Cervical Cancer Cell Growth, Drug Resistance, and Epithelial-Mesenchymal Transition Are Suppressed by γ-Secretase Inhibitor RO4929097

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Background: The Notch signaling pathway has been reported to play a pivotal role in tumorigenesis. Emerging evidence has demonstrated that the Notch signaling pathway regulates several cellular processes. The present study investigated the effect of the Notch signaling pathway on cell growth, invasiveness, and drug resistance, as well as epithelial-mesenchymal transition (EMT), of cervical cancer cells.

Material/Methods: We used quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis to measure the expression level of Notch2. CCK-8, clonality, wound healing, and Transwell assays were used to evaluate the effect of γ-secretase inhibitor (GSI) RO4929097 on cervical cancer cell lines HeLa and Caski. To explore the role of the Notch signaling pathway in EMT, the epithelial and mesenchymal markers were detected by qRT-PCR and Western blot after cervical cancer cell lines were treated with GSI RO4929097.

Results: The expression of Notch2 was found to increase in cervical cancer cell lines compared with the normal immortalized human cervical epithelial cells. GSI RO4929097 was confirmed to inhibit the Notch signaling pathway and impaired the proliferation, drug resistance, migration, and invasion abilities of cervical cancer cells. The protein expression levels of the mesenchymal biomarkers Snail, Twist, and neural cadherin (N-cadherin) decreased; however, the expression of the epithelial biomarker epithelial cadherin (E-cadherin) increased in the cervical cancer cells treated with GSI RO4929097.

Conclusions: Notch signaling pathway plays an important role in the development and progression of cervical cancer. Blockade of the Notch pathway using GSI RO4929097 inhibited cell growth and reduced chemoresistance, invasion, metastasis, and EMT in cervical cancer cells.

MeSH Keywords: Epithelial-Mesenchymal Transition • Receptors, Notch • Uterine Cervical Neoplasms

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Background

Cervical cancer is one of the common malignant tumors in women worldwide [1]. Despite advances in early screening and prevention, the prognosis of cervical cancer is particularly poor and the 1-year survival of advanced/recurrent patients is only 10–20% [2]. The occurrence of drug resistance, invasion, and metastasis, which results in treatment failure and is the main cause of most cervical cancer-related deaths. Despite a great number of clinical trials with targeted and conventional therapies, current treatments can only provide limited benefits. Therefore, strategies are required to overcome this life-threatening disease.

Although the precise mechanism by which cervical cancer cells acquire chemoresistance, invasion, and metastasis abilities is unclear, several studies have suggested that epithelial-mesenchymal transition (EMT), metastasis, recurrence, and drug resistance are closely associated with tumor progression [3,4]. Research found that multidimensional growth factors secreted by the cancer cells and host cells in local microenvironments may indicate the chemoresistance, metastases, and recurrence of cervical cancer, which are associated with the EMT process [5–8]. During this process, epithelial cadherin (E-cadherin), a kind of epithelial-specific junction protein, is downregulated and mesenchymal proteins, including neural cadherin (N-cadherin), Snail, and Twist, are upregulated [9]. Consequently, epithelial phenotype cells transform into individual, non-polarized, motile, and invasive mesenchymal phenotype cells, which show increased potential of metastasis and invasion [9,10]. Recent studies also shown that EMT can cause overexpression of ATP-binding cassette transporters, thereby promoting drug resistance in tumor cells [11–13].

EMT is a dynamic biological process triggered by the interplay of extracellular signals and occurring under specific conditions. Previous studies have suggested the Notch signaling pathway has a critical role during the EMT process in cancer cells [14–16].

The Notch signaling pathway is known to regulate a series of cellular processes, including cell proliferation, apoptosis, cell cycle, chemoresistance, migration, invasion, and stem cell maintenance [17,18]. Moreover, several studies have reported that Notch expression was upregulated in many human malignancies [17,19–21]. However, the function of Notch in the EMT biological processes of cervical cancer remains poorly understood. Therefore, the present study investigated the function of the Notch signaling pathway in the EMT in cervical cancer cells.

Material and Methods

Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM), antibiotics (penicillin and streptomycin), and fetal bovine serum (FBS) were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primary antibodies were obtained from Abcam (Cambridge, MA, USA), which included anti-Notch2, anti-Hey1, anti-Snail, anti-N-cadherin, anti-Twist, anti-E-cadherin, and anti-GAPDH antibodies. RO4929097 and DMSO (dimethyl sulfoxide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo (Kumamoto, Japan) and all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell culture

HPV-16-immortalized cervical epithelial cell line CRL2614 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cervical cancer cell lines HeLa and Caski were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The cells were cultured in DMEM containing 10% FBS and 1% antibiotics (penicillin and streptomycin). Cells were propagated at 37°C with 5% CO₂ and 100% humidity. Culture medium was replaced every 3 days.

CCK-8 assay

Cell viability was measured with the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer’s recommendations. In brief, cells in each well containing 100 μl medium were incubated with 10 μl CCK-8 solution at 37°C for 1.5 h. The optical density (OD) of each well was then measured at 450 nm using a microplate reader.

 Colony-forming assay

Cervical cancer cell lines were plated in 6-well plates at a density of 400 cells per well and treated with RO4929097 (5 μmol/L) and DMSO as a control for 48 h. The culture medium was replaced every 3 days. After another 14 days, the colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet staining solution (Servicebio, Wuhan). The visible colonies (≥50 cells) were counted and the typical images were photographed by a common NIKON camera.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated and cDNA was synthesized according to the manufacturer’s protocol and as we described previously [22]. Amplifications were performed in an ABI 7500 Detection
System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Target sequences were amplified at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All assays were performed in triplicate. Gene expression levels were calculated using the 2-ΔΔCt method and normalized to GAPDH. The gene-specific primers are listed in Table 1.

### Western blot analysis

Proteins were extracted with protein lysis buffer. Lysates were centrifuged at 12 000 g at 4°C for 5 min, and supernatants collected. Cell lysates containing 40 μg protein were separated by 10% SDS–PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes using a transfer apparatus according to the manufacturer’s protocol (Bio-Rad). The membranes were blocked with 5% (w/v) nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 2 h at room temperature and incubated at 4°C overnight with the indicated antibodies. The membranes were washed twice with PBS and incubated with a 1: 2000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 2 h at room temperature. Blots were washed with TBST 3 times and developed with the ECL system (Amersham Biosciences) according to the manufacturer’s protocol.

### Wound healing assay

Briefly, when adherent cells reached about 90–95% confluency, a scratch was made using a 1-mL sterile pipette tip. The cells were washed 3 times with PBS and further incubated in DMEM without FBS for 24 h in specified conditions. The migration distance was photographed and measured at zero time and 36 h.

### Transwell invasion assay

Cells (1×10⁴ cells/well) were seeded onto the upper chamber of a Matrigel (Sigma-Aldrich, USA)-coated polycarbonate membrane insert (6.5 mm in diameter with 8.0-μm pores) in a Transwell apparatus (Corning Incorporated, Corning, NY, USA) and maintained in DMEM medium without FBS. The lower chambers were filled with DMEM medium with 10% FBS. After culturing for 24 h at 37°C, cells on the upper surface of the insert were removed and cells that migrated to the bottom surface of the insert were fixed. The filters were stained with 0.1% crystal violet for 10 min and peeled off after washing twice with PBS. Cells were counted based on 5-field digital images taken randomly at ×200 magnification.

### Statistical analysis

Each experiment was performed at least 3 times and the results were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as means ± standard deviation (SD). Two-tailed t tests (paired and unpaired) were performed for statistical analysis. P values less than 0.05 were considered statistically significant.

### Results

#### Notch2 is upregulated in cervical cancer cell lines

Initially, we investigated Notch2 expression in human cervical cell lines using qRT-PCR and Western blot. The HPV-16-immortalized human cervical epithelial cells (CRL2614) and 2 cervical cancer cell lines (HeLa and Caski) were used. As shown in Figure 1, increasing Notch2 was found in cervical cancer cells when compared with the normal cervical epithelial cells (CRL2614). The HeLa cells were derived from non-metastatic tissue and the Caski cells were derived from metastatic tissue. It is noteworthy that there was gradual Notch2 upregulation in the normal cervical epithelial cell and in cervical cancer cell lines without and with metastatic properties. As shown in Figure 1, Notch2 mRNA and protein expression was highest in metastatic cervical cancer cell line Caski cells and was intermediate in non-metastatic cervical cancer cell line HeLa cells and was lowest in normal cervical epithelial cell line CRL2614 cells. These findings suggest that Notch2 is upregulated in cervical cancer cell lines and may play a cardinal role in cervical cancer genesis and metastasis.

#### Blocking the Notch signaling pathway inhibits cell proliferation, migration and invasion

In order to explore the role of Notch signaling pathway in the progression of cervical cancer, HeLa and Caski cells were treated with RO4929097, a γ-secretase inhibitor (GSI). As shown in

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**Table 1. Primer sequences used for real-time PCR analysis.**

| Genes      | Primer sequence                  |
|------------|----------------------------------|
| Notch2     | Forward: 5'-CCAGGAGAGGTGTCTGTG-3' |
|            | Reverse: 5'-AATGCCTTGATGGAATAAG-3'|
| Snail      | Forward: 5'-TTACCTTCCAGAGGCTAGCA-3'|
|            | Reverse: 5'-GAGGCTTTCCACTGTCTCTAT-3'|
| N-cadherin | Forward: 5'-CGAATGGGATGAAACCCACCT-3'|
|            | Reverse: 5'-TAGCGCTTCAAGGCAGAAGTTC-3'|
| Twist      | Forward: 5'-ATGGACCGAACATGCCAG-3' |
|            | Reverse: 5'-TGGCTGAGCAATGAG-3' |
| E-cadherin | Forward: 5'-AAGGCACAGCTTGCAAGAACA-3'|
|            | Reverse: 5'-ACGTGTTCGCTGGTGTCTACCT-3'|
| GAPDH      | Forward: 5'-ACCTTTGATCTGTTGAAGGACTCAT-3'|
|            | Reverse: 5'-GTTTTTCTAGACGCGAGTG-3' |
Figure 1. Notch2 expression was upregulated in cervical cell lines. (A) Notch2 mRNA expression was increased in cervical cell lines (HeLa and Caski) compared with the normal human cervical epithelial cell line CRL2614. GAPDH was used as the internal control. (B, C) Western blot analysis of Notch2 protein expression normalized to GAPDH. Data are presented as means ±SD. * P<0.05, ** P<0.01, *** P<0.001.

Figure 2. Blocking the Notch signaling pathway inhibits cell proliferation, migration, and invasion. (A, B) HeLa and Caski cells treated with RO4929097 and the DMSO control showed downregulation of the Hey1 protein as determined by Western blot analysis; (C–E) RO4929097 markedly inhibited the proliferation and colony-forming abilities; (F, G) reduced the migration and invasion capacities of HeLa and Caski cells compared with the controls. Data are presented as means ±SD. * P<0.05, ** P<0.01, *** P<0.001.
Figure 2A and 2B, treatment with 5 μmol/L RO4929097 for 24 h significantly suppressed the protein expression level of Hey1, which is an important target gene of the Notch pathway. This result proves the effectiveness of GSI RO4929097. Then, the CCK-8 and colony-forming assay were performed, and both of verified that the proliferation potential in the cells treated with 5 μmol/L RO4929097 were markedly decreased when compared with the control (Figure 2C–2E). Subsequently, the effect of GSI RO4929097 on the migration and invasiveness of cervical cancer cells was also evaluated. The wound-healing and Transwell invasion assays showed that 5 μmol/L RO4929097 significantly reduces the migration and invasion capacities of HeLa and Caski cells, respectively (Figure 2F, 2G). These outcomes confirmed that GSI RO4929097 blocks the Notch pathway and suppresses the proliferation, migration, and invasion of cervical cancer cell lines.

**Inhibition of the Notch signaling pathway reduces the drug resistance of cervical cancer cells**

Following treatment with the RO4929097 (1 μM) non-toxic dose for 48 h, the HeLa and Caski cervical cancer cells were treated with different doses of cisplatin, a chemotherapeutic drug. Then, the CCK-8 assay was performed to detect cell viability. We found that RO4929097-treated cells exhibited an increased sensitivity to cisplatin-induced cytotoxicity compared with that of the DMSO control cells (Figure 3). These data indicate that suppressing the Notch signaling pathway increases sensitivity of cervical cancer cells to chemotherapeutic drugs.

**Inhibition of the Notch signaling pathway prevents EMT in cervical cancer cell lines**

To further investigate the potential molecular mechanism of the GSI RO4929097 on EMT in cervical cancer cell lines, epithelial- and mesenchymal-related markers in the presence of 5 μmol/L RO4929097 for 24 h or DMSO control were detected by qRT-PCR and Western blot, respectively. The mRNA expression levels of the epithelial biomarker E-cadherin was increased when treated with GSI compared with the control group. In contrast, mesenchymal markers, including N-cadherin, Snail, and vimentin, were decreased compared with the control group (Figure 4A). Western blot analysis demonstrated similar results to that of qRT-PCR (Figure 4B, 4C). This observed up-regulation of epithelial markers and downregulation of mesenchymal markers suggests that inhibition of the Notch pathway impairs the EMT in cervical cancer cells.

**Discussion**

Accumulating evidence shows that the Notch signaling pathway is overactive in lung, head and neck, pancreatic, bladder, and prostate cancer [18,19,23–26]. Furthermore, the high expression levels of Notch receptors and ligands have been associated with a poor prognosis in many malignant tumors [17,19,27]. In prostate cancer cells, activation of the Notch signaling pathway has been found to contribute to invasion and metastasis by EMT [23]. In bladder cancer, Notch3 was more highly expressed in human urothelial cancer tissues than in non-tumorous bladder tissue samples, with Notch3 overexpression being associated with poor clinical outcome. Notch3 overexpression promotes growth and chemoresistance in urothelial cancer [28]. The expression level of Notch signaling is correlated with malignant behavior and prognosis in cervical cancer, and Yousif et al. [29] demonstrated that the Notch signaling pathway play an important role in invasiveness and metastasis of cervical cancer, with patients who have high Notch1/JAG1 expression tending to have worse overall survival in comparison to patients with tumor of low Notch1/JAG1 expression. In this work, the expression of Notch2 increased in the cervical cancer HeLa and Caski cell lines compared with the normal cervical epithelial cells CRL2614 cell line. In addition, it is noteworthy that...
the expression level of Notch2 mRNA and protein in the metastatic cervical cancer cell line Caski was higher than that of the non-metastatic carcinoma cell line HeLa. These results demonstrate that the Notch signaling pathway correlates with the invasion and metastasis of cervical cancer, consistent with the results of some other studies [30]. Similarly, in other types of tumors, the activity of the Notch signaling pathway is positively correlated with malignant behavior of tumors (such as metastasis). For example, Zou et al. [31] found that Notch2 is highly expressed in laryngeal squamous cell carcinoma tissues compared with the levels in vocal cord polyp samples. Additionally, the expression of Notch2 was increased in tissue samples from laryngeal squamous cell carcinoma with lymph node metastasis compared with those without metastasis, which indicates

**Figure 4.** Inhibition of the Notch signaling pathway prevents EMT in cervical cancer cell lines. (A) Effect of inhibiting the Notch signaling pathway on mRNA levels of EMT-associated biomarkers Snail, N-cadherin, Twist, and E-cadherin in HeLa and Caski cells. (B, C) Effect of inhibiting the Notch signaling pathway on the protein expression levels of EMT-associated proteins Snail, N-cadherin, Twist, and E-cadherin in HeLa and Caski cells. Western blot results are shown in B and quantification of protein levels in C. Data are presented as mean ±SD. * P<0.05, ** P<0.01, *** P<0.001.
that Notch2 may be important in metastasis of laryngeal squamous cell carcinoma.

Activation of the ligand-dependent Notch signaling pathway allows subsequent proteolytic cleavage of the Notch receptor, which is cleaved by the metalloprotease tumor necrosis factor-α-converting enzyme and the γ-secretase complex [32], then releasing the intracellular domain of the Notch receptor (NICD). Therefore, blocking the γ-secretase function is likely to prevent the formation of NICD and inhibit the Notch signaling pathway [32].

Furthermore, in laryngeal squamous cell carcinoma, the inhibition of Notch1 suppresses cell growth, migration, and invasion [33]. In renal cell carcinoma, blockage of Notch1 or Notch2 signaling pathway using pharmacological inhibitor MRK-003 or its endogenous inhibitor Numb resulted in partial loss of chemoresistance, invasive, and migratory potential, and tumorigenesis in vivo [34]. In the present study, after treatment of cervical cancer cell lines with the GSI RO4929097, the expression of the Notch2 target gene, Hey1, was markedly decreased. Blocking the Notch2 signaling pathway with GSI RO4929097 markedly inhibited cell proliferation and decreased colony formation, migration, and invasion of cervical cancer cell lines compared with the cells treated with the DMSO control (Figure 2). Moreover, when the Notch signaling pathway was blocked by RO4929097, the drug resistance of cervical cancer cells was also significantly reduced (Figure 3). In addition, epithelial markers E-cadherin was upregulated and mesenchymal proteins such as Snail, N-cadherin, and Twist were downregulated in cervical cancer cell lines treated with RO4929097 compared to the cells treated with the DMSO control (Figure 4).

Tumor metastasis is the process of adhesion, migration, and invasion. During this process, some epithelial-derived tumor cells lose their polarity, and the connection between cells becomes loose; this is the occurrence of EMT, which allows the tumor cells to gain elevated migratory properties and increased invasiveness, which makes the tumor more conducive to metastasis and spread. The biological process of EMT is accompanied by an upregulation of the more plastic mesenchymal protein N-cadherin and reduction in the cell–cell adhesion molecule E-cadherin. A number of transcription factors (TFs), including Snail and Twist, are also involved in this biological process [9,35]. It was demonstrated that the processes that govern the acquisition of EMT is stimulated and regulated by many biologic stimuli, including TFs, and signal transduction pathways. Recently, the Notch signaling pathway was found to be an important regulator in the process of EMT [14]. Notch signaling was activated during EMT in hepatic carcinoma, colorectal cancer, and gastric cancer progression [36–38]. Zhang et al. [23] demonstrated that overexpression of Notch-1 induced EMT, whereas downregulation of Notch-1 retarded EMT in prostate cancer cells. Wang et al. [18] showed that GSI inhibitor inhibits bladder cancer cell drug resistance and invasion by reducing EMT. Hence, we evaluated the effect of GSI RO4929097 on cervical cancer cells. In our study, the expression of Hey1, a downstream target gene of the Notch pathway, was also found to markedly decrease following treatment with the GSI RO4929097. In addition, E-cadherin expression was upregulated and N-cadherin, Snail, and Twist were downregulated in cervical cancer cells treated with RO4929097. Taken together, these results indicate that the biological process of EMT is impaired, at least in part, through blocking the Notch signaling pathway.

Nonetheless, there remain several limitations in our study. Firstly, it is not clinically relevant because we did not carry out a comparative study of human cervical cancer specimens. Secondly, we did not carry out xenograft animal model in vivo, which could provide more clinical importance. Thirdly, further analysis of molecular mechanism needs to be continued in future experiments.

**Conclusions**

In conclusion, in the present study we found the Notch signaling pathway is closely associated with the proliferation, migration, invasion, and drug resistance of cervical cancer cells. Furthermore, the results demonstrated that blockage of the Notch signaling pathway with the GSI RO4929097 impairs the growth, chemoresistance, migration, and invasion of cervical cancer cells by suppressing EMT.

**Competing interests**

None.

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