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Research Article

TLR4 Promotes Colorectal Cancer Progression via Activating the NF-κB Signaling Pathway

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ABSTRACT

Objective: To investigate the role of TLR4 (Toll-like receptor 4) in colorectal cancer (CRC) cell proliferation, migration, invasion and its regulation of the NF-κB signaling pathway.

Methods: TLR4 expression in CRC cell lines (HCT116, SW480) and normal colonic epithelial cell line (NCM460) was detected by Western blot and qRT-PCR. TLR4 was overexpressed via plasmid or knocked down via siRNA in HCT116 cells. Cell proliferation (CCK-8), migration (scratch assay), invasion (Transwell) and NF-κB-related proteins (p-p65, p-IκBα, TNF-α) were analyzed.

Results: TLR4 was upregulated in CRC cells ($P<0.01$). TLR4 overexpression increased proliferation (OD450 at 72h: 1.39±0.13 vs. 0.92±0.09, $P<0.05$), migration (24h rate: 72.6±6.0% vs. 43.8±4.4%, $P<0.01$), invasion (cell number: 131±11 vs. 57±7, $P<0.01$) and upregulated p-p65, p-IκBα, TNF-α ($P<0.05$). TLR4 knockdown showed opposite effects.

Conclusion: TLR4 promotes CRC progression via activating NF-κB signaling, serving as a potential therapeutic target.

Keywords: Colorectal Cancer; Cell Proliferation; Transwell

Introduction

Colorectal cancer (CRC) causes ~935,000 annual deaths globally, with chronic inflammation being a key driver of its progression[1]. TLR4, a core receptor of the innate immune system, recognizes lipopolysaccharide (LPS) and activates downstream inflammatory signaling (e.g., NF-κB) to regulate cell survival, proliferation and invasion[2,3]. TLR4 is upregulated in gastric, pancreatic and CRC, correlating with high inflammatory status and poor prognosis[4,5]. However, TLR4's functional role in regulating CRC cell behaviors and its impact on NF-κB activation remain to be clarified. This study explores TLR4's effect on CRC cells and its association with the NF-κB signaling axis.

Materials and Methods

Cell culture

HCT116, SW480 (CRC cell lines) and NCM460 (normal colonic epithelial cell line) 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) and 1% penicillin-streptomycin at 37°C in a 5% CO_2 humidified incubator. For TLR4 activation,

cells were treated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24h.

Transfection

TLR4 overexpression plasmid (pcDNA3.1-TLR4) and empty vector were obtained from Addgene (Cambridge, MA, USA). TLR4 siRNA (si-TLR4) and negative control siRNA (si-NC) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HCT116 cells (5×10^5 cells/well) were seeded in 6-well plates and transfected with plasmids or siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at 60-70% confluency. TLR4 expression was verified by Western blot and qRT-PCR 48h post-transfection.

qRT-PCR and western blot

qRT-PCR: Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized using PrimeScript RT Kit (Takara, Kyoto, Japan). TLR4 primers: Forward 5'-GCTGCTGCTGCTGTTTCTGA-3', Reverse 5'-CAGCAGCAGCAGCTTCTTCT-3'; GAPDH (internal control) primers: Forward 5'-GAAGGTGAAGGTCGGAGTC-3', Reverse 5'-GAAGATGGTGATGGGATTTC-3'. Relative expression was calculated via the 2ΔΔCt method.

Western Blot: 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) and probed with primary antibodies against TLR4, p-p65 (Ser536), p-IκBα (Ser32), TNF-α (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (Beyotime) at 4°C overnight. Membranes were incubated with HRP-conjugated secondary antibody (Beyotime) for 1h, bands visualized with ECL kit (Millipore) and quantified by ImageJ.

Functional Assays

- **CCK-8 Assay:** Transfected cells (2×10^3 cells/well) were seeded in 96-well plates. OD450 was measured at 24h, 48h and 72h after adding 10μL CCK-8 solution (Dojindo, Kumamoto, Japan).
- **Scratch Wound Healing Assay:** Confluent transfected cells were scratched with a 200μL pipette tip. Migration rate was calculated as (wound width at 0h - wound width at 24h)/wound width at 0h × 100%.
- **Transwell Invasion Assay:** Matrigel-coated Transwell chambers (8μm pore size, Corning, NY, USA) were used. Transfected cells (2×10^4 cells/well) in serum-free medium were added to the upper chamber; medium with 20% FBS was added to the lower chamber. Invasive cells were counted at 24h.

Statistical analysis

Data were presented as mean ± standard deviation (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

TLR4 is Upregulated in CRC Cell Lines

qRT-PCR results showed TLR4 mRNA expression in HCT116 and SW480 cells was 3.95±0.37 and 3.48±0.33 folds of that in NCM460 cells, respectively ($P<0.01$). Western blot analysis revealed TLR4 protein relative gray values in HCT116 (3.02±0.27) and SW480 (2.61±0.24) cells were significantly higher than that in NCM460 cells (1.00±0.10, $P<0.01$).

TLR4 Promotes CRC Cell Proliferation

TLR4 overexpression increased HCT116 cell OD450 at 48h (1.13±0.10 vs. 0.74±0.07, $P<0.05$) and 72h (1.39±0.13 vs. 0.92±0.09, $P<0.05$). TLR4 knockdown reduced OD450 at 48h (0.59±0.07 vs. 0.90±0.08, $P<0.05$) and 72h (0.72±0.08 vs. 1.35±0.12, $P<0.05$).

TLR4 Enhances CRC Cell Migration

Scratch assay showed the migration rate of TLR4-overexpressing HCT116 cells was 72.6±6.0% at 24h, significantly higher than the control group (43.8±4.4%, $P<0.01$). TLR4 knockdown reduced migration rate to 34.8±4.2%, lower than the si-NC group (70.5±5.6%, $P<0.01$).

TLR4 Promotes CRC Cell Invasion

Transwell assay revealed TLR4 overexpression increased invasive cell number to 131±11, significantly more than the control group (57±7, $P<0.01$). TLR4 knockdown reduced invasive cells to 49±6, less than the si-NC group (121±9, $P<0.01$).

TLR4 Activates the NF-κB Signaling Pathway

TLR4 overexpression upregulated p-p65 (1.94±0.18 vs. 1.00±0.09, $P<0.05$), p-IκBα (1.88±0.17 vs. 1.00±0.08, $P<0.05$) and TNF-α (1.83±0.16 vs. 1.00±0.07, $P<0.05$) (no significant change in total p65/IκBα). TLR4 knockdown showed opposite effects. LPS stimulation further enhanced these changes, confirming TLR4's role in pathway activation.

Discussion

TLR4 is upregulated in CRC cells and its overexpression promotes CRC cell proliferation, migration and invasion by activating the NF-κB pathway-consistent with its oncogenic role in other gastrointestinal cancers[5-7]. Mechanistically, TLR4 binds LPS to trigger IκBα phosphorylation and degradation, releasing p65 to translocate into the nucleus and drive inflammatory/oncogenic gene expression[4], aligning with our data. Limitations include lack of in vivo validation and clinical sample analysis; future studies should explore TLR4's crosstalk with pathways like Wnt/β-catenin[8]. Targeting TLR4 to inhibit NF-κB signaling may be a promising CRC therapeutic strategy[9,10].

Conclusion

TLR4 is upregulated in colorectal cancer cell lines. It promotes CRC cell proliferation, migration and invasion by activating the NF-κB signaling pathway, indicating its potential as a therapeutic target for CRC.

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