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ORIGINAL ARTICLE

Notch2 regulates matrix metallopeptidase 9 *via* PI3K/AKT signaling in human gastric carcinoma cell MKN-45

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Abstract

AIM: To clarify the role of activated Notch2 in the invasiveness of gastric cancer.

METHODS: To investigate the invasiveness of silencing *Notch2* gene expression, we established a Notch2 small interfering RNA (siRNA) transfected cell line using the MKN-45 gastric cancer cell line. After the successful transfection confirmed by real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, migration and invasion assays were employed to evaluate the aggressiveness of the gastric cancer. RT-PCR and Western blottings were employed to confirm the down-regulation of Notch2 and to evaluate the expression of epithelial mesenchymal transition-related gene matrix metallopeptidase 9 (MMP9), Akt, p-Akt. To confirm the relationship between PI3K-Akt and MMP9, the PI3K inhibitor LY294002 was used to treat MKN-45 cells.

RESULTS: Notch2 expression was dramatically decreased after Notch2 siRNA transfection (100.00% ± 9.74% *vs* 11.61% ± 3.85%, $P < 0.01$ by qRT-PCR). There was also a marked reduction of Notch target gene Hes1 (100.00% ± 4.74% *vs* 61.61% ± 3.58%, $P < 0.05$) at the mRNA, indicating an inhibition of Notch signaling. Inhibition of Notch signaling was also confirmed by the marked reduction of Notch2 intracellular domain at the protein levels (100.00% ± 9.74% *vs* 65.61% ± 7.58%, $P < 0.05$). Down-regulation of Notch2 by siRNA enhanced tumor cell invasion (100.00% ± 21.64% *vs* 162.22% ± 16.84%, $P < 0.05$) and expression of MMP9 (1.56 fold, $P < 0.05$), and activated the pro-MMP9 protein to its active form (1.48 fold, $P < 0.05$). There was no significant difference in the protein levels of Akt between the two groups (100.00% ± 10.87% *vs* 96.61% ± 7.33%, $P > 0.05$), while down-regulation of Notch2 elevated p-Akt expression (100.00% ± 9.87% *vs* 154.61% ± 13.10%, $P < 0.05$). Furthermore, p-Akt and MMP9 was down-regulated in response to the inhibitor LY294002 (p-Akt 100.00% ± 8.87% *vs* 58.27% ± 5.01%, $P < 0.05$; MMP9 100.00% ± 9.17% *vs* 50.03% ± 4.88%, $P < 0.05$).

CONCLUSION: Notch2 may negatively regulate cell invasion by inhibiting the PI3K-Akt signaling pathway

in gastric cancer.

Key words: Notch2; Stomach; Cancer; Invasion; Epithelial mesenchymal transition; Matrix metallopeptidase 9; RNA interference

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INTRODUCTION

Gastric carcinoma is one of the most common malignancies and one of the most important causes of the cancer-related death worldwide[1]. Most of the current available therapeutic methods for advanced gastric cancer, such as chemotherapy and chemoembolization, are less optimal, thus the prognosis of gastric cancer is rather poor. This is largely attributed to a lack of complete understanding of the exact cause and mechanisms for this malignancy. Hence, identification of critical molecular pathways of gastric cancer development would greatly facilitate the development of more effective therapies.

Notch signaling pathway is involved in several cellular processes, such as proliferation, differentiation, apoptosis, cell fate decision, and maintenance of stem cells[2-5]. It also plays an important role in the control of tumorigenesis. Activated Notch receptor can be oncogenic or tumor suppressive depending on the tumor type and cellular context[6]. However, the mechanisms by which Notch signaling activates or suppresses tumorigenesis remain unclear. Recently, activation of Notch signaling pathway has been found to stimulate tumorigenesis *via* regulating epithelial mesenchymal transition (EMT)[7] .

EMT is a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to mesenchymal phenotype leading to increased motility and invasion[4]. During the acquisition of EMT characteristics, epithelial cells loose cell-cell junction, which is associated with actin cytoskeleton reorganization, expression of proteins that promote cell-cell contact, and appearance of the expression of mesenchymal markers. Recently, Notch signaling pathway was found to be involved in the acquisition of EMT in both physiological and pathological processes[8]. However, how Notch signaling regulates EMT is largely unknown.

The Notch pathway includes a conserved family of transmembrane receptors (Notch1-4) that interact with a number of specific ligands (DLL1, DLL3, DLL4, Jagged1 and Jagged2) to regulate cell fate. Notch signaling initiates following the binding of the Notch ligands to the Notch receptors, causing an enzymatic cleavage of Notch receptors by γ-*secretase* to release the intracellular domain of the Notch receptor (NICD). NICD is the active form of Notch receptors which can translocate into the nucleus, where it assembles a large transcriptional activation complex that interacts with the conserved transcription factor CSL [CBF-1, Su (H) and Lag-2], and then activates the transcription of CSL-dependent downstream targets[9]. Many target genes of Notch signaling have been identified in various cell contexts, but the Hairy/Enhancer of Split (Hes) family of basic helix-loop-helix (bHLH) proteins are believed to be the direct Notch targets, including Hes1 and Hey1. Among the Notch signaling genes, Notch2 appears to function as a biological antagonist for Notch1 in many cancers, such as breast cancer[10], colorectal cancer[11], malignant mesothelioma[12], multiple myeloma[13], and embryonal brain tumors[14]. Although major advances have been made in the understanding of the opposite effects of Notch2 and Notch1 in cancer development, the exact molecular mechanisms underlying a biological interaction between Notch1 and Notch2 remain unclear, and few studies have been done on the possible relationship between Notch1 and Notch2 in gastric cancer.

Notch2 signal pathway plays a potential oncogenic role in several malignancies, such as hematologic malignancies including multiple myeloma[15], B cell chronic lymphocytic leukemia, and B cell and T cell acute lymphoblastic leukemia[16,17], and solid tumors including glioblastoma[18], and colon cancer[19]. It also plays a tumor suppressive role in some solid tumors, such as breast cancer[20], and small cell lung cancer[21,22]. In gastric cancer, Notch2 has been proved to be overexpressed by Sun *et al*[23] and He[24].

In this study, we aimed to address whether Notch2 is also involved in control of gastric cancer progression and investigate the effects of Notch2 signaling on gastric cancer aggressiveness.

MATERIALS AND METHODS

Cell culture and transfections

Human gastric cancer cell line MKN-45 (Cell Collection of the Chinese Academy of Sciences, Shanghai, China) was cultured in RPMI 1640 (HyClone Laboratories Inc., Logan, United States) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, United States), 100 U/mL penicillin and streptomycin, in a 5% CO_2 atmosphere at 37 ℃.

Small interfering RNA knockdown of Notch2

MKN-45 cells were transfected with small interfering RNA (siRNA) against Notch2 and scrambled siRNA (Santa Cruz Biotechnology, CA, United States) constructs using the commercial transfection reagent (Santa Cruz Biotechnology, CA, United States) according to the manufacturer's instructions. Following transfection, cells were incubated at 37 ℃ in a CO_2 incubator for 48 h before being harvested for the assays described below.

Real-time quantitative reverse transcription-polymerase chain reaction analysis for gene expression

Total RNA was isolated by the RNAiso plus reagent (TaKaRa Biotechnology Co., Dalian, China) and then reverse transcribed into complimentary DNA (cDNA) using the Primescript™ reverse transcription Master Mix (TaKaRa Biotechnology Co., Dalian, China) according to manufacturer's instructions. Reverse transcription reaction was performed at 37 ℃ for 15 min followed by 85 ℃ for 5 s. The primers used in the polymerase chain reaction (PCR) reactions are described in Table 1. One mL of reverse transcription reaction product was used for quantitative reverse transcription-PCR (qPCR) reaction in a total volume of 20 μL. The qPCR cycles were as follows: 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 34 s. PCR amplifications were undertaken with the Applied Biosystems 7500/7500 Fast Real-Time PCR Software (Applied Biosystems, CA, Unite States) using the SYBR® Premix Ex Taq™ Ⅱ (TaKaRa Biotechnology Co., Dalian, China). Data were analyzed according to the comparative Ct method and were normalized to glyceraldehyde-3-phosphate dehydrogenase expression in each sample. All qPCR assays were performed in triplicate.

Protein extraction and Western blotting analysis

Total protein was extracted from the treated cells using RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride. The protein concentration was measured by BCA protein assay system (Beyotime Biotechnology, Haimen, China). Total proteins (40-50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidenefluoride (PVDF) membranes. The blots were blocked with 5% skim milk in Tris buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature, incubated with primary antibodies against N2ICD (Abcam Inc., Cambridge, United Kingdom) (1:1000), Hes1 (Abcam Inc., Cambridge, United Kingdom) (1:500), matrix metallopeptidase 9 (MMP9) (Abcam Inc., Cambridge, United Kingdom) (1:1000), Akt (Cell Signaling Technology, Inc., Danvers, MA, United States) (1:1000), and p-Akt (Cell Signaling Technology, Inc., Danvers, MA, United States) (1:1000). β-actin (Zhongshan Golden Bridge Biotech, Beijing, China) (1:10 000) was used as a loading control. The membranes were reacted with respective primary antibodies overnight at 4 ℃. After being washed in TBST for three times, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Zhongshan Golden Bridge Biotech, Beijing, China) (1:10 000) for 1 h at room temperature. The protein bands were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, United States) and imaged using a VersaDoc Imaging System (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States). Densitometric analysis was performed using Quantity One Software v4.62 (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States) and the results were presented as the mean of three independent experiments.

Table 1 Primer sequences used for the real-time polymerase chain reaction analysis

Primer	Sense (5′-3′)	Anti-sense (5′-3′)
Notch2	CCTGGGCTATACT-GGGAGCTACTG	ACACCCTGATAGCCTGGGA-CAC
MMP9	ACGCACGACGTCTTC-CAGTA	CCACCTGGTTCAACTCACTCC
Hes1	AGCGGGCGCAGATGAC	CGTTCATGCACTCGCTGAA
GAPDH	GCACCGTCAAGGCT-GAGAAC	TGGTGAAGACGCCAGTGGA

MMP9: Matrix metallopeptidase 9; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Migration and invasion assays in vitro

The effects of Notch2 siRNA on the migratory and invasive abilities of MKN-45 cells were assayed in 24-well plates using relevant kits (BD Biosciences, United States). Approximately 3×10^4 cells were seeded for the 12 h migration assay, and 3×10^5 cells for the 24 h invasion assay and the invasive activity of the Notch-2 siRNA-transfected MKN-45 cells was tested using BD Falcon™ Cell culture inserts coated with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, United States). Briefly, transfected MKN-45 cells were resuspended in serum-free medium and seeded into the upper chamber of the assay system. The bottom wells of the system were filled with complete growth medium. After 12 and 24 h incubation, the migrated and invaded cells were washed twice with ice-cold PBS and then fixed with 4% paraformaldehyde for 15 min and stained with methyl violet (0.01% v/v) for 30 min. The numbers of migrated or invaded cells were then counted from 5 random fields under 200 and 400 magnification.

MMP9 activity assay

The culture media from Notch2 siRNA- and scrambled siRNA-transfected MKN-45 cells grown in 6-well plates were collected, spun at 12 000 × *g* for 10 min at 4 ℃ to remove cell debris, and the supernatant collected for MMP9 assay using a commercial enzyme-linked immunosorbent assay kits (RD Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

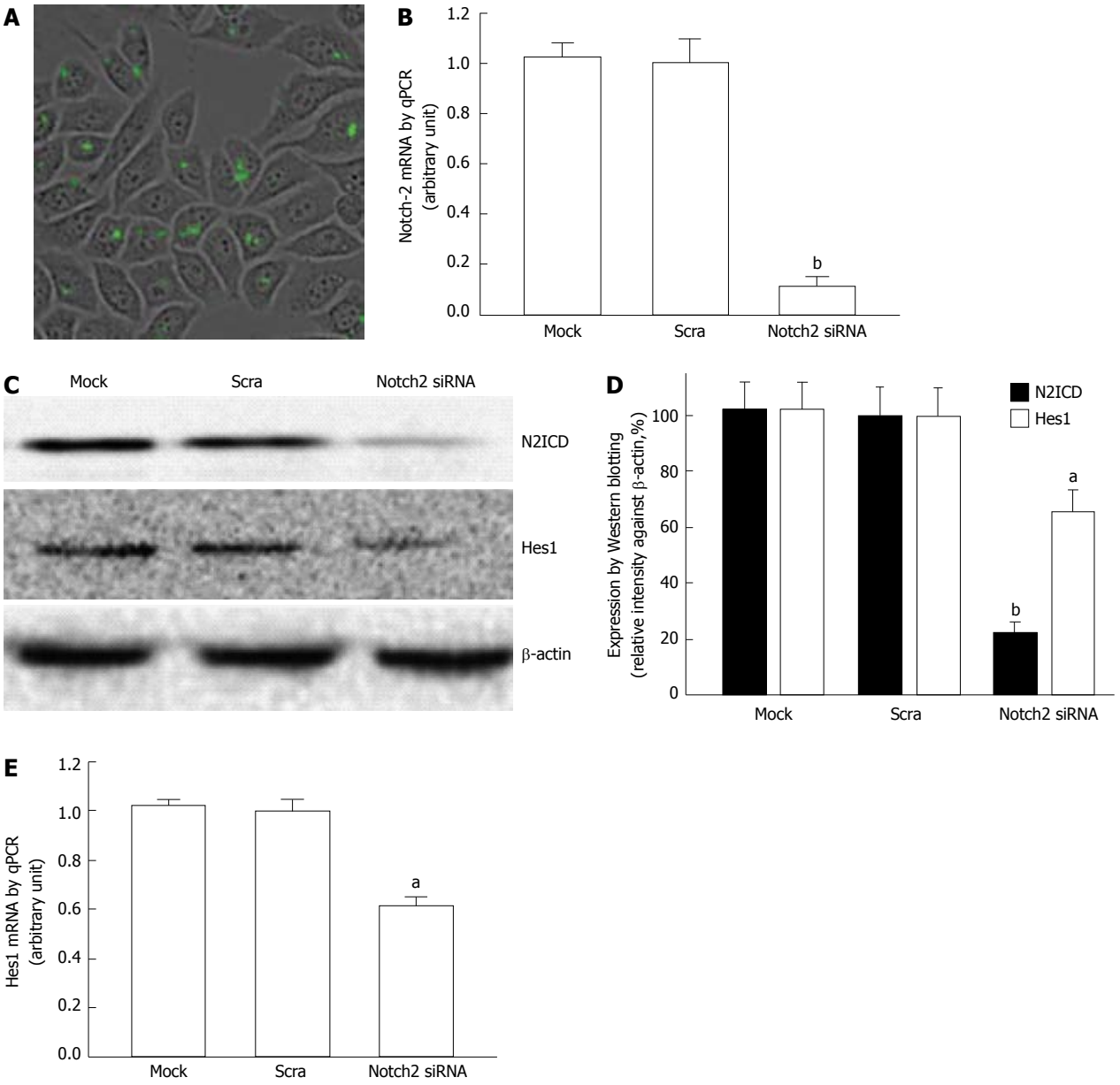

Figure 1 Verification of successful transfection and knockdown of Notch2. [a]*P* < 0.05, [b]*P* < 0.01 *vs* the Mock or Scra groups. Scra: Scrambled small interfering RNA (siRNA); qPCR: Quantitative reverse transcription polymerase chain reaction.

Statistical analysis

Data analysis was performed using SPSS19. 0 (IBM, Armonk, New York, United States) and displayed using Sigma Plot10. 0 (Systat Software Inc, San Jose, CA, United States). Comparison of the differences between the groups was performed using a one-way analysis of variance followed by the Bonferroni correction. All data were expressed as the mean ± SD. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Knockdown of Notch2 enhanced the migration and invasion of MKN-45 cells

After successful transfection of Notch2 siRNA into MKN-45 cells (Figure 1A) and a marked knockdown of Notch2 (> 90%) at mRNA (Figure 1B) and protein (Figure 1C and D) levels, there was a marked reduction of Notch target gene Hes1 at the mRNA (Figure 1E), indicating an inhibition of Notch signaling. Inhibition of Notch signaling was also confirmed by the marked reduction of Notch2 intracellular domain (N2ICD) at the mRNA (Figure 1D) and protein (Figure 1C) levels.

In the cells with confirmed knockdown of Notch2, the ability of cells to migrate and invade was evaluated as described in the "Materials and Methods". MKN-45 cells with Notch2 knockdown showed an increased cell migration (Figure 2B and E) compared with the cells transfected with scrambled siRNA (Scra, Figure 2A and E). Similarly, MKN-45 cells with Notch2 knockdown showed an increased cell invasion (Figure 2D and F) compared with the cells transfected with scrambled siRNA (Scra,

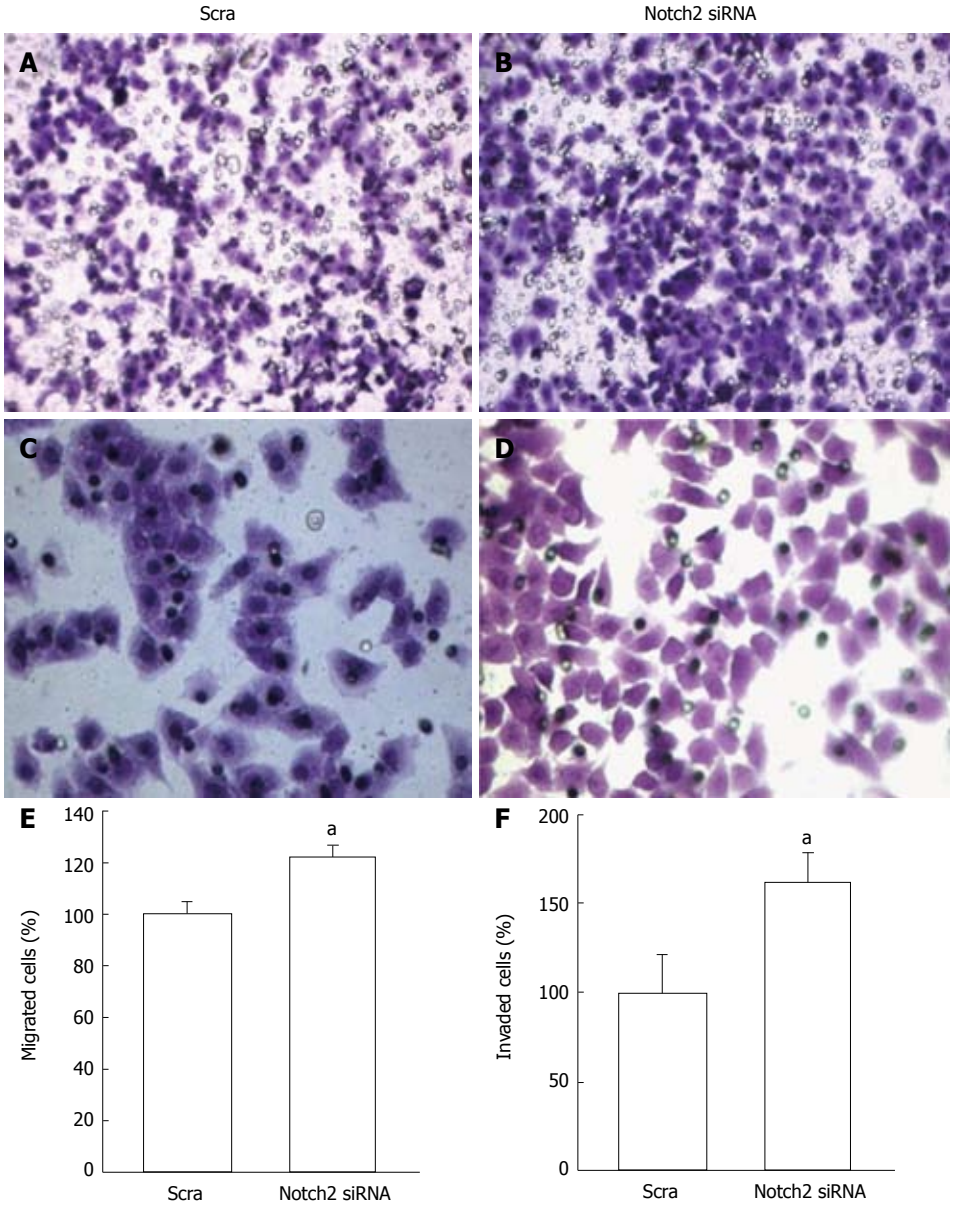

Figure 2 Knockdown of Notch2 led to an increased migration and invasion of MKN-45 cells. Cells were transfected with scrambled small interfering RNA (siRNA) (Scra, A, C) or Notch2 siRNA (B, D) for 48 h, and the effect of the migration (A, B, E) and invasion (C, D, F) were assayed as described in "Materials and Methods". The number of migrated cells or invaded cells were quantitated (E, F). $^{a}P < 0.05$ *vs* the Scra groups. A, B, ×200; C, D, ×400.

Figure 2C and F).

Knockdown of Notch2 enhanced the expression and activity of MMP9 in MKN-45 cells

Tumor metastasis occurs by a series of steps, including cell invasion, degradation of basement membranes, and the stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis. The MMPs are a family of related enzymes that degrade extracellular matrix, which are considered to be important factors in facilitating tumor invasion and metastasis. Among these MMPs, MMP9 is considered an important factor involved in the degradation of basement membrane collagen in facilitating invasion and metastases in gastric cancer. Knockdown of Notch2 in MKN-45 cells markedly enhanced the expression of MMP9 at mRNA (Figure 3A) and protein (Figure 3B and C) levels. Additionally, knockdown of Notch2 led to a 1.48-fold increase in MMP9 activity (Figure 3D).

Effect of Notch2 knockdown on the expression of PI3K/Akt pathway in MKN-45 cells

In order to elucidate the mechanisms of Notch2 mediated alteration in MMP9, we measured the expression of PI3K downstream target Akt in the MKN-45 cells transfected with or without Notch2 siRNA. Knockdown of Notch2 by siRNA increased the Akt phosphorylation (Figure 4A and B). Blocking the PI3K/Akt pathway by PI3K inhibitor LY294002 resulted in a reduced expression of MMP9 (Figure 4C and D).

DISCUSSION

Aberrant expression of Notch pathway has been found in a variety of human cancers, including cancers of breast, brain, cervix, lung, colon, head and neck, kidney, bone marrow, lymph nodes and stomach[25-27]. Abnormal Notch signaling is also linked to EMT. Notch signaling is known to suppress apoptosis and promote cell

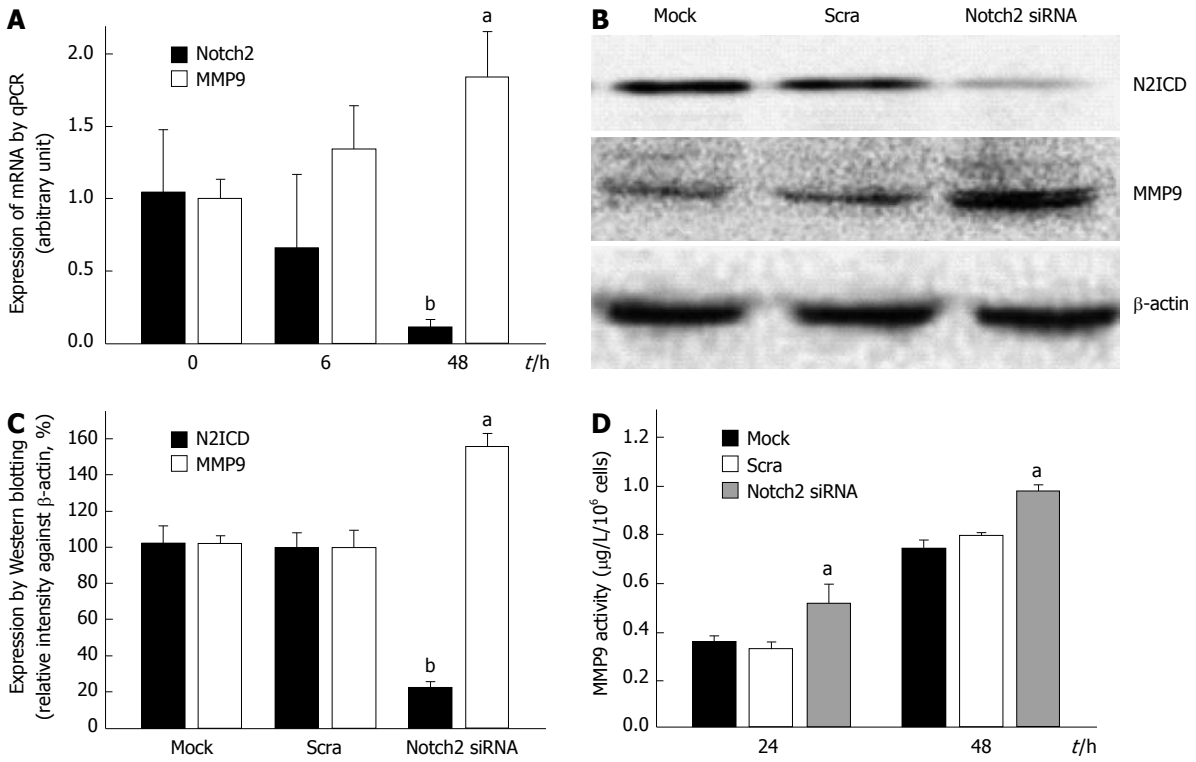

Figure 3 Knockdown of Notch2 enhanced the expression and activity of matrix metallopeptidase 9. The small interfering RNA (siRNA) mediated knockdown of Notch2 (A) and Notch2 intracellular domain (N2ICD) (B, C) was associated with a marked increase in the expression (A, B, C) and activity (D) of matrix metallopeptidase 9 (MMP9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the blank control groups. qPCR: Quantitative reverse transcription polymerase chain reaction.

proliferation through a growth factor-mediated survival pathway[28-30]. However, the precise role and mechanism of Notch for tumor invasion remains unclear. In this study, we found that siRNA mediated down-regulation of Notch2 in gastric cancer cells could (1) increase tumor cell invasion; (2) enhance MMP9 expression and its activities; and (3) promote the phosphorylation of PI3K pathway as demonstrated by increased p-Akt level.

Tumor metastasis occurs *via* a series of steps, including cell invasion, degradation of basement membranes and the stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis. The MMPs are a family of related enzymes that degrade extracellular matrix, which are considered to be important factors in facilitating tumor invasion and metastasis[31-33]. Among these MMPs, MMP9 has been considered an important factor involved in the degradation of basement membrane collagen in facilitating invasion and metastases in gastric cancer[34,35]. MMP9 is a downstream target for PI3K/Akt pathway, which is an important signaling pathway in controlling cell proliferation[36,37]. In physiological circumstances, MMP9 plays an important role in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis[38]. MMP9 is required for maintaining normal tissue structure and epithelial integrity. Under pathological conditions, particularly in various cancers, abnormal expression and activity of MMP9 have been reported[34,39-42]. Abnormal function of MMP9 is linked to tumor cell migration, invasion and metastasis[35,39]. The role of MMP9 in the development of gastric cancer has been reported and the expression of metalloproteinase-9 or its inhibitor is related to a more aggressive phenotype of gastric cancer or correlated with lymph node metastasis in advanced gastric carcinoma[35,43], but how MMP9 is regulated in gastric cancer is unclear.

Based on our study, we propose that physiological cellular level of Notch2 may be required for the maintenance of normal MMP9 function. Reduced Notch2 may enhance the proliferative and invasive potential of cancer cells, likely through activation of PI3K/Akt pathway and ensuing increase in MMP9 activities. In this perspective, Notch2 appears to function as a tumor suppressor gene in gastric cancer.

Here, we showed that the facilitation of MMP9 expression by down-regulation of Notch2 may be mediated by the up-regulation of p-Akt. Thus, these results suggest that up-regulation of Notch2 could potentiate the anti-tumor and anti-metastasis activities partly through the up-regulation of MMP expression. Because we observed that down-regulation of Notch2 promoted MMP9 expression, we tested the effects of Notch2 on the invasion of MKN-45 cells. We found that down-regulation of Notch2 promoted the invasion of MKN-45 cells. These results were consistent with MMP9 data, showing that down-regulation of Notch2 could promote cancer cell invasion partly through up-regulation of MMP9. On the basis of our results, we propose a hypothetical pathway by which Notch2 may inhibit invasion of MKN-45 cells, partly through PI3K-Akt signaling pathway.

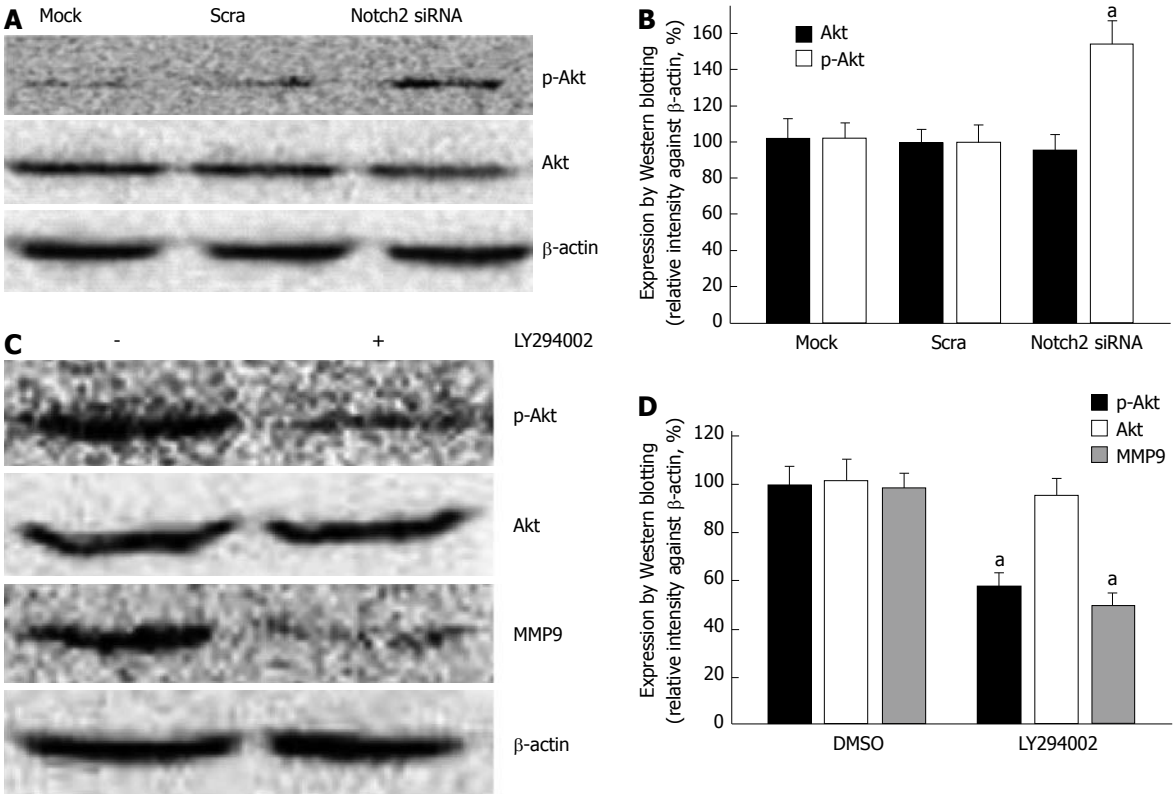

Figure 4 Knockdown of Notch2 enhanced the expression of matrix metallopeptidase 9 *via* increased phosphorylation of p-Akt in MKN-45 cells. A, B: Knockdown of Notch2 led to an increased phosphorylation of Akt (p-Akt); C, D: Blockade of PI3K/Akt pathway by LY294002 (20 μmol/L) abolished the effect of Notch small interfering RNA (siRNA) in Akt phosphorylation and matrix metallopeptidase 9 (MMP9). $^{a}P < 0.05$ *vs* the all control groups. DMSO: Dimethyl sulfoxide.

Notch has been reported to cross-talk with other major cell growth and apoptotic regulatory pathways, including the PI3K-Akt pathway[44]. Hyperactivation of PI3K/Akt pathway has previously been observed in human gastric cancer[45]. It has recently been shown that activation of Notch1 enhanced the survival of melanoma cells[29] and leukemia cells *via* activation of the PI3/Akt pathway[46]. In our study, down-regulation of Notch2 by siRNA led to the activation of PI3K/Akt pathway, which is associated with an increased expression and function of MMP9, suggesting that Notch2 can regulate MMP9 *via* PI3K/Akt pathway and increased Akt phosphorylation. Interestingly, we also observed that inactivation of Akt by LY294002 eliminated Akt phosphorylation and MMP9 expression. These results suggest that Notch2 can induce Akt signaling.

In summary, the role of Notch2 in malignancies is uncertain. Although the overexpression of Notch2 has been confirmed, Notch2 appeared to function as a tumor suppressor gene in gastric cancer in this study. Further studies are warranted before Notch inhibitor based therapeutic approaches are employed in the treatment of advanced gastric cancer.

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COMMENTS

Background

Notch is one of the most important signaling pathways involved in cell fate determination. Activation of the Notch pathway requires the binding of a membrane-bound ligand to the Notch receptor in the adjacent cell which induces proteolytic cleavages and the activation of the receptor. A unique feature of the Notch signaling is that the processes such as modification, endocytosis or recycling of the ligand, have been reported to play critical roles in Notch signaling, however, the underlying molecular mechanism appears context-dependent and often controversial.

Research frontiers

There are four Notch receptors (Notch 1-4) and five ligands [Jagged 1, Jagged 2, delta-like ligand-1, -3 and -4 (DLL1, DLL3 and DLL4)] in mammals. Recently, it is reported to be involved in tumorigenesis as oncogenes or tumor suppressors, and proposed as prognostic factors or anti-cancer targets in aggressive or advanced cancers. This study was undertaken to investigate whether Notch2 is also involved in the control of gastric cancer progression, and the effects of Notch2 signaling in gastric cancer aggressiveness.

Innovations and breakthroughs

Abnormal Notch signaling has been reported in many human solid tumors. This is the first study to characterize the role of Notch signaling in gastric cancer aggressiveness. The findings indicated that Notch2 may negatively regulate the cell invasion of human gastric carcinoma.

Terminology

Four Notch receptors (Notch 1-4) and five ligands (Jagged 1, Jagged 2, DLL1, DLL3, and DLL4) are found in mammals. Ligand-receptor interaction between two neighboring cells is involved in developmental, physiologic and pathologic processes.

Peer review

The Notch signaling pathway plays a crucial role in the maintenance and the development of several tissues. Ectopic expression of Notch has been found in a variety of human cancers. In this work, the authors indicate that Notch2 could negatively regulate the cell invasion of human gastric carcinoma. By this way, the authors described Notch2 as a tumor suppressor gene in gastric cancers. At the same time, the authors detected an increased expression and activity of matrix metallopeptidase 9, arguing that such increase could be related to the enhanced migration and invasiveness. The results are clear and support the authors' hypothesis.

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