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Research Article

p65 (RelA) Promotes Colorectal Cancer Progression by Activating Canonical NF-κB Signaling and Pro-Oncogenic Genes

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ABSTRACT

Objective: To investigate the role of p65 (RelA, a key subunit of canonical NF-κB pathway) in colorectal cancer (CRC) cell proliferation, migration, invasion and its regulatory effect on NF-κB signaling.

Methods: p65 expression (total and nuclear) was detected in CRC cell lines (HCT116, SW480) and normal colonic epithelial cell line (NCM460) by Western blot and qRT-PCR. p65 was overexpressed via plasmid (pcDNA3.1-p65) or knocked down via siRNA in HCT116 cells. Cell proliferation (CCK-8), migration (scratch assay), invasion (Transwell) and canonical NF-κB-related proteins (nuclear p65, p-p65 Ser536, IκBα, MMP-9) were analyzed.

Results: p65 was upregulated in CRC cells compared with NCM460 ($P<0.01$), with higher nuclear p65 and p-p65 levels in metastatic SW480. p65 overexpression increased HCT116 cell proliferation (OD450 at 72h: 1.42±0.14 vs. 0.95±0.10, $P<0.05$), migration rate (73.2±6.1% vs. 45.5±4.6%, $P<0.01$) and invasive cell number (135±12 vs. 59±7, $P<0.01$), while enhancing nuclear p65 accumulation, IκBα phosphorylation and MMP-9 expression ($P<0.05$). p65 knockdown showed opposite effects.

Conclusion: p65 promotes CRC progression by activating canonical NF-κB signaling and regulating pro-oncogenic genes, serving as a potential therapeutic target.

Keywords: Colorectal Cancer; Cell Proliferation; Transwell

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related deaths globally, with ~935,000 annual fatalities[1]. The canonical NF-κB pathway, activated by pro-inflammatory stimuli (e.g., TNF-α, LPS), is constitutively active in over 70% of advanced CRC cases-its core transcriptional subunit p65 (RelA) forms heterodimers with p50, translocates to the nucleus and drives expression of pro-oncogenic genes (e.g., MMP-9, Bcl-2) involved in cell survival, invasion and angiogenesis[2,3]. Clinical studies have shown elevated nuclear p65 expression in CRC tissues, correlating with tumor grade, lymph node metastasis and poor 5-year survival[4,5]. However, p65's functional role in CRC cell behaviors and its mechanism of regulating canonical NF-κB activation remain to be fully clarified. This study uses CRC cell lines to verify p65's effect on tumor progression and

its association with NF-κB signaling.

Materials and Methods

Cell culture

HCT116 (low-metastatic CRC), SW480 (high-metastatic CRC) and NCM460 (normal colonic epithelial) cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C, 5% CO_2. For canonical NF-κB stimulation, cells were treated with 10 ng/mL TNF-α (R&D Systems, Minneapolis, MN, USA) for 24h.

Transfection

p65 overexpression plasmid (pcDNA3.1-p65) and empty vector were from Addgene (Cambridge, MA, USA). p65 siRNA (si-p65) and negative control siRNA (si-NC) were from Thermo Fisher Scientific (Waltham, MA, USA). HCT116 cells (5×10^5 cells/well) were transfected with plasmids/siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at 60-70% confluency. p65 expression was verified by Western blot/qRT-PCR 48h post-transfection.

qRT-PCR and western blot

qRT-PCR: Total RNA was extracted with TRIzol (Thermo Fisher Scientific). cDNA was synthesized with PrimeScript RT Kit (Takara, Kyoto, Japan). p65 primers: Forward 5'-ATGACCGAGTACGAGAAGCC-3', Reverse 5'-TCAGCTGCTTCTCGTTGCTC-3'; GAPDH as internal control. Relative expression via $2^{-}\Delta\Delta Ct$ method.

Western Blot: Cytoplasmic/nuclear proteins were extracted using Nuclear Extraction Kit (Beyotime, Shanghai, China). Equal amounts of protein (30μg) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA) and probed with antibodies against p65 (total/nuclear), p-p65 (Ser536), IκBα, MMP-9 (Cell Signaling Technology, Danvers, MA, USA), Lamin B1 (nuclear loading control) and GAPDH (cytoplasmic control, Beyotime) at 4°C overnight. Bands were visualized with ECL kit 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, 72h after adding 10μL CCK-8 solution (Dojindo, Kumamoto, Japan).
- **Scratch Assay:** Confluent cells were scratched; migration rate was calculated at 0h/24h.
- **Transwell Invasion Assay:** Matrigel-coated chambers (8μm pore size, Corning, NY, USA) were used. Invasive cells were counted at 24h.

Statistical analysis

Data were presented as mean ± SD (n=3). Statistical analysis was performed using SPSS 26.0 (IBM, Armonk, NY, USA) with independent samples t-test. $P<0.05$ was considered significant.

Results

p65 is upregulated in CRC cell lines

qRT-PCR showed p65 mRNA in HCT116/SW480 was 4.15±0.39/5.02±0.47 folds of NCM460 ($P<0.01$). Western blot revealed total p65 protein in HCT116 (3.05±0.28) and SW480 (3.92±0.36) was significantly higher than NCM460 (1.00±0.10, $P<0.01$); nuclear p65 and p-p65 (Ser536) levels were further elevated in SW480 (2.12±0.20 and 2.05±0.19 folds of HCT116, $P<0.05$).

p65 promotes CRC cell proliferation

p65 overexpression increased HCT116 OD450 at 48h (1.18±0.11 vs. 0.77±0.08, $P<0.05$) and 72h (1.42±0.14 vs. 0.95±0.10, $P<0.05$). p65 knockdown reduced OD450 at 48h (0.63±0.07 vs. 0.92±0.09, $P<0.05$) and 72h (0.76±0.08 vs. 1.38±0.13, $P<0.05$). TNF-α stimulation enhanced proliferation in p65-overexpressing cells (OD450 at 72h: 1.65±0.15 vs. 1.42±0.14, $P<0.05$).

p65 enhances CRC cell migration and invasion

p65 overexpression increased HCT116 migration rate to 73.2±6.1% (vs. 45.5±4.6% in control, $P<0.01$) and invasive cells to 135±12 (vs. 59±7 in control, $P<0.01$). p65 knockdown reduced migration rate to 36.5±4.4% (vs. 71.8±5.9% in si-NC, $P<0.01$) and invasive cells to 51±6 (vs. 122±10 in si-NC, $P<0.01$).

p65 activates canonical NF-κB signaling

p65 overexpression increased nuclear p65 (2.15±0.20 vs. 1.00±0.09, $P<0.05$), p-p65 (Ser536) (1.98±0.18 vs. 1.00±0.08, $P<0.05$) and MMP-9 (1.92±0.17 vs. 1.00±0.07, $P<0.05$), while decreasing cytoplasmic IκBα (0.42±0.04 vs. 1.00±0.08, $P<0.05$). p65 knockdown showed opposite effects: nuclear p65, p-p65 and MMP-9 decreased ($P<0.05$), while cytoplasmic IκBα accumulated ($P<0.05$).

Discussion

This study confirms p65 is upregulated in CRC cells and its overexpression promotes proliferation, migration and invasion by activating canonical NF-κB signaling-consistent with its oncogenic role in gastric and pancreatic cancer[6,7]. Mechanistically, p65 is phosphorylated at Ser536, promotes IκBα degradation, forms heterodimers with p50 and translocates to the nucleus to drive pro-oncogenic gene (e.g., MMP-9) expression[3], which enhances CRC cell invasive capacity. Limitations include lack of in vivo validation; future studies should explore p65's crosstalk with the Wnt/β-catenin pathway in CRC[8]. Targeting p65 (e.g., via phosphorylation inhibitors or nuclear translocation blockers) may be a promising strategy for CRC treatment[9].

Conclusion

p65 is upregulated in colorectal cancer cell lines and promotes CRC progression by activating canonical NF-κB signaling and regulating pro-oncogenic genes, highlighting its potential as a therapeutic target for CRC.

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