNA-transfected cells that migrated. In addition, the use of SMMC-7721 and MHCC97H cells showed similar results (Fig. 5). To confirm that the inhibitory effects of downregulated Notch1 on cell invasion were independent of apoptosis, we used the MTT assay to detect Notch1 siRNA-transfected cells. According to the results of the MTT assay, downregulated Notch1 did not affect the HCC cell viability (Fig. 6). Thus, these data indicated that the downregulation of Notch1 by siRNA reduced the invasion capacity of HCC cells. Downregulated Notch1 decreased the protein expression and proteolytic activity of MMP-2 and MMP-9.

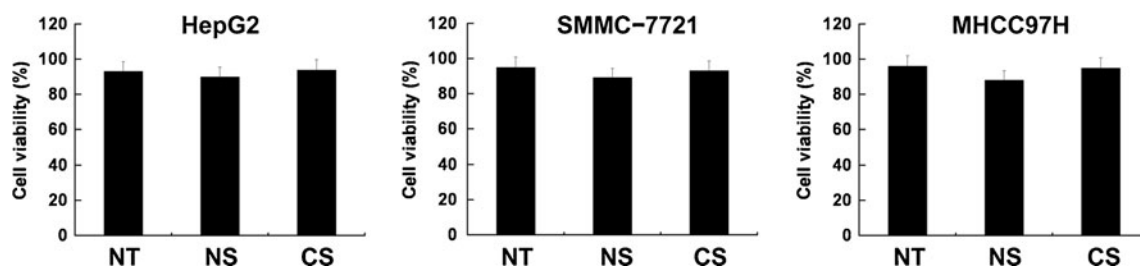
To determine the potential mechanism for the role of Notch1 in HCC cell invasion, we examined the effect of downregulated Notch1 on MMP-2 and MMP-9. Using western blot and ELISA, we found that the protein expression levels and proteolytic activity of MMP-2 and MMP-9 were decreased in Notch1 siRNA-transfected HepG2 cells (Fig. 5a, b). In addition, the use of SMMC-7721 and MHCC97H cells showed similar results (Fig. 5a–c). These results indicated that Notch1 may participate in HCC cell invasion by regulating the expression and activity of MMP-2 and MMP-9. However, additional studies are needed to address whether increased Notch1 expression is capable of activating MMP-2 and MMP-9.

## Discussion

Surgery, including transplantation, remains the only potentially curative modality for HCC, yet recurrence rates are high, and long-term survival is poor. Therefore, the ability to predict individual recurrence risk and patient prognosis would help guide surgical and chemotherapeutic treatments. Currently, physicians rely heavily on traditional pathologic variables, such as tumor size, tumor grade, metastasis, and TNM stage, and tumor markers, particularly AFP, have also been found to be prognostic indicators for HCC. However, these variables cannot accurately predict the probability of tumor recurrence following surgery, and it is therefore difficult to apply tailored treatment to individual patients. As a

result, many patients receive unnecessary adjuvant treatments that can be harmful. Therefore, it is necessary to further investigate other variables that could be used as an adjunct to the TNM staging system or AFP levels to improve the prognosis of individual patients.

The Notch signaling pathway regulates a wide variety of cellular processes during development and also plays an important role in tumor development [26, 27]. Notch1 is upregulated in many types of tumors and is involved in the invasiveness of tumor cells, including HCC cells [10, 14–18]. It has also been reported that high levels of Notch1 expression are related to poor overall survival among patients with breast cancer and colorectal cancer [24, 25]. However, the relationship between Notch1 expression and survival in patients with HCC remains unknown. In the present study, we examined the expression of Notch1 by immunohistochemistry in HCC samples. Our results indicated that high levels of Notch1 expression in HCC tumor tissues correlated with tumor size, tumor grade, metastasis, venous invasion, and TNM stage, each of which is an indication of advanced tumor status. These results strongly suggest that Notch1 may play a key role in the progression of human HCC. But in part of HCC samples, Notch1 staining was negative or weak positive staining. These results also were the same with the data of Villanueva who showed that Notch signaling promotes liver carcinogenesis in a genetically engineered mouse model, and that this pathway is activated in one third of human HCCs [11]. Prognostic molecular biomarkers are invaluable for evaluating patient status and promoting tumor control. Kaplan–Meier analysis of the survival curves from patients in the current study showed a significantly worse overall survival rate for patients whose tumors had high Notch1 expression levels (log-rank test,  $P < 0.001$ ), indicating that high levels of Notch1 protein may serve as a marker of poor prognosis for patients with HCC. Moreover, the multivariate analysis found Notch1 expression to be an indicator of worse patient outcome, independently of known clinical prognostic indicators such as TNM stage. These data suggest that high Notch1 expression is correlated with worse patient outcome and may serve as an independent prognostic factor for patients with HCC. Moreover, Notch1 expression may



**Fig. 6** Following different treatments in HepG2, SMMC-7721, and MHCC97H cells, cell viability was measured using the MTT assay. *NT* non-transfection, *NS* Notch1 siRNA transfection, *CS* control siRNA transfection

constitute a useful prognostic marker to be used in addition to the TNM staging system or AFP level for HCC patients, and these factors together may help to correctly assign patients to receive aggressive adjuvant chemotherapeutic treatment. Moreover, this is the first report to show that Notch1 expression can be used as a prognostic marker for HCC.

One significant finding of the current study was that metastasis and venous invasion were detected more frequently in Notch1-positive tumors compared to Notch1-negative cases. Invasion and metastasis are the processes by which tumors spread from the location of the primary tumor to distant locations in the body. These processes consist of a series of sequential steps, including tumor invasion and the establishment of metastatic foci at the secondary site, and involve various molecules [28, 29]. The MMPs family of proteins are the proteolytic enzymes in the extracellular matrix which contribute to tumor invasion, angiogenesis, and metastasis [30]. Among the previously reported human MMPs, MMP-2 and MMP-9 have been implicated in invasion and metastasis because of their role in the degradation of basement membrane collagen [31, 32]. In addition, it was reported that MMP-2 and MMP-9 were associated with an aggressive, invasive, or metastatic tumor phenotype [33, 34]. It is well known that MMP inhibitors can block endothelial cell activities that are essential for new vessel development, leading to proliferation and invasion [35]. Therefore, MMP-2 and MMP-9 are thought to be therapeutic targets of anticancer drugs based on the degrading actions of both enzymes on gelatins which are major components of the basement membrane. Our previous studies also showed that in HCC, inhibition of Notch signaling pathway could suppress the invasion of HCC cells via the downregulation of MMP-2 and MMP-9 [23]. Previous studies have also shown that in some tumors, the Notch1 signaling pathway can regulate MMP-2 and MMP-9 [16–18], which are important for the processes of tumor invasion and metastasis. But the mechanisms that Notch regulated MMP are complex. Our previous studies showed that the Notch signaling pathway inhibitor could suppress invasion of HCC cells via the extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathways, resulting in the downregulation of MMP-2 and MMP-9 [23]. In pancreatic cancer cells, Notch1 could be an effective approach for the inactivation of NF- $\kappa$ B and downregulation of its target genes, such as MMP-9 expression, resulting in the inhibition of invasion and metastasis [16]. However, it remains unknown whether Notch1 participates in the invasion and metastasis of HCC cells via the regulation of MMP-2 and MMP-9. In the current study, we showed that the invasion capabilities of Notch1 siRNA-transfected HCC cells were decreased, and that the downregulation of Notch1 decreased the protein expression and proteolytic activity of MMP-2 and MMP-9. These results suggest that in HCC cells, the

Notch1-MMP-2/MMP-9 axis may participate in tumor cell invasion. However, further study is necessary to elucidate the mechanism of the Notch1–MMP interaction in HCC.

While performing the current study, we found additional interesting results. For example, downregulated Notch1 did not affect the cell growth or viability of HepG2, SMMC7721, or MHCC97H cells. However, Li et al. showed that downregulation of Notch1 inhibited tumor growth in the human HCC cell lines HEP3B, SK-Hep-1, and SNU449 [36], whereas Qi et al. showed that the over-expression of Notch1 was able to inhibit the growth of SMMC7721 cells [37]. These results also indicate that Notch1 plays a complex role in tumor cells which depends on the tissue and cell type involved. Thus, the Notch signaling pathway likely plays a critical role in maintaining the balance between cell proliferation and apoptosis. Moderate changes to the Notch signaling pathway may be caused by intrinsic cellular regulation mechanisms, which can protect cells from damage. However, artificial fluctuations in the Notch signaling pathway may mislead the results of an experiment, and this represents one limitation of the current research.

In summary, our findings strongly suggest that high levels of Notch1 expression significantly correlate with tumor progression and an unfavorable patient prognosis. Thus, Notch1 expression may be used as an adjunct to the TNM staging system or AFP levels to improve the prognostication of individual patients. In vitro, downregulated Notch1 expression decreased the invasiveness of HCC cells via the regulation of MMP-2 and MMP-9. Therefore, Notch1 may be regarded as not only a novel candidate marker for prognosis but also a molecular target for HCC therapy. However, the underlying mechanisms responsible for these observations require further elucidation.

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**Conflicts of interest** None.

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