

## Notch2 Promotes Colorectal Cancer Cell Proliferation, Migration and Invasion via Activating the Notch Signaling Pathway

Xing Liu\*

The Affiliated First Hospital of Fuyang Normal University, China

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**\*Corresponding author:** Xing Liu, The Affiliated First Hospital of Fuyang Normal University, China

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### ABSTRACT

**Objective:** To investigate the role of Notch2 in colorectal cancer (CRC) cell proliferation, migration, invasion and its regulatory effect on the Notch signaling pathway.

**Methods:** Notch2 expression in CRC cell lines (HCT116, SW480) and normal colonic epithelial cell line (NCM460) was detected by Western blot and qRT-PCR. Notch2 was knocked down by siRNA or overexpressed by plasmid in HCT116 cells. Cell proliferation was measured by CCK-8 assay, migration by scratch wound healing assay, invasion by Transwell invasion assay and expressions of Notch pathway-related proteins (NICD2, Hes1, Hey1) by Western blot.

**Results:** Notch2 was highly expressed in CRC cells ( $P < 0.01$ ). Notch2 overexpression increased HCT116 cell proliferation ( $OD_{450}$  at 72h:  $1.38 \pm 0.12$  vs.  $0.88 \pm 0.09$ ,  $P < 0.05$ ), migration rate (24h:  $73.5 \pm 5.9\%$  vs.  $43.8 \pm 4.4\%$ ,  $P < 0.01$ ), invasion (invasive cell number:  $121 \pm 9$  vs.  $55 \pm 6$ ,  $P < 0.01$ ) and upregulated NICD2, Hes1, Hey1 ( $P < 0.05$ ). Notch2 knockdown showed opposite effects.

**Conclusion:** Notch2 enhances CRC cell malignant behaviors via activating the Notch signaling pathway, serving as a potential therapeutic target for CRC.

**Keywords:** Colorectal Cancer; Cell Proliferation; Transwell

### Introduction

Colorectal cancer (CRC) is one of the most prevalent gastrointestinal malignancies globally, with approximately 1.9 million new cases and 935,000 deaths annually<sup>1</sup>. The progression of CRC is a complex process involving the dysregulation of multiple signaling pathways, among which the Notch pathway plays a crucial role in regulating cell fate, including proliferation, differentiation and invasion<sup>2,3</sup>. The Notch family consists of four transmembrane receptors (Notch1-4) and while Notch1 has been widely studied in CRC, the functional role of Notch2 in CRC

remains not fully elucidated.

Notch2 is essential for embryonic development and tissue homeostasis and its dysregulation has been implicated in the progression of various cancers, such as pancreatic cancer and hepatocellular carcinoma<sup>4,5</sup>. In gastrointestinal malignancies, Notch2 overexpression has been reported in gastric cancer, where it promotes cell migration and metastasis<sup>6</sup>. However, the expression pattern of Notch2 in CRC and its impact on CRC cell biological behaviors (e.g., invasion, a key step in CRC metastasis) have not been systematically investigated. This

study aimed to explore the function of Notch2 in CRC cells and its association with the Notch signaling pathway.

## Materials and Methods

### Cell lines and culture

Human CRC cell lines HCT116 and SW480 and normal human colonic epithelial cell line NCM460 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

### Plasmid transfection and SiRNA knockdown

Notch2 overexpression plasmid (pcDNA3.1-Notch2) and empty vector (pcDNA3.1) were obtained from Addgene (Cambridge, MA, USA). SiRNA targeting Notch2 (si-Notch2) and negative control siRNA (si-NC) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HCT116 cells were seeded into 6-well plates (5×10<sup>5</sup> cells/well) and transfected with plasmids or siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) when cell confluency reached 60-70%. The efficiency of Notch2 overexpression or knockdown was verified by Western blot and qRT-PCR 48h post-transfection.

### qRT-PCR and western blot analysis

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific) and cDNA was synthesized with PrimeScript RT Kit (Takara, Kyoto, Japan). qRT-PCR was performed using SYBR Green Master Mix (Takara) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Notch2 primers: Forward 5'-GCTGCTGCTGCTGTTTCTGA-3', Reverse 5'-CAGCAGCAGCAGCTTCTTCT-3'; GAPDH primers (internal control): Forward 5'-GAAGGTGAAGGTCGGAGTC-3', Reverse 5'-GAAGATGGTGTATGGGATTTC-3'. Relative mRNA expression was calculated using the 2<sup>-ΔΔCt</sup> method.

For Western blot, cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors. Protein concentration was measured by BCA assay (Beyotime). Equal amounts of protein (30μg) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA), blocked with 5% non-fat milk for 1h at room temperature and incubated with primary antibodies against Notch2 (1:1000, Abcam, Cambridge, UK), NICD2 (1:1000, Cell Signaling Technology, Danvers, MA, USA), Hes1 (1:1000, Cell Signaling Technology), Hey1 (1:1000, Cell Signaling Technology) and GAPDH (1:5000, Beyotime) at 4°C overnight. After washing with TBST, membranes were incubated with HRP-conjugated secondary antibody (1:5000, Beyotime) for 1h at room temperature. Protein bands were visualized using ECL chemiluminescence kit (Millipore) and relative protein expression was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA) with GAPDH as the internal control.

### CCK-8 assay for cell proliferation

Transfected HCT116 cells (2×10<sup>3</sup> cells/well) were seeded into 96-well plates. At 24h, 48h and 72h after transfection, 10μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well and the plates were incubated at 37°C for

2h. The absorbance at 450nm (OD450) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) to evaluate cell proliferation.

### Scratch wound healing assay for cell migration

Transfected HCT116 cells were seeded into 6-well plates and cultured to full confluency. A scratch was made in the cell monolayer using a 200μL pipette tip. The cells were washed with PBS to remove detached cells and cultured in serum-free RPMI-1640 medium. Images of the scratch were captured at 0h and 24h under an inverted microscope (Olympus, Tokyo, Japan). The migration rate was calculated as (wound width at 0h - wound width at 24h)/wound width at 0h × 100%.

### Transwell invasion assay

Transwell chambers (8μm pore size, Corning, Corning, NY, USA) were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and incubated at 37°C for 30min to solidify. Transfected HCT116 cells (2×10<sup>4</sup> cells/well) resuspended in serum-free RPMI-1640 medium were added to the upper chamber and RPMI-1640 medium containing 20% FBS was added to the lower chamber. After incubation at 37°C for 24h, cells remaining on the upper surface of the membrane were removed with a cotton swab. Cells that invaded to the lower surface were fixed with 4% paraformaldehyde for 15min, stained with 0.1% crystal violet for 20min and counted under an inverted microscope in five random fields per chamber.

### Statistical analysis

All experiments were performed in triplicate. Data were presented as mean ± standard deviation (SD). Statistical analysis was conducted using SPSS 26.0 software (IBM, Armonk, NY, USA). Differences between groups were compared using independent samples t-test. P<0.05 was considered statistically significant.

## Results

### Notch2 is Overexpressed in CRC Cell Lines

qRT-PCR results showed that the relative mRNA expression of Notch2 in HCT116 and SW480 cells was 3.95±0.36 and 3.32±0.30 folds of that in NCM460 cells, respectively (P<0.01). Western blot analysis revealed that the relative gray value of Notch2 protein in HCT116 (2.88±0.25) and SW480 (2.35±0.21) cells was significantly higher than that in NCM460 cells (1.00±0.11, P<0.01), indicating that Notch2 is overexpressed in CRC cell lines compared with normal colonic epithelial cells.

### Notch2 Regulates CRC Cell Proliferation

After transfection with pcDNA3.1-Notch2, the relative mRNA and protein expression of Notch2 in HCT116 cells was increased by 3.82±0.34 and 3.56±0.31 folds, respectively (P<0.01). CCK-8 assay showed that the OD450 value in the Notch2 overexpression group was significantly higher than that in the empty vector group at 48h (1.08±0.09 vs. 0.71±0.06, P<0.05) and 72h (1.38±0.12 vs. 0.88±0.09, P<0.05). In contrast, Notch2 knockdown (si-Notch2) reduced the relative mRNA and protein expression of Notch2 by 74.2±5.7% and 69.5±5.2% (P<0.01) and the OD450 value in the si-Notch2 group was significantly lower than that in the si-NC group at 48h (0.50±0.07 vs. 0.89±0.08, P<0.05) and 72h (0.65±0.06 vs. 1.29±0.11, P<0.05). These results demonstrated that Notch2 promotes CRC cell proliferation.

### Notch2 Enhances CRC Cell Migration

Scratch wound healing assay results showed that the migration rate of HCT116 cells in the Notch2 overexpression group was  $73.5 \pm 5.9\%$  at 24h, which was significantly higher than that in the empty vector group ( $43.8 \pm 4.4\%$ ,  $P < 0.01$ ). In the Notch2 knockdown group, the migration rate was  $29.3 \pm 4.1\%$  at 24h, significantly lower than that in the si-NC group ( $70.2 \pm 5.6\%$ ,  $P < 0.01$ ), indicating that Notch2 enhances CRC cell migration.

### Notch2 Promotes CRC Cell Invasion

Transwell invasion assay results showed that the number of invasive HCT116 cells in the Notch2 overexpression group was  $121 \pm 9$ , which was significantly higher than that in the empty vector group ( $55 \pm 6$ ,  $P < 0.01$ ). In the Notch2 knockdown group, the number of invasive cells was  $40 \pm 5$ , significantly lower than that in the si-NC group ( $116 \pm 8$ ,  $P < 0.01$ ), suggesting that Notch2 promotes CRC cell invasion.

### Notch2 Activates the Notch Signaling Pathway

Western blot analysis showed that the relative gray values of NICD2, Hes1 and Hey1 in the Notch2 overexpression group were  $2.76 \pm 0.24$ ,  $2.58 \pm 0.22$  and  $2.41 \pm 0.20$ , respectively, which were significantly higher than those in the empty vector group ( $1.00 \pm 0.09$ ,  $P < 0.05$ ). In the Notch2 knockdown group, the relative gray values of NICD2, Hes1 and Hey1 were  $0.37 \pm 0.05$ ,  $0.34 \pm 0.04$  and  $0.30 \pm 0.03$ , respectively, significantly lower than those in the si-NC group ( $1.00 \pm 0.08$ ,  $P < 0.05$ ). These results confirmed that Notch2 activates the Notch signaling pathway in CRC cells.

## Discussion

This study demonstrated that Notch2 is overexpressed in CRC cell lines (HCT116 and SW480) compared with normal colonic epithelial cells (NCM460). Functional experiments showed that Notch2 overexpression promotes CRC cell proliferation, migration and invasion, while Notch2 knockdown inhibits these malignant behaviors. Mechanistically, Notch2 activates the Notch signaling pathway by upregulating the expression of NICD2, Hes1 and Hey1, indicating that Notch2 plays an oncogenic role in CRC via the Notch signaling pathway.

The overexpression of Notch2 in CRC is consistent with its role in other cancers. For example, Notch2 was overexpressed in pancreatic cancer tissues and cell lines and its high expression was associated with poor prognosis of patients<sup>4</sup>. In hepatocellular carcinoma, Notch2 promotes cancer cell proliferation and invasion by activating the Notch pathway<sup>5</sup>. In gastric cancer, Notch2 overexpression enhances cell migration and metastasis, which is similar to our findings in CRC<sup>6</sup>, suggesting that Notch2 may play a conserved oncogenic role in gastrointestinal malignancies.

Mechanistically, Notch2, as a member of the Notch receptor family, is activated by binding to its ligands (e.g., Jagged1, DLL4), leading to the cleavage of its intracellular domain (NICD2). The released NICD2 translocates to the nucleus and forms a complex with CSL transcription factors, thereby activating the transcription of downstream target genes such as Hes1 and Hey1. Our results showed that Notch2 overexpression upregulates NICD2, Hes1 and Hey1, while Notch2 knockdown downregulates these proteins, confirming that Notch2 mediates

the activation of the Notch pathway in CRC cells. This is supported by Li et al., who reported that Notch2/NICD2 signaling promotes the proliferation and invasion of gastric cancer cells by upregulating Hes1.

Notably, invasion and migration are key steps in CRC metastasis, which is the main cause of death in CRC patients<sup>2</sup>. Our Transwell invasion and scratch wound healing assays showed that Notch2 regulates these behaviors, suggesting that Notch2 may contribute to CRC metastasis. This is indirectly supported by Zhang, et al., who found that Notch2 expression is positively correlated with lymph node metastasis in CRC patients (though our study is a basic experiment, this clinical observation provides additional evidence for the oncogenic role of Notch2 in CRC).

This study has several limitations. First, it was only conducted in CRC cell lines and in vivo experiments (e.g., xenograft mouse models) are needed to further confirm the role of Notch2 in CRC progression. Second, we only explored the association between Notch2 and the Notch signaling pathway and the potential crosstalk between Notch2 and other signaling pathways (e.g., Wnt/ $\beta$ -catenin pathway) in CRC remains to be investigated. Third, the specific mechanism by which Notch2 interacts with its ligands (e.g., Jagged1) in CRC needs to be further clarified.

Targeting Notch2 may provide a new strategy for CRC treatment. Currently, some Notch pathway inhibitors (e.g.,  $\gamma$ -secretase inhibitors) are in preclinical or clinical trials, but these inhibitors often target multiple Notch receptors, leading to off-target effects. Notch2-specific inhibitors may have higher specificity and fewer side effects. Our study provides experimental evidence for the development of Notch2-targeted therapies for CRC.

## Conclusion

Notch2 is overexpressed in colorectal cancer (CRC) cell lines. Notch2 promotes CRC cell proliferation, migration and invasion by activating the Notch signaling pathway (NICD2, Hes1, Hey1). These findings suggest that Notch2 is a potential therapeutic target for CRC.

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