

Downregulation of Notch1 inhibits the invasion and metastasis of human gastric cancer cells SGC7901 and MKN74 *in vitro* through PTEN activation and dephosphorylation of Akt and FAK

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Abstract. Migration and invasion are both vital causes of mortality in patients with gastric cancer. Therefore, the inhibition of these tumour cell processes is of great importance in gastric cancer therapy. Activation of Notch has been reported in many cancers. The critical role of Notch and its regulation in tumorigenesis has been noted. Although the studies on Notch in the field of cancer have been performed extensively, the role of Notch1 signalling in gastric cancer requires further study. Inactivation of PTEN has been observed in the development of many malignant tumors, and loss of PTEN function has been implicated in tumorigenic processes. Notch acts as an upstream signalling pathway that regulates PTEN activities. However, the effect of Notch on invasion and metastasis in gastric cancer and the regulation of PTEN during this process remain poorly understood. In the present study, small interfering RNA (siRNA) was used to knock down Notch1 expression in gastric cancer cell lines SGC7901 and MKN74. The mRNA and protein expression of Notch1, PTEN, Akt and FAK were measured upon depletion of Notch1. phospho-PTEN, phospho-Akt and phospho-FAK expression were measured using western blot analysis. Migration and invasion assays were also used after Notch1 depletion. Our results showed that the knockdown of Notch1 leads to the inhibition of cell invasion and metastasis of human gastric cancer cells SGC7901 and MKN74 *in vitro*. Compared to control and mock groups, PTEN activities were significantly

promoted following depletion of Notch1, and the expression of Phospho-Akt and Phospho-FAK were downregulated. Taken together, our findings suggest that Notch1 could be used as a therapeutic target to inhibit cell invasion and migration in gastric cancer.

Introduction

Gastric cancer is one of the most malignant, aggressive and common cancers worldwide (1). According to recent statistics, the yearly estimated number of new gastric cancer cases was 26,370, and the yearly estimated number of gastric cancer-related deaths was 10,730 in the USA alone (2). These numbers were much higher in China: 679,100 and 498,000, respectively (3). Although the diagnosis and management of gastric cancer have greatly improved, this disease still accounts for 10% of all deaths caused by malignant tumors annually (4,5). The primary curative treatment of gastric cancer is surgical resection. However, Chinese gastric cancer patients are often diagnosed at advanced stages, making surgery much more difficult and less effective (6). Because advanced stages of gastric cancer are generally associated with greater invasion and metastasis, studies have suggested that inhibition of cell signalling pathways could greatly affect the invasion and metastasis of gastric cancer cells (7-12). Therefore, the new prevention strategy of targeting cell signalling pathways of invasion and metastasis in gastric cancer will likely be of great clinical value.

PTEN, which is also known as TGF- β -regulated and epithelial cell enriched phosphatase (TEP1) or muted in multiple advanced cancers (MMAC1), was isolated and identified by three laboratories in 1997 (13-15). As a tumor suppressor gene, its protein is ubiquitously expressed among human cells. PTEN can function as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) into the biphosphate product PIP₂ (16). PIP₃ is a primary activator of Akt, and dephosphorylation of PIP₃ by PTEN results in inhibition of Akt signalling, which is critical in various cancer cellular functions, including cell transcription, proliferation, metastasis and invasion (16). PTEN also functions as a protein phosphatase by dephosphorylating FAK, restricting cancer

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cell invasion and metastasis (17,18). Studies have suggested that upstream signalling regulates invasion and metastasis of cancer cells through regulation of PTEN (19,20).

Notch is vitally important in controlling cell fate. The critical roles of Notch in tumorigenesis have been reported in many malignant tumors (21,22). Studies have indicated that Notch signalling has variable effects on different cancer cells. Notch signalling can either act as a tumor suppressor or tumor promoter (23-25). The function of Notch signalling in gastric cancer, especially its effect on invasion and metastasis, remains unclear. Evidence has shown that Notch signalling regulates PTEN in cancer cells (12,26,27). However, the relationships between Notch and PTEN in gastric cancer cells require further research.

The aim of this study was to investigate the role and mechanism of the Notch1 signalling pathway on cell invasion and metastasis and possible downstream regulation during this process in gastric cancer cells *in vitro*.

In this study we determined that downregulation of Notch1 signalling using siRNA inhibits invasion and metastasis of gastric cancer cell lines SGC7901 and MKN74 *in vitro*. PTEN activation and decreased expression of phosphorylated forms of FAK and Akt was also observed after Notch1 depletion. Our data suggest that the Notch1 signalling pathway may provide an effective treatment in gastric cancer patients.

Materials and methods

Cell culture. The gastric cell line SGC7901 was kindly provided by the Second Affiliated Hospital of Harbin Medical University. The gastric cell line MKN74 was obtained from Harbin Medical University Cancer Hospital. Both cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences; Hyclone, Logan, UT, USA) containing 10% foetal bovine serum (FBS; Biowest SAS, Nuaillé, France). All cells were incubated in 5% CO₂ and 37°C in a humidified chamber.

RNA extraction and quantitative PCR. Total RNA was harvested from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription were performed using the Golden 1st cDNA Synthesis kit (HaiGene, Harbin, China). Quantitative real-time PCR was performed with Mini-Opticon2 (MJ) by using the Golden HS SYBR-Green qPCR Mix (HaiGene). β -actin was used as an internal control and β -actin qPCR primer was obtained from HaiGene. The specific primers for each gene were synthesized by HaiGene. Specific primer sequences used were as follows: Notch1 forward, 5'-TCG GAG TGT GTA TGC CAA GAG-3' and reverse, 5'-TGA TGC CTA CAT TTC AAG AAC GG-3'; PTEN forward, 5'-GTG TGG AAT GAA GTG AGG CTT G-3' and reverse, 5'-TTG GAC AAC TGG ATA GAG TAG GC-3'; Akt forward, 5'-CCA TCA CAC CAC CTG ACC AAG-3' and reverse, 5'-CGC CTC TCC ATC CCT CCA AG-3'; FAK forward, 5'-GAG ATT GAC ATG GCA CAG AAG C-3' and reverse, 5'-TGA GCA GCA GTC AGC ATT TG-3'. Specificity of amplification products was determined by melting curve analysis. Independent experiments were done in triplicate. The 2^{- $\Delta\Delta$ Ct} was presented as the relative expression of the gene expression.

Antibodies and reagents. The following primary antibodies were purchased from Cell Signaling Technology (CST; Danvers, MA, USA): Notch1, PTEN, Akt, FAK, phospho-PTEN (Ser380/Thr382/383), phospho-Akt (Ser473) and phospho-FAK (Tyr397). β -actin and all secondary antibodies were provided by Santa Cruz Biotechnology (Dallas, Texas, USA). Lipofectamine RNAiMAX, Notch1 small interfering RNA (siRNA), control siRNA, and related chemicals were purchased from Invitrogen.

siRNA transfection. The putative Notch1 candidate sequences and the control sequence were designed and provided by Invitrogen. The siRNA sequences are as follows: Sequence 1 forward, 5'-CCG CCU UUG UGC UUC UGU UCU UCG U-3' and reverse, 5'-ACG AAG AAC AGA AGC ACA AAG GCG G-3'; sequence 2 forward, 5'-CCA CCA GUU UGA AUG GUC AAU GCG A-3' and reverse, 5'-UCG CAU UGA CCA UUC AAA CUG GUG G-3'; sequence 3 forward, 5'-CCG CCA AAU UCA ACG GGC UCU UGU G-3' and reverse, 5'-CAC AAG AGC CCG UUG AAU UUG GCG G-3'). Control duplexes using Invitrogen stealth RNAi negative control duplexes (High GC Duplex, cat no. 12935-400) were utilized. The transfection procedure was performed following manufacturer's instructions. Cells were harvested at the same time for investigation after 24 to 48 h of growth.

Wound healing assay. Wound-healing assays were performed to assess the effect of migration. Gastric cancer cells were seeded into 6-well plates and treated with mitomycin C (Santa Cruz Biotechnology, Dallas, Texas, USA) to inhibit cell proliferation. The cell monolayer was disrupted with a pipette tip. DMEM medium was used to wash away floating cells. Photographs were captured using an inverted microscope (same magnification) at the same time 48 h after the scratch. Six fields for each point were recorded. Relative wound size was calculated to assess migration activity.

Cell invasion assay. For invasion assay, a Transwell assay was performed (8 μ m; Corning Inc., New York, NY, USA). The membranes were coated with 200 μ l Matrigel at 200 μ g/ml. The upper chamber was seeded with cells in serum-free DMEM medium, and DMEM with 10% FBS was added in the lower chamber. After incubation for 24 h, cells were removed at the same time from the upper surface of the filter by scraping gently with a swab. Cells that invaded the bottom of membrane were fixed and stained. The numbers of invaded cells were calculated.

Western blot analysis. Cells were lysed in buffer [1% nonidet P-40, 100 mg/l phenylmethylsulfonyl fluoride, 50 mmol/l Tris-Cl (pH 8.0), 0.02% sodium azide, and 1 mg/l aprotinin]. After centrifugation for 20 min, the supernatant was collected, and the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to measure protein concentrations following manufacturer's instructions. Equivalent amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis. Then, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA) and blocked for 2 h at 37°C. The membranes were then incubated overnight

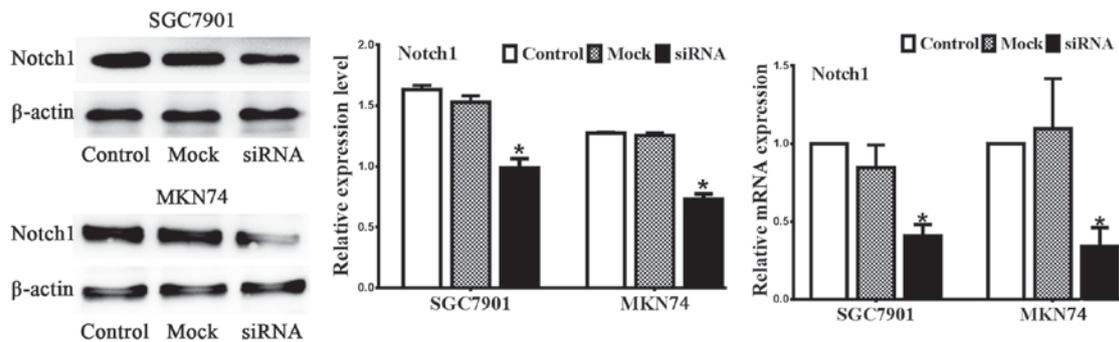


Figure 1. Notch1 was expressed in both SGC7901 and MKN74 cell lines. Knockdown efficiency of siRNA was tested in both gastric cancer cell lines by western blot and real-time PCR analysis. Notch1 expression was decreased compared to the control and mock groups in both gastric cancer SGC7901 and MKN74 cell lines following transfection with Notch1 siRNA (* $P < 0.05$ siRNA vs. control; siRNA vs. mock). All data are expressed as the means \pm SD; (n=3) for each group.

at 4°C with primary antibodies. Immunocomplexes were incubated at room temperature with anti-mouse or anti-rabbit IgG for 1 h (diluted at 1:1,000). The results were visualized using an ECL kit (Amersham Biosciences). All antibodies and reagents were used based on manufacturer's instruction.

Statistical analysis. Data were analysed and presented as the means \pm standard deviation (SD) of at least 3 independent experiments using one-way analysis of variance (one-way ANOVA). All analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered to indicate a significant difference.

Results

Notch1 is silenced by siRNA. The gastric cancer cell lines SGC7901 and MKN74 were transiently transfected with Notch1 siRNA and mock siRNA. We designed candidate Notch1 siRNA and negative control sequences (mock). Real-time PCR and western blot analysis were performed to assess the efficiency of Notch1 siRNA. As illustrated in Fig. 1, Notch1 was expressed in both SGC7901 and MKN74 cell lines, and the candidate sequence inhibited both Notch1 mRNA and protein expression compared to the control and mock treatments. Collectively, the expression of Notch1 protein was markedly decreased in the cells transfected with Notch1 siRNA compared with control (no siRNA) and mock (negative control siRNA) treatments in both cell lines (n=3, $P < 0.05$).

The metastasis and invasion of gastric cancer cell lines were inhibited after downregulation of Notch1 expression. To determine whether the migratory abilities of SGC7901 and MKN74 cell lines were affected by Notch1 depletion, we performed wound-healing assays as presented in Fig. 2. The metastasis of SGC7901 was significantly suppressed after downregulation of Notch1 (Fig. 2A). The relative wound size of the Notch1 siRNA group (0.79 ± 0.06 mm) was larger than the control (0.23 ± 0.14 mm) and mock groups (0.19 ± 0.12 mm) (n=6, $P < 0.05$). A similar result was observed for MKN74 (Fig. 2B). The relative wound size of the Notch1 siRNA group (0.66 ± 0.06 mm) was larger than the

control (0.32 ± 0.04 mm) and mock groups (0.33 ± 0.05 mm) (n=6, $P < 0.05$). These data demonstrated that Notch1 depletion inhibits the migration of SGC7901 and MKN74 cells. The results of Transwell invasion assays were consistent with the wound-healing assay results (Fig. 3). The number of invaded SGC7901 cells transfected with Notch1 siRNA (62 ± 4.1) was significantly reduced compared with the control (148.5 ± 11.4) and mock groups (147.3 ± 8.0) (n=6 $P < 0.05$) (Fig. 3A). The number of invaded MKN74 cells transfected with Notch1 siRNA (51.3 ± 6.0) was also significantly reduced compared with the control (142.3 ± 10.0) and mock groups (152.7 ± 10.4) (n=6 $P < 0.05$) (Fig. 3B). Taken together, our data indicate the role of Notch1 regarding invasion and metastasis in SGC7901 and MKN74 gastric cancer cells.

Inhibition of Notch1 alters expression of PTEN, pPTEN, pAkt and pFAK. As shown in Fig. 4, both mRNA and protein expression of PTEN was upregulated in the Notch1 siRNA group compared with the control and mock groups, whereas phospho-PTEN expression was downregulated after inhibition of Notch1 (n=3, $P < 0.05$) in SGC7901 and MKN74 gastric cancer cell lines. The mRNA and protein expression of total Akt and FAK showed no significant changes. These results demonstrate that PTEN function is activated by the depletion of Notch1. Decreased expression of phospho-Akt and phospho-FAK but not total expression of Akt and FAK was also observed following reactivation of PTEN in both cell lines as shown in Fig. 4 (n=3, $P < 0.05$).

Discussion

Invasion and metastasis are both vital causes of mortality in gastric cancer patients. Therefore, developing new treatments targeting invasion and metastasis are of great importance. Inhibition of cell signalling pathways shows great promise. As an upstream signalling pathway, the importance of Notch activation has been reported in numerous cancers (21,22). Increasing evidence has indicated that Notch1 is aberrantly activated and highly expressed in gastric cancer tissue (22,28). Notch1 plays an important tumor progression role in gastric cancer (22,29). Several studies have found that Notch1 promotes invasion and metastasis in cancer

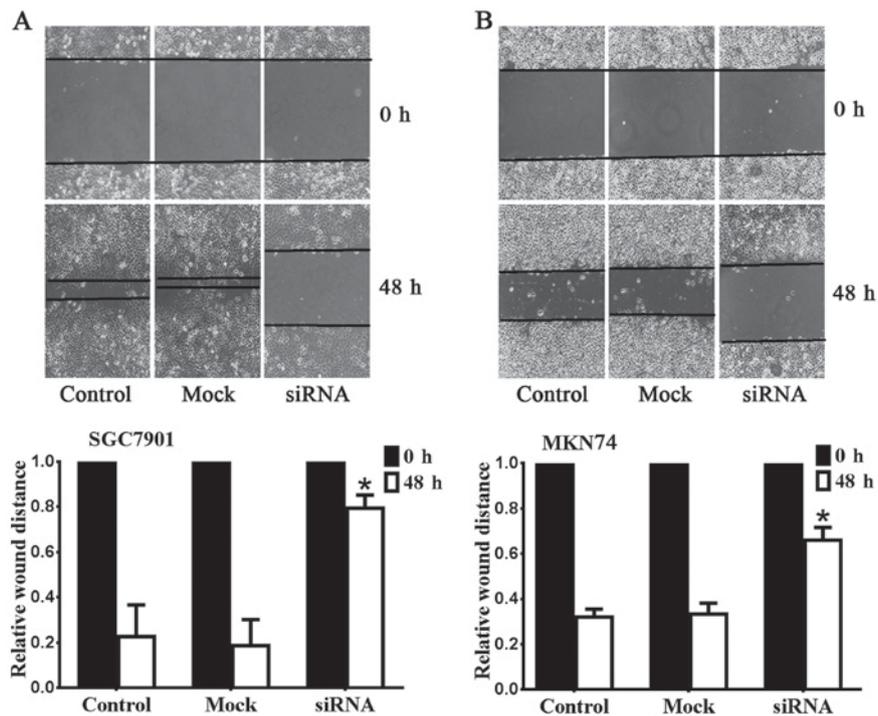


Figure 2. Downregulation of Notch1 mediated by siRNA inhibits cell migration in SGC7901 and MKN74 gastric cancer cell lines. (A) Representative images from the wound-healing assay of SGC7901 cells showed that the metastasis of the siRNA-transfected group was inhibited compared to the control and mock groups ($P < 0.05$ siRNA vs. control; siRNA vs. mock). (B) The results of wound-healing assays of MKN74 cells are consistent with SGC7901 cells. The relative wound size of the siRNA group was significantly larger than the control and mock groups ($P < 0.05$ siRNA vs. control; siRNA vs. mock). All data are expressed as the means \pm SD; (n=6) for each group.

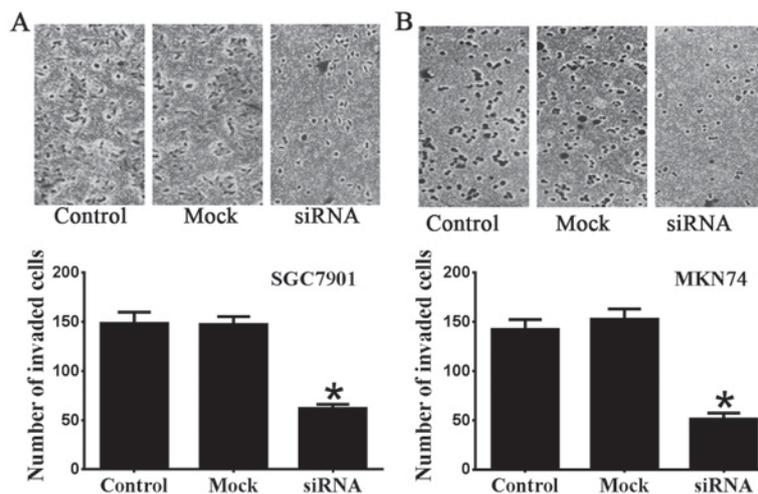


Figure 3. Notch1 depletion by siRNA inhibits gastric cancer cell invasion. (A) Representative pictures showed that SGC7901 cell invasion was decreased in the Notch1 siRNA group compared to the control and mock groups ($P < 0.05$ siRNA vs. control; siRNA vs. mock). (B) Invasion ability of the MKN74 cell line was suppressed in Notch1 siRNA compared to the control and mock groups ($P < 0.05$ siRNA vs. control; siRNA vs. mock). All data are expressed as the means \pm SD; (n=6) for each group.

cells (30-34). To examine whether Notch1 affects invasion and metastasis in gastric cancer cells, we used siRNA to inhibit Notch1 signalling in SGC7901 and MKN74. Notch signalling can be inhibited by prevention of ligand binding using gamma-secretase inhibitors (GSIs) or transcriptional activity inhibition. The methods we used to inhibit Notch1 in this study are widely effective, and we investigated the effect of inhibition using real-time PCR and western blot analysis. We also employed wound-healing and Transwell assays to

assess the effect of cell invasion and metastasis after Notch1 downregulation. The data indicate that siRNA downregulates the expression of Notch1 mRNA and protein following the suppression of cell invasion and metastasis of SGC7901 and MKN74 gastric cancer cells. This result indicates the role of Notch1 in gastric cancer cell lines SGC7901 and MKN74 regarding invasion and metastasis *in vitro* and also suggests that Notch1 could be a potential therapeutic target in gastric cancer treatment.

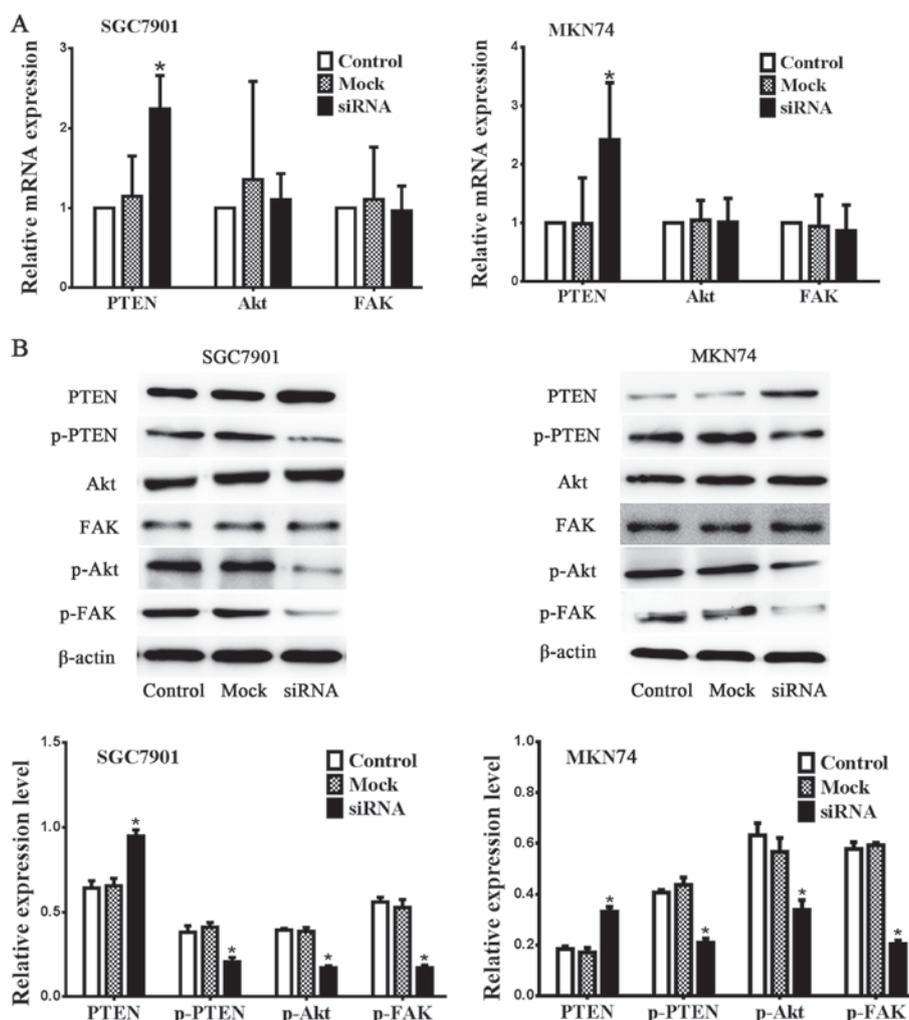


Figure 4. (A) Relative mRNA expression level of PTEN, Akt and FAK in the gastric cancer cell lines SGC7901 and MKN74 after downregulation of Notch1. In both cell lines, the levels of PTEN mRNA expression were increased following Notch1 depletion ($P < 0.05$ siRNA vs. control; siRNA vs. mock). The mRNA expression level of Akt and FAK showed no significant changes. (B) PTEN, pPTEN, Akt, FAK, pAkt and pFAK protein expression levels in the gastric cancer cell lines SGC7901 and MKN74 after downregulation of Notch1. In both cell lines, the levels of PTEN expression were increased following Notch1 depletion, whereas the levels of pPTEN, pAkt and pFAK were decreased ($P < 0.05$ siRNA vs. control; siRNA vs. mock). Protein expression level of Akt and FAK showed no significant changes. All data are expressed as the means \pm SD; (n=3) for each group.

To explore the mechanism by which Notch1 affects invasion and metastasis in SGC7901 and MKN74 cells, we focused on expression of PTEN and phospho-PTEN (Ser380/Thr382/383). PTEN acts as a tumor suppressor by functioning as a dual-specificity protein and phospholipid phosphatase (35). Its function depends on its protein structure, which has five distinct domains: N-term, Phosphatase domain, C2 domain, C-tail and PDZ. N-term contains the PIP binding domain. The phosphatase domain is responsible for its enzymatic and phosphatase activity. The C2 domain is responsible for its cellular location and protein-protein interactions. The C-tail domain is less defined but may be critical for the stability of PTEN and the C-terminal PDZ domain (36). PTEN is involved in numerous biological processes, and its regulation is very complex. One of its important regulations is posttranslational. The C-terminal tail of PTEN can be phosphorylated at Ser380, Thy382 and Thy383. The result of this regulation is inhibition of PTEN's critical phosphatase activity, thus leading to cell growth promotion (37). Phospho-PTEN (Ser380/Thr382/383) protein expression may inhibit PTEN activation.

In this study, increased total PTEN mRNA and protein expression and decreased phospho-PTEN expression was observed following Notch1 depletion. Notch can either be a tumor promoter or tumor suppressor via differential regulation of PTEN protein expression in different situations (38,39). In this situation, Notch1 acts as a tumor promoter that negatively correlates with PTEN expression and positively correlates with phospho-PTEN (Ser380/Thr382/383) protein expression.

We hypothesize that Notch1 negatively regulates PTEN activation not only by suppressing total PTEN expression but also by phosphorylating the PTEN C-terminal tail at Ser380, Thy382 and Thy383, thus causing inhibition of PTEN's phosphatase activity in gastric cancer cell lines. This hypothesis is supported by Kim *et al* research; they determined that Notch signalling disables PTEN by phosphorylation and contributes to tumorigenesis (40). We will investigate relative mechanism of this regulation and focus on testing if PTEN activation is required by inhibition of migration in gastric cancer cells upon depletion of Notch1 in our further study.

Cancer cell invasion and metastasis involves many mechanisms. Activation of Akt and FAK signalling pathways through phosphorylation promotes invasion, metastasis and proliferation (41-43). One of the classic PTEN functions involves dephosphorylating PIP₃, thus antagonizing the (PI3K)/Akt signalling pathway (16). PTEN also downregulates the activity of FAK by dephosphorylation (18). In this study, decreased expression of phosphorylated Akt and FAK was observed after the inhibition of Notch1. We hypothesized that decreased expression of phosphorylated Akt and FAK directly leads to suppression of cell invasion and metastasis and correlates with re-activation of PTEN. However, further investigations are required.

Collectively, our results demonstrate that invasion and metastasis in SGC7901 and MKN74 gastric cancer cells are inhibited *in vitro* after downregulation of the Notch1 signalling pathway by siRNA. Depletion of Notch1 leads to increased PTEN and decreased phospho-PTEN (Ser380/Thr382/383) protein expression in gastric cancer cells. Re-activation of PTEN by inhibition of Notch1 leads to decreased expression of phosphorylated Akt and FAK. The Notch1-PTEN-Akt&FAK signalling axis may serve as a further treatment of gastric cancer targeting invasion and metastasis.

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