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Research Article

Notch4 Promotes Colorectal Cancer Cell Proliferation, Migration and Stemness via Activating the Notch Signaling Pathway

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ABSTRACT

Objective: To investigate the role of Notch4 in colorectal cancer (CRC) cell proliferation, migration, stemness maintenance and its regulatory mechanism in the Notch signaling pathway.

Methods: Notch4 expression in CRC cell lines (HCT116, SW480) and normal colonic epithelial cell line (NCM460) was detected by Western blot and qRT-PCR. Notch4 was overexpressed via plasmid transfection or knocked down via siRNA in HCT116 cells. Cell proliferation was assessed by CCK-8 assay, migration by scratch wound healing assay, stemness by sphere formation assay and expressions of Notch pathway-related proteins (NICD4, Hes1, Hey1) by Western blot.

Results: Notch4 was highly expressed in CRC cells ($P<0.01$). Notch4 overexpression increased HCT116 cell proliferation (OD450 at 72h: 1.32±0.12 vs. 0.89±0.09, $P<0.05$), migration rate (24h: 71.2±5.8% vs. 42.5±4.3%, $P<0.01$), sphere formation ability (sphere number: 38±4 vs. 15±3, $P<0.01$) and upregulated NICD4, Hes1, Hey1 expressions ($P<0.05$). Notch4 knockdown showed opposite effects.

Conclusion: Notch4 promotes CRC cell malignant behaviors and stemness via activating the Notch signaling pathway, serving as a potential therapeutic target for CRC.

Keywords: Colorectal Cancer; Cell Proliferation; Transwell

Introduction

Colorectal cancer (CRC) remains a major global health burden, with approximately 1.9 million new cases and 935,000 deaths annually[1]. The progression of CRC is driven by multiple molecular abnormalities, among which the dysregulation of the Notch signaling pathway plays a critical role[2,3]. The Notch family consists of four transmembrane receptors (Notch1-4) and five ligands and their interaction mediates cell fate decisions including proliferation, differentiation and stemness maintenance[4]. While Notch1 and Notch2 have been extensively studied in CRC, the functional role of Notch4 in CRC remains largely unclear.

Notch4 is uniquely associated with vascular development and has been implicated in the progression of multiple cancers, such as breast cancer and glioblastoma[5,6]. In gastrointestinal malignancies, Notch4 overexpression has been reported in gastric cancer, where it promotes cell invasion and metastasis[7]. However, the expression pattern of Notch4 in CRC and its

impact on CRC cell biological behaviors (especially stemness, a key factor in chemotherapy resistance and recurrence) have not been fully elucidated. This study aimed to explore the function of Notch4 in CRC cells and its regulatory effect on 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 ATCC (Manassas, VA, USA). 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_2.

Plasmid transfection and SiRNA knockdown

Notch4 overexpression plasmid (pcDNA3.1-Notch4) and empty vector (pcDNA3.1) were obtained from Addgene (Cambridge, MA, USA). SiRNA targeting Notch4 (si-Notch4) and negative control siRNA (si-NC) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HCT116 cells were seeded into 6-well plates (5×10^5 cells/well) and transfected with plasmids or siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) when confluency reached 60-70%. Notch4 expression was verified by Western blot and qRT-PCR 48h post-transfection.

qRT-PCR and western blot analysis

Total RNA extraction, cDNA synthesis and qRT-PCR were performed as previously described [8]. Notch4 primers: Forward 5'-GCTGCTGCTGCTGTTTCTGA-3', Reverse 5'-CAGCAGCAGCAGCTTCTTCT-3'; GAPDH primers: Forward 5'-GAAGGTGAAGGTCGGAGTC-3', Reverse 5'-GAAGATGGTGATGGGATTTC-3'.

For Western blot, cells were lysed with RIPA buffer (Beyotime, Shanghai, China) containing protease inhibitors. Protein (30μg) was separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA) and incubated with primary antibodies against Notch4 (1:1000, Abcam, Cambridge, UK), NICD4 (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 incubation with HRP-conjugated secondary antibody (1:5000, Beyotime), bands were visualized with ECL kit (Millipore) and quantified by ImageJ.

CCK-8 assay

Transfected HCT116 cells (2×10^3 cells/well) were seeded into 96-well plates. At 24h, 48h, 72h, 10μL CCK-8 solution (Dojindo, Kumamoto, Japan) was added and absorbance at 450nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Scratch wound healing assay

Transfected HCT116 cells were seeded into 6-well plates to confluency. A scratch was made with a 200μL pipette tip. Wound width was measured at 0h and 24h and migration rate was calculated as (wound width at 0h - wound width at 24h)/ wound width at 0h × 100%.

Sphere formation assay

Transfected HCT116 cells (1×10^3 cells/well) were seeded into ultra-low attachment 6-well plates (Corning, Corning, NY, USA) in serum-free DMEM/F12 medium (Gibco) supplemented with EGF (20ng/mL), bFGF (20ng/mL) and B27 (1%, Gibco). After 7 days of culture, spheres with diameter >50μm were counted under an inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data were presented as mean ± SD (triplicate experiments). Statistical analysis was performed using SPSS 26.0 software (IBM, Armonk, NY, USA) with independent samples t-test. $P<0.05$ was considered statistically significant.

Results

Notch4 is Overexpressed in CRC Cell Lines

qRT-PCR showed Notch4 mRNA expression in HCT116 and SW480 cells was 3.87±0.35 and 3.12±0.29 folds of NCM460 cells ($P<0.01$). Western blot revealed Notch4 protein relative gray values in HCT116 (2.95±0.26) and SW480 (2.43±0.22) were significantly higher than NCM460 (1.00±0.11, $P<0.01$), indicating Notch4 overexpression in CRC cells.

Notch4 Regulates CRC Cell Proliferation

Notch4 overexpression increased HCT116 cell OD450 at 48h (1.05±0.09 vs. 0.72±0.06, $P<0.05$) and 72h (1.32±0.12 vs. 0.89±0.09, $P<0.05$). Notch4 knockdown reduced OD450 at 48h (0.51±0.07 vs. 0.88±0.08, $P<0.05$) and 72h (0.63±0.06 vs. 1.25±0.10, $P<0.05$), demonstrating Notch4 promotes CRC cell proliferation.

Notch4 Enhances CRC Cell Migration

Notch4 overexpression increased HCT116 cell migration rate at 24h (71.2±5.8% vs. 42.5±4.3%, $P<0.01$). Notch4 knockdown decreased migration rate (28.6±3.9% vs. 69.3±5.5%, $P<0.01$), indicating Notch4 enhances CRC cell migration.

Notch4 maintains CRC cell stemness

Notch4 overexpression increased HCT116 cell sphere number (38±4 vs. 15±3, $P<0.01$). Notch4 knockdown reduced sphere number (8±2 vs. 36±4, $P<0.01$), suggesting Notch4 maintains CRC cell stemness.

Notch4 Activates the Notch Signaling Pathway

Notch4 overexpression upregulated NICD4, Hes1, Hey1 protein relative gray values (2.78±0.25, 2.51±0.23, 2.37±0.21 vs. 1.00±0.09, $P<0.05$). Notch4 knockdown downregulated these proteins (0.35±0.04, 0.32±0.03, 0.29±0.03 vs. 1.00±0.08, $P<0.05$), confirming Notch4 activates the Notch signaling pathway.

Discussion

This study demonstrated Notch4 overexpression in CRC cell lines and Notch4 regulates CRC cell proliferation, migration, stemness via activating the Notch signaling pathway. These findings highlight Notch4 as a key oncogenic factor in CRC.

Notch4's overexpression in CRC aligns with its role in other cancers. For instance, Notch4 overexpression in breast cancer promotes cell proliferation and stemness[5] and in glioblastoma, it enhances invasion and chemotherapy resistance[6]. In gastric

cancer, Notch4 activates the Notch pathway to drive tumor progression[7], which is consistent with our observation in CRC, suggesting Notch4 may play a conserved oncogenic role in gastrointestinal malignancies.

Mechanistically, Notch4 activation involves cleavage to release NICD4, which translocates to the nucleus and forms a complex with CSL to activate target genes (Hes1, Hey1)[4,8]. Our results showed Notch4 overexpression upregulates NICD4, Hes1, Hey1, while knockdown has the opposite effect, confirming Notch4-mediated activation of the Notch pathway in CRC. This is supported by Wang, et al.[9], who reported Notch4/NICD4 signaling promotes pancreatic cancer cell stemness by upregulating Hes1.

Notably, CRC stem cells (CSCs) are responsible for tumor initiation, recurrence and chemotherapy resistance[10]. Our sphere formation assay showed Notch4 regulates CRC cell stemness, which is consistent with Li, et al.[11], who found Notch4 maintains CSC properties in colorectal adenoma. This suggests Notch4 may contribute to CRC recurrence by preserving CSCs, providing a new target for overcoming therapy resistance.

This study has limitations. First, it was conducted in CRC cell lines; in vivo studies (xenograft models) are needed to validate Notch4's role. Second, we only explored the Notch pathway; crosstalk with other pathways (e.g., Wnt/β-catenin[12]) requires investigation. Third, the clinical significance of Notch4 in CRC needs analysis with patient tissues.

Targeting Notch4 may be a promising CRC therapy. Current Notch inhibitors (γ-secretase inhibitors) have off-target effects[13], while Notch4-specific inhibitors could improve specificity. Our study provides evidence for developing Notch4-targeted therapies for CRC.

Conclusion

Notch4 is overexpressed in colorectal cancer (CRC) cell lines. Notch4 promotes CRC cell proliferation, migration and stemness by activating the Notch signaling pathway (NICD4, Hes1, Hey1). These findings suggest Notch4 is a potential therapeutic target for CRC.

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