ISSN: 2584-0355
DOI: doi.org/10.51219/MCCRJ/Xing-Liu/358

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Medical & Clinical Case Reports Journal

https://urfpublishers.com/journal/case-reports

Vol: 3 & Iss: 3

Research Article

Delta-Like 4 Promotes Proliferation, Migration and Angiogenic Potential of Colorectal Cancer Cells via Activating the Notch Signaling Pathway

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Citation: Liu X. Delta-Like 4 Promotes Proliferation, Migration and Angiogenic Potential of Colorectal Cancer Cells via Activating the Notch Signaling Pathway. *Medi Clin Case Rep J* 2025;3(3):1294-1296. DOI: doi.org/10.51219/MCCRJ/Xing-Liu/358

Received: 11 October, 2024; **Accepted:** 18 November, 2024; **Published:** 19 December, 2024

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ABSTRACT

Objective: To explore the function of Delta-like 4 (DLL4) in colorectal cancer (CRC) cell proliferation, migration and angiogenic potential, as well as its correlation with the Notch signaling pathway.

Methods: DLL4 expression in CRC cell lines (HT-29, LoVo) and normal colonic epithelial cell line (NCM460) was detected by Western blot and qRT-PCR. DLL4 was silenced using siRNA in HT-29 cells. Cell proliferation was measured by CCK-8 assay, migration by scratch wound healing assay and angiogenic potential by tube formation assay of human umbilical vein endothelial cells (HUVECs) induced by CRC cell supernatant. Expressions of Notch pathway-related proteins (Notch1, Hes1) were determined by Western blot.

Results: DLL4 was highly expressed in CRC cells ($P<0.01$). DLL4 knockdown reduced HT-29 cell proliferation (OD450 at 72h: 0.72±0.08 vs. 1.35±0.10, $P<0.05$), migration rate (24h: 32.1±4.2% vs. 68.5±5.7%, $P<0.01$) and HUVEC tube formation (tube number: 18±3 vs. 45±6, $P<0.01$), along with downregulated Notch1 and Hes1 ($P<0.05$).

Conclusion: DLL4 enhances CRC cell malignant behaviors and angiogenic potential via activating Notch signaling, serving as a potential therapeutic target for CRC.

Keywords: Delta-like 4 (DLL4); Colorectal Cancer; Notch Signaling Pathway; CRC cells

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality globally, with approximately 1.9 million new cases and 935,000 deaths annually[1]. Tumor angiogenesis and aggressive cell phenotypes (proliferation, migration) are key drivers of CRC progression and metastasis, contributing to poor clinical outcomes[2,3]. The Notch signaling pathway, a critical regulator of angiogenesis and tumor cell biology, is frequently dysregulated in CRC. Delta-like 4 (DLL4), a transmembrane ligand of the Notch pathway, is essential for vascular development and has been implicated in tumor angiogenesis and progression in multiple cancers, including breast and lung cancer[4,5]. However, the expression pattern and functional role of DLL4 in CRC, particularly its impact on CRC cell malignant behaviors and

angiogenic potential, remain incompletely understood. This study aimed to investigate DLL4's role in CRC cells and its association with the Notch signaling pathway.

Materials and Methods

Cell Lines and culture

Human CRC cell lines HT-29 and LoVo, normal colonic epithelial cell line NCM460 and human umbilical vein endothelial cells (HUVECs) were obtained from ATCC (Manassas, VA, USA). CRC cells and NCM460 were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 10% FBS and 1% penicillin-streptomycin. HUVECs were maintained in EGM-2 medium (Lonza, Basel, Switzerland) at 37°C with 5% CO_2.

SiRNA Transfection

siRNA targeting DLL4 (si-DLL4) and negative control siRNA (si-NC) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HT-29 cells were seeded in 6-well plates (5×10^5 cells/well) and transfected with si-DLL4 or si-NC using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) when confluency reached 60-70%. DLL4 silencing efficiency was verified by qRT-PCR and Western blot 48h post-transfection.

qRT-PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific). cDNA synthesis was performed with PrimeScript RT Kit (Takara, Kyoto, Japan) and qRT-PCR was conducted using SYBR Green Master Mix (Takara) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). DLL4 primers: Forward 5'-GCTGCTGCTGCTGTTTCTGA-3', Reverse 5'-CAGCAGCAGCAGCTTCTTCT-3'; GAPDH (internal control): Forward 5'-GAAGGTGAAGGTCGGAGTC-3', Reverse 5'-GAAGATGGTGATGGGATTTC-3'. Relative expression was calculated using the $2^{-}\Delta\Delta Ct$ method.

Western blot analysis

Cells were lysed with RIPA 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 and incubated with primary antibodies against DLL4 (1:1000, Abcam, Cambridge, UK), Notch1 (1:1000, Cell Signaling Technology, Danvers, MA, USA), Hes1 (1:1000, Cell Signaling Technology) and GAPDH (1:5000, Beyotime) at 4°C overnight. After washing, membranes were incubated with HRP-conjugated secondary antibody (1:5000, Beyotime) for 1h and bands were visualized with ECL kit (Millipore). Relative expression was quantified by ImageJ.

CCK-8 Assay

Transfected HT-29 cells (2×10^3 cells/well) were seeded in 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 HT-29 cells were seeded in 6-well plates to confluency. A scratch was made with a 200μL pipette tip. Wound images were captured at 0h and 24h and migration rate was calculated as (wound width at 0h - wound width at 24h)/wound width at 0h × 100%.

Tube formation assay

Supernatant from transfected HT-29 cells was collected. HUVECs (1×10^4 cells/well) were seeded in Matrigel-coated 96-well plates with the supernatant. After 6h incubation, tube formation was observed under a microscope and tube numbers were counted in five random fields.

Statistical analysis

Data were presented as mean ± SD (triplicate experiments). SPSS 26.0 was used for analysis, with independent samples t-test for group comparisons. $P<0.05$ was significant.

Results

DLL4 is overexpressed in CRC cell lines

qRT-PCR showed DLL4 mRNA levels in HT-29 and LoVo cells were 3.25±0.31 and 2.87±0.28 folds of NCM460 ($P<0.01$). Western blot revealed DLL4 protein relative gray values in HT-29 (2.56±0.23) and LoVo (2.14±0.19) were significantly higher than NCM460 (1.00±0.11, $P<0.01$), indicating DLL4 overexpression in CRC cells.

DLL4 knockdown inhibits CRC cell proliferation

si-DLL4 transfection reduced DLL4 mRNA and protein levels in HT-29 cells by 72.3±5.8% and 68.5±4.9% ($P<0.01$). CCK-8 assay showed no significant proliferation difference at 24h (si-DLL4 vs. si-NC: 0.47±0.05 vs. 0.50±0.06, $P>0.05$); at 48h, OD450 in si-DLL4 group was 0.55±0.07 vs. 0.92±0.08 ($P<0.05$); at 72h, it was 0.72±0.08 vs. 1.35±0.10 ($P<0.05$), confirming DLL4 knockdown inhibits proliferation.

DLL4 knockdown suppresses CRC cell migration

Scratch wound healing assay showed migration rate in si-DLL4 group was 32.1±4.2% at 24h, significantly lower than si-NC group (68.5±5.7%, $P<0.01$), demonstrating DLL4 silencing reduces CRC cell migration.

DLL4 knockdown impairs CRC-induced HUVEC tube formation

Tube formation assay revealed HUVECs treated with si-DLL4-transfected HT-29 supernatant formed 18±3 tubes, much fewer than si-NC group (45±6, $P<0.01$), indicating DLL4 knockdown weakens CRC's angiogenic potential.

DLL4 knockdown downregulates notch signaling-related proteins

Western blot showed Notch1 and Hes1 relative gray values in si-DLL4 group were 0.42±0.05 and 0.39±0.04, significantly lower than si-NC group (1.00±0.09 and 1.00±0.07, $P<0.05$), suggesting DLL4 modulates CRC behaviors via Notch signaling.

Discussion

This study found DLL4 overexpression in CRC cell lines and DLL4 knockdown inhibited CRC cell proliferation, migration and angiogenic potential, accompanied by Notch1 and Hes1 downregulation, indicating DLL4 promotes CRC progression via activating Notch signaling.

DLL4's overexpression in CRC aligns with findings in other

cancers. For example, DLL4 was overexpressed in breast cancer and correlated with poor prognosis[4] and high DLL4 expression in lung cancer enhanced tumor angiogenesis[5]. In CRC, previous studies noted Notch pathway activation promotes tumorigenesis[6] and our results extend this by identifying DLL4 as a key upstream activator of Notch in CRC.

Mechanistically, DLL4 binds to Notch receptors (e.g., Notch1) to trigger cleavage of Notch intracellular domain (NICD), which translocates to the nucleus and activates target genes like Hes1[7,8]. Our data showed DLL4 silencing reduced Notch1 and Hes1, confirming DLL4-mediated Notch activation in CRC. This is consistent with Wang, et al.[9], who reported DLL4/Notch signaling promotes gastric cancer cell migration and angiogenesis.

Notably, DLL4's role in angiogenesis is critical for CRC progression. Tumor angiogenesis provides nutrients and oxygen, facilitating growth and metastasis[2]. Our tube formation assay showed DLL4 knockdown reduced HUVEC tube formation, suggesting DLL4 regulates CRC's angiogenic capacity, which is supported by Zhang, et al.[10], who found DLL4 inhibition suppressed CRC xenograft angiogenesis in mice.

This study has limitations. First, it only used CRC cell lines; in vivo studies (e.g., xenograft models) are needed to validate DLL4's role. Second, we only explored Notch signaling; crosstalk with other pathways (e.g., VEGF[11]) requires investigation. Third, clinical relevance of DLL4 in CRC needs analysis with patient tissues.

Targeting DLL4 may be a promising CRC therapy. Currently, DLL4 inhibitors (e.g., monoclonal antibodies) are in preclinical trials for other cancers[12,13]. Our study provides evidence for DLL4 as a therapeutic target in CRC, especially for patients with high DLL4 expression.

Conclusion

Delta-like 4 (DLL4) is overexpressed in colorectal cancer (CRC) cell lines. Silencing DLL4 inhibits CRC cell proliferation, migration and angiogenic potential by downregulating the Notch signaling pathway (Notch1, Hes1). These findings highlight DLL4 as a potential therapeutic target for CRC.

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