

## p65 (RelA) Promotes Colorectal Cancer Progression by Activating Canonical NF- $\kappa$ B Signaling and Pro-Oncogenic Genes

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### ABSTRACT

**Objective:** To investigate the role of p65 (RelA, a key subunit of canonical NF- $\kappa$ B pathway) in colorectal cancer (CRC) cell proliferation, migration, invasion and its regulatory effect on NF- $\kappa$ B 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- $\kappa$ B-related proteins (nuclear p65, p-p65 Ser536, I $\kappa$ B $\alpha$ , 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 (OD<sub>450</sub> at 72h:  $1.42\pm0.14$  vs.  $0.95\pm0.10$ ,  $P<0.05$ ), migration rate ( $73.2\pm6.1\%$  vs.  $45.5\pm4.6\%$ ,  $P<0.01$ ) and invasive cell number ( $135\pm12$  vs.  $59\pm7$ ,  $P<0.01$ ), while enhancing nuclear p65 accumulation, I $\kappa$ B $\alpha$  phosphorylation and MMP-9 expression ( $P<0.05$ ). p65 knockdown showed opposite effects.

**Conclusion:** p65 promotes CRC progression by activating canonical NF- $\kappa$ B 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<sup>1</sup>. The canonical NF- $\kappa$ B pathway, activated by pro-inflammatory stimuli (e.g., TNF- $\alpha$ , 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<sup>2,3</sup>. Clinical studies have shown elevated nuclear p65 expression in CRC tissues, correlating with tumor grade, lymph node metastasis and poor 5-year survival<sup>4,5</sup>. However, p65's functional role in CRC cell behaviors and its mechanism of regulating canonical NF- $\kappa$ B 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- $\kappa$ B 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<sub>2</sub>. For canonical NF- $\kappa$ B stimulation, cells were treated with 10 ng/mL TNF- $\alpha$  (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 ( $5 \times 10^5$  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 (30 $\mu$ g) 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), I $\kappa$ B $\alpha$ , 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 ( $2 \times 10^3$  cells/well) were seeded in 96-well plates. OD450 was measured at 24h, 48h, 72h after adding 10 $\mu$ L 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 (8 $\mu$ m pore size, Corning, NY, USA) were used. Invasive cells were counted at 24h.

### Statistical analysis

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

### p65 promotes CRC cell proliferation

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

### p65 enhances CRC cell migration and invasion

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

### p65 activates canonical NF- $\kappa$ B signaling

p65 overexpression increased nuclear p65 (2.15 $\pm$ 0.20 vs. 1.00 $\pm$ 0.09, P<0.05), p-p65 (Ser536) (1.98 $\pm$ 0.18 vs. 1.00 $\pm$ 0.08, P<0.05) and MMP-9 (1.92 $\pm$ 0.17 vs. 1.00 $\pm$ 0.07, P<0.05), while decreasing cytoplasmic I $\kappa$ B $\alpha$  (0.42 $\pm$ 0.04 vs. 1.00 $\pm$ 0.08, P<0.05). p65 knockdown showed opposite effects: nuclear p65, p-p65 and MMP-9 decreased (P<0.05), while cytoplasmic I $\kappa$ B $\alpha$  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- $\kappa$ B signaling-consistent with its oncogenic role in gastric and pancreatic cancer<sup>6,7</sup>. Mechanistically, p65 is phosphorylated at Ser536, promotes I $\kappa$ B $\alpha$  degradation, forms heterodimers with p50 and translocates to the nucleus to drive pro-oncogenic gene (e.g., MMP-9) expression<sup>3</sup>, which enhances CRC cell invasive capacity. Limitations include lack of in vivo validation; future studies should explore p65's crosstalk with the Wnt/ $\beta$ -catenin pathway in CRC<sup>8</sup>. Targeting p65 (e.g., via phosphorylation inhibitors or nuclear translocation blockers) may be a promising strategy for CRC treatment<sup>9</sup>.

## Conclusion

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

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