Selective suppression of Notch1 inhibits proliferation of renal cell carcinoma cells through JNK/p38 pathway

KERONG WU[1,2], LINKUN HU[1] and JIANQUAN HOU[1]

[1]Department of Urology, First Affiliated Hospital, Soochow University, Suzhou, Jiangsu 215000;
[2]Department of Urology, Ningbo First Hospital, Ningbo, Zhejiang 315000, P.R. China

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Abstract. The present study was performed to explore the effects of Notch1 inhibition selectively by siRNA on the proliferation and cell cycle of renal carcinoma cells. Human renal carcinoma cell lines, 786-0 and Caki-1, were treated with Si-Notch1 or negative control (NC). RT-PCR and western blotting were used to confirm the efficiency of siRNA on Notch1 expression. MTT, cell cycle analysis, colony formation as well as migration and invasion assays were performed. The expression levels of p38 and SAPK/JNK were measured by western blotting. For both cell lines, as compared with the NC group, the cell growth was markedly reduced, and colony formation was restricted in the Si-Notch1-treated group. After incubated with Si-Notch1 or NC for 48 h, Si-Notch1-treated cells arrested the cell cycle at G1/S phase. The Si-Notch1 group also had a reduced rate of migration as well as invasion. Moreover, we observed a reduction in p-SAPK/JNK and p-p38 in Si-Notch1 transfected cells. The present study indicated that Notch signaling is important in the tumorigenesis of renal cell carcinoma. Notch1 may be a potential therapeutic regimen towards renal cell carcinoma, and JNK/p38 may serve as an important molecular mechanism for Notch1-mediated carcinogenesis.

Introduction

Renal cell carcinoma (RCC) is the most lethal urologic cancer, representing 2-3% of all cancers (1). Although surgery is considered the primary curative therapy for patients with RCC, the prognosis for patients with metastatic disease is poor, with a 5-year survival rate of <10% (2).

Clear cell renal cell carcinoma (ccRCC), which accounts for ~80% of all RCCs, is characterized by inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene (3). Drugs targeting the HIF axis (including mTOR inhibitors) have been approved for advanced RCC (4). However, the efficacy is thought to be limited, and treatment response is not long lasting; therefore, the overall survival of ccRCC remains poor (5,6). Since the pathogenesis of ccRCC is quite complicated, it is unrealistic to expect that any single mechanism will uncover the full process. Additional tumorigenic events are supposed to contribute to the genesis and development of ccRCCs (7,8).

The Notch signaling controls a variety of cellular processes (9). Notch signaling is initiated through the interactions between the plasma-embedded Notch receptors (Notch1-4) and cell surface ligands (Jagged1 and Jagged2, and δ-like 1, 2 and 4) present on adjacent cells. The intracellular domain of Notch (ICN), is cleaved from the plasma membrane and translocates into the nucleus, leading to transcription of its downstream targets such as Hes and Hey (10).

In RCC, Notch seems to play an 'oncogenic' role; it was previously reported that the Notch signaling cascade was constitutively active in cell lines (8), and high expression of Notch was associated with increased risk of metastasis (11). Our previous study revealed the elevated level of Notch1 and Jagged1 in ccRCC tissues compared with in normal kidney tissues (12). We also reported that Jagged1 overexpression may predict poor outcome in RCC patients (13). However, how Notch plays the oncogenic function and whether Notch pathway is a target in RCC remains unclear.

Since Notch1 was detected overexpressed in ccRCC (13), in the present study, we exclusively suppressed Notch1, explored how the inhibition would influence the survival of ccRCC cells, and detected the potential mechanism. The present study provides the information on the mechanism of tumorigenesis of RCC and highlights the potential use of Notch inhibition as a novel treatment for RCC.

Materials and methods

Reagents and antibody. The antibody used against Notch1 (polyclonal rabbit anti-human Notch1; ab27526) was purchased from Abcam (Cambridge, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Tissue culture media and fetal bovine serum (FBS) were purchased from Gibco (Fullerton, CA, USA). Cell cycle

Correspondence to: Dr Jianquan Hou, Department of Urology, First Affiliated Hospital, Soochow University, 188 Shizi Avenue, Suzhou, Jiangsu 215000, P.R. China
E-mail: wkr1983@163.com

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analysis was performed using the Coulter DNA Prep™ reagents kit (Beckman Coulter, Fullerton, CA, USA). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Beckman Coulter. Human renal carcinoma cell lines, 786-0 and Caki-1, were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Other materials will be introduced in the following context.

Cell culture. Human renal carcinoma cell lines, 786-0, and Caki-1, were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 incubator at 37˚C.

Western blotting. The cells were solubilized in a lysis buffer on ice. All lysates were centrifuged at 4˚C at 10,000 x g for 10 min. The protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). An amount of 100 mg protein content was electrophoresed in 8% SDS-PAGE and blotted on a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin in 1X Tris-buffered saline (TBS) buffer at room temperature for 2 h and incubated Notch1 (1:200) at 4˚C overnight. After three washes for 15 min in TBS, the membrane was incubated with the peroxidase-conjugated mouse anti-goat IgG antibody for 2 h at room temperature. Immunoreactive proteins were visualized by an enhanced chemiluminescence system (Immobilon, Millipore, Billerica, MA, USA) and GAPDH was used as the control for protein loading.

Reverse transcriptase-polymerase chain reaction. Total RNA from the cells was isolated with RNAiso Plus (Takara, Japan). The RNA was reverse transcribed with a PrimeScript RT reagent kit (Takara). All the procedures were conducted in accordance with the manufacturer's instructions. The resulting cDNA was quantified by RT-PCR using SYBR Premix Ex Taq (Takara). GAPDH was used as a housekeeping gene. The primer sequences were: GAGGCGTGGCAGACTATGC (forward) and CTTGTACTCCGTCAGCGTGA (reverse).

RNA interference. siRNA duplexes were produced by Shanghai GenePharma Co., Inc. (Shanghai, China) against human Notch1. Scrambled control siRNA, which was used as a negative control (NC), was also designed and obtained from GenePharma Co., Inc. (Table I).

Growing cells were seeded at $2x10^5$ cells/well into a 6-well tissue culture dish. The transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. In the preliminary test, different concentrations of siRNA 0, 25, 50 and 100 nM were tested. We found 50 nM Si-Notch1 achieved an efficient inhibition of Notch1. When we increased the concentration of Si-Notch1, the efficacy did not improve significantly. The final concentration of siRNA added to the cells was determined to be 50 nM. The cells were cultured in the presence of transfection mixture for 24 h; the transfection mixture was replaced by fresh RPMI-1640 medium.

Table I. Primers used in the present study.

Gene name	Primer sequence
Si-Notch1	GCACGCGGAUUAAUUUGCAdTdT
	UGCAAAUUAAUCCGCGUGCdTdT
Negative control	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAATT

Cell viability assay. Cell viability assay was performed as previously described (12). Briefly, cells were seeded into 96-well plates at a density of $1.0x10^4$ cells/well. Following overnight incubation, the cells were treated with siRNA (50 nM) or NC for 48 h. Thereafter, the medium was removed and 20 μl MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well. Following incubation for 4 h at 37˚C, the supernatant was removed and the formazan crystals were solubilized by adding 150 μl dimethyl sulfoxide (DMSO). Viable cells were detected by measuring absorbance at 490 nm using MRX II absorbance reader (Dynex Technologies, Chantill, VA, USA).

Colony formation assay. Cells were counted and plated at 500 cells/well in a 6-well plate. After 2 weeks, the cells were washed with PBS, fixed with methanol for 15 min at room temperature, and stained with crystal violet for 30 min and after that the number of colonies was counted.

Apoptosis. Detection of apoptotic cells by flow cytometry. Cells were plated in 6-well plates (2 ml/well) at a density of $5x10^5$ cells/ml and incubated overnight. After siRNA treatment for 48 h, the cells were collected and washed with PBS, followed by resuspension in binding buffer at a concentration of $1x10^6$ cells/ml. A total of 100 ml ($1x10^5$ cells) of the solution was removed and mixed with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. The mixed solution was incubated in the dark at room temperature for 15 min, 400 μl dilution buffer was then added to each tube and cell apoptosis analysis was performed using the FC500 flow cytometry system (Beckman Coulter) within 1 h.

Analysis of cell cycle distribution. After exposed to Si-Notch1 or NC (50 nM) for 48 h at 37˚C, cells were harvested, washed with cold PBS, fixed with 70% ethanol and stored at 4˚C for subsequent cell cycle analysis. For detecting DNA content, cells were incubated in the dark at room temperature with 0.5 ml RNase A for 20 min and with 1 ml PI for 20 min. The DNA content of the cells was measured using the Beckman Coulter FC500 flow cytometry system. The percentage of cells in G1, S and G2/M phases was calculated.

Cell migration and invasion assays. For the *in vitro* migration and invasion assays, $5x10^4$ cells were resuspended in serum-free medium and placed in the upper Transwell chamber (8.0-μm PC; Corning Inc., Corning, NY, USA). The lower chamber contained medium with 10% FBS. After 24 h of incubation, the migrating cells in the lower chamber or invading cells on the bottom of each well were stained with

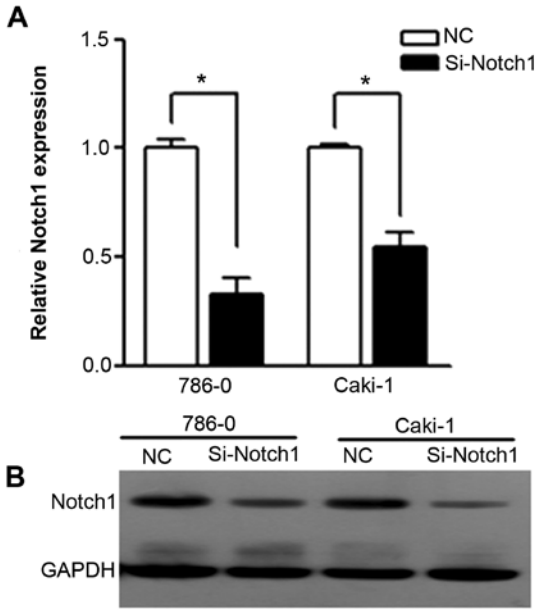

Figure 1. RNA interference efficiency. Suppression of Notch1 expression by siRNA. A) Cells were transfected with the NC and Si-Notch1 for 48 h, and total RNA was analyzed by RT-PCR. B) Total protein was analyzed by western blotting with specific antibody. *P<0.05, the results were statistically significant compared to the NC group. The data are from three separate experiments.

4',6-diamidino-2-phenylindole (1 mg/ml; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) followed by fixation in methyl alcohol for 30 min. Then, the number of cells in six randomly selected microscopic fields (magnification, x100) was counted with a BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using a statistical software package (SPSS, version 16.0; SPSS, Inc., Chicago, IL, USA). For MTT results, cell cycle analysis, colony-formation as well as migration and invasion assays, data from three independent experiments are presented as the mean values with standard deviations. The differences were evaluated using two-tailed Student's t-tests. P<0.05 was considered to indicate a statistically significant difference and all P-values were two-sided.

Results

Notch1 expression is markedly decreased by siRNA. We used RT-PCR and western blotting to confirm the efficiency of siRNA on Notch1 expression. After transfection, expression of Notch1 in Si-Notch1 group was reduced as compared with in the NC group (Fig. 1). For PCR, we detected a 67.3 and 45.2% reduction of Notch1 in 786-0 and Caki-1 cell lines, respectively. For western blotting, the protein of Notch1 was expressed with specific bands at 80 kDa. Notch1 protein was found to be downregulated in Si-Notch1-treated cell lines compared with the NC group for both cell lines.

Notch1 inhibition by RNA interference inhibits growth of tumor cells. To determine whether Notch1 signaling was responsible for cell proliferation in ccRCC, we interrupted the signaling pathway by RNA interference of Notch1. An MTT assay was carried out to measure the proliferation status of the cells. As shown in Fig. 2, inhibition of cell proliferation was detected in Si-Notch1-treated cell lines. After 48 h, in NC group, percentage of cell viability was 94.4±4.1 and 91.6±3.5% in 786-0 and Caki-1 cell lines, while in Si-Notch1 group it was 70.2±2.3 and 61.6±5.1% in 786-0 and Caki-1 cell lines. Compared to the NC group, the cell growth of Si-Notch1 group was decreased by 24.0 and 32.8% after 48 h of incubation, in 786-0 and Caki-1 cells, respectively.

We subsequently confirmed the inhibitory effects of Si-Notch1 through colony formation assays. The assay further confirmed that Si-Notch1 treatment inhibited cell proliferation. After 14 days, the colony formation rates of Si-Notch1 transfected cells were 7.1±1.2 and 5.7±1.2%, in 786-0 and Caki-1, respectively. In comparison, the colony formation rates of NC transfected cells were 19.7±2.2 and 15.9±1.4%, respectively. The cell colony formation rate of Si-Notch1 group was clearly suppressed as compared to the NC group, in both 786-0 and Caki-1 cell lines (P=0.003 and P=0.001, respectively) (Fig. 2).

To determine whether the Notch1 inhibition would cause cell apoptosis, flow cytometry was further used to identify the cell death types. The 786-0 and Caki-1 RCC cells treated with Si-Notch1 did not show obvious apoptosis.

Notch1 inhibition by RNA interference induces G1/S phase cell cycle arrest. Based on the growth inhibitory response of siRNA treatment in cells, its effect on cell cycle distribution was next examined. Both cell types were incubated with Si-Notch1 or NC for 48 h after which cell cycle analysis was performed. Si-Notch1-treated cells arrested the cell cycle at the G1/S phase (Table II, Fig. 3), suggesting that the MTT assay indicated cell cycle arrest.

Notch1 inhibition by RNA Interference inhibits cell migration and invasion. We used Transwell migration and invasion

Table II. Negative control (NC) and Si-Notch1 cells.

	786-0 (%)			Caki-1 (%)	
Phase	NC	Si-Notch1	Phase	NC	Si-Notch1
G1	41.29±2.31	53.06±2.54	G1	61.38±3.19	73.32±4.99
S	43.62±1.34	37.47±4.94	S	26.84±1.13	15.76±3.12
G2	15.09±1.08	9.47±3.14	G2	12.11±1.93	10.91±2.73

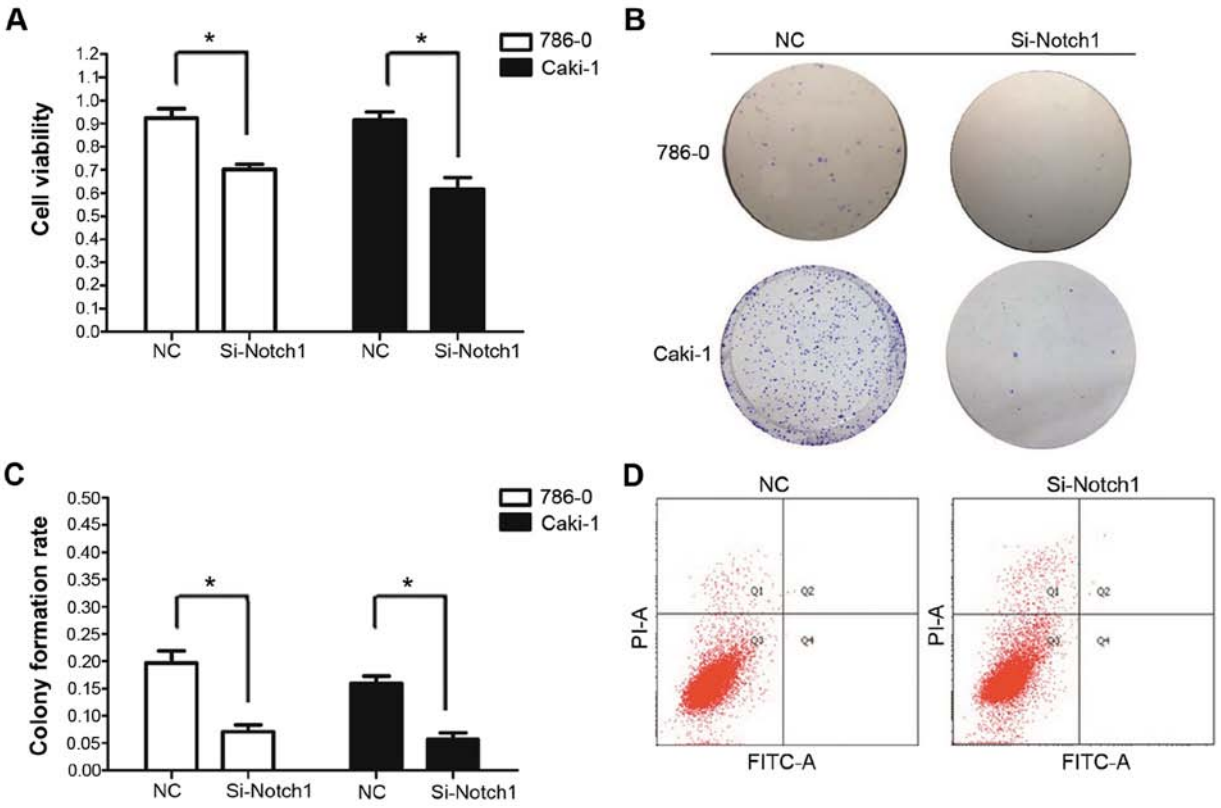

Figure 2. Cell proliferation and colony formation. (A) Cell proliferation assay using MTT was performed to evaluate the growth of the two cell lines. (B) Si-Notch1 transfection inhibited the colony formation detected by colony-forming assay. The number of colonies was diminished in both cell lines following Si-Notch1 treatment. (C) Colony formation rate for 786-0 and Caki-1 cell lines following Si-Notch1 or NC transfection. (D) No obvious apoptosis was detected in either cell lines using a double-staining method with Annexin V-FITC/PI. FITC, fluorescein isothiocyanate; PI, propidium iodide. *P<0.05, the results were statistically significant compared to the NC group. The data are from three separate experiments.

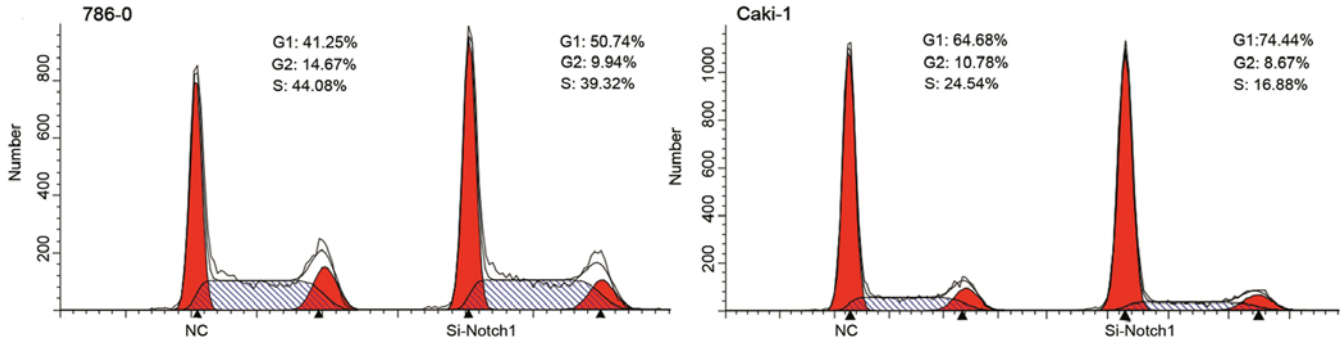

Figure 3. Cell cycle analysis. Notch1 inhibition arrested the cell cycle at the G1/S phase, analyzed by FACS.

assays to compare the cell migration and invasive capacity in each group. Fig. 4 shows results for 786-0 and Caki-1 cell lines, the Si-Notch1 group had a reduced rate of migration as well as invasion. For 786-0 cell line, the migration cell count of Si-Notch1 group was 316±58, while it was 1,271±198 in NC group (P=0.009). In addition, the invasive cell count of Si-Notch1-treated cells was 223±22, compared to 1,046±180 with the NC group (P=0.015). For Caki-1 cell line, the migration cell count was 306±53 and 1,380±102 in Si-Notch1 and NC groups, respectively (P<0.001). Moreover, the invasive cell count was 427±97 and 1,229±116 in Si-Notch1 and NC group, respectively (P=0.001).

MAPK pathway is regulated by Notch1 inhibition. The cell lines were treated with Si-Notch1 or NC for 48 h, respectively. In addition, the levels of p38 and JNK were measured by western blotting. As shown in Fig. 5, the levels of phospho-p38 and phospho-JNK were downregulated at 48 h in Si-Notch1 group as compared with the NC group, for both 786-0 and Caki-1 cell lines.

Discussion

RCC is globally the 13th most common cancer (14,15). Over the past several years, targeted therapies have increasingly

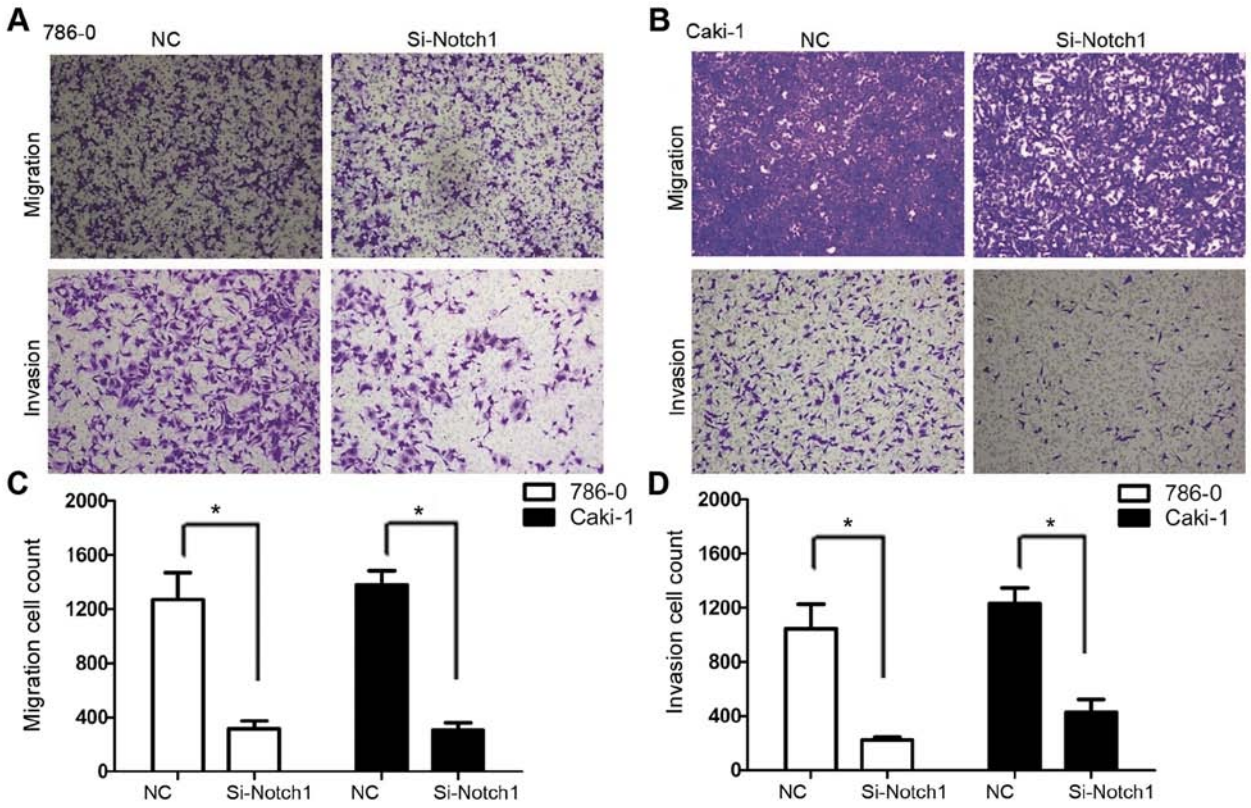

Figure 4. Cell invasion and migration. (A and B) Notch1 interference inhibited tumor migration as well as invasion to the lower chamber (magnification, x100). (C and D) *P<0.05, the results were statistically significant compared to the NC group. The data are from three separate experiments.

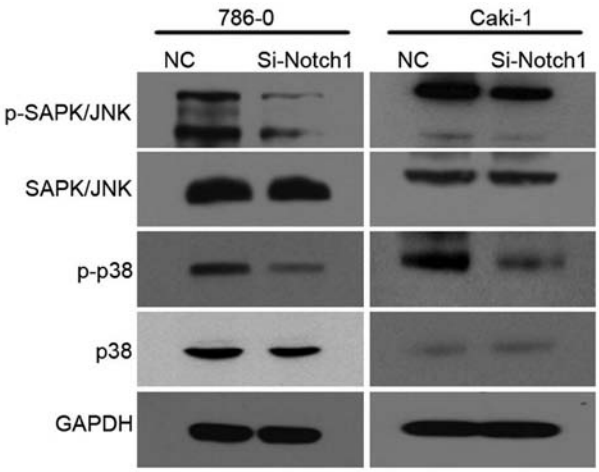

Figure 5. Expression of SAPK/JNK, p38 by western blotting. The levels of p38 and JNK did not show significant difference in Si-Notch1 group when compared with NC group in either cell line. While the levels of phospho-p38 and phospho-JNK were lower in Si-Notch1 groups.

become available and have shown considerable promise for the treatment of RCC; however, even with such therapies life expectancy is generally only extended by less than one year (16). New therapeutic targets are needed and thus identification of such targets could lead to the design of new drugs for use in RCC patients.

The Notch pathway is critical in the determination of cell fates by regulating cell growth, differentiation and apoptosis (9). In RCC, Notch signaling cascade was active in human ccRCC cell lines independent of the VHL/HIF pathway; Notch1 and the Notch ligand Jagged1 were expressed at significantly higher levels in ccRCC tumors than in normal human renal tissue (8). The expression level of Notch was correlated with the Furman grading, TNM staging as well as prognosis in RCC cases (13). As a basis for the present study, we discovered that Notch1 was overexpressed in RCC cell lines as well as kidney cancer tissues (12,13). Thus, we took Notch1 as our target to test how the inhibition of Notch1 exclusively would influence the biological behavior of RCC cells.

In the present study, inhibition of Notch1 with siRNA led to a considerable decrease of cell proliferation and induced cell cycle arrest. Cell colony forming was also inhibited by Si-Notch1 interference. Moreover, the capacity of invasion and migration were suppressed by Si-Notch1 treatment. The results support the therapeutic effect of Si-Notch1 for ccRCC. Though apoptosis analysis did not show a positive result, we suppose the inhibitory effect was mainly achieved by cell cycle arrest. The present study implied that the receptor Notch1, at least played a significant role in Notch pathway and thus could be considered as a promising therapeutic target. In fact, Notch pathway has been taken as a treatment target in various malignancies, and the results seemed to be encouraging (17,18). Though non-selective inhibition like Notch γ-secretase inhibitor has been reported, the side-effects could be serious when it is applied in experiments *in vivo* (18). Considering that, a selective inhibition of Notch seemed to be more reasonable, and given the limitation of kinase inhibitors, a comprehensive evaluation of Notch inhibition would be an alternative choice for ccRCC.

The mechanism involved with the oncogenic role of Notch may be multiple (19,20). The MAPKs comprise a well-studied family of serine threonine kinases that play important regulatory roles in the cells (21). Among the MAPK signaling pathways, JNKs and p38-MAPKs were identified to be activated in response to a variety of cellular and environmental stress such as changes in osmolarity, DNA damage, heat shock, ischemia, cytokines, UV irradiation and oxidative stress (22,23).

Although JNKs are primarily attributed to proapoptotic cell death or tumor suppression in response to a variety of stress, emerging evidence suggests that JNKs, play a role in the malignant transformation of cells and in tumorigenesis (24). Research suggests that JNK signaling contributed to cancer development (25,26). The roles of p38 in invasion and metastasis have also been reported (27), and activation of p38 has been shown in various cancers to be the mechanism promoting expression of MMP-2 (28,29). In the present study, we observed a reduction in p-SAPK/JNK and p-p38 in Si-Notch1 transfected cells, suggesting that JNK/p38 may serve downstream of the Notch pathway. This result may partly illustrate why inhibition of the Notch pathway leads to considerable inhibition of cell proliferation, and clear restriction of the invasion and migration capability. We demonstrated that the oncogenic effect of Notch1 is at least partially mediated through regulation of the JNK/p38 pathway in two ccRCC cell lines.

Deficiencies remain in the present study. Firstly, though we blocked the Notch pathway with a specific receptor, we consider that knockout of Notch1 would make the results stronger. Secondly, as a conservative pathway, Notch has a comprehensive network of its targets. To detect the downstream targets of Notch pathway, more research needs to be carried out in our following study.

In conclusion, the present study indicated that Notch signaling is important in the tumorigenesis of RCC. JNK/p38 may serve as an important molecular mechanism for Notch1-mediated carcinogenesis of ccRCC. Notch1 inhibition has the potential of being a novel therapeutic regimen for RCC.

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