

LRP5/6 Promote Colorectal Cancer Progression by Activating Wnt/ β -Catenin Signaling and Pro-Metastatic Genes

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ABSTRACT

Objective: To investigate the role of LRP5/6 (low-density lipoprotein receptor-related proteins 5/6, core co-receptors of Wnt/ β -catenin pathway) in colorectal cancer (CRC) cell proliferation, migration, invasion and their regulatory effect on Wnt signaling.

Methods: LRP5/6 expression (total and membrane-bound) was detected in CRC cell lines (HCT116, SW480) and normal colonic epithelial cell line (NCM460) by Western blot and qRT-PCR. LRP5/6 were overexpressed via plasmids (pcDNA3.1-LRP5, pcDNA3.1-LRP6) or knocked down via siRNA in HCT116 cells. Cell proliferation (CKK-8), migration (scratch assay), invasion (Transwell), sphere formation (stemness assay) and Wnt/ β -catenin-related proteins (active β -catenin, GSK-3 β , MMP-7) were analyzed.

Results: LRP5/6 were upregulated in CRC cells compared with NCM460 ($P < 0.01$), with higher membrane-bound LRP5/6 and active β -catenin levels in metastatic SW480. Co-overexpression of LRP5/6 increased HCT116 cell proliferation (OD_{450} at 72h: 1.52 ± 0.15 vs. 0.99 ± 0.10 , $P < 0.05$), migration rate ($76.8 \pm 6.5\%$ vs. $48.2 \pm 4.9\%$, $P < 0.01$), invasive cell number (145 ± 13 vs. 65 ± 7 , $P < 0.01$) and sphere formation efficiency (3.2 ± 0.3 folds vs. control, $P < 0.01$), while enhancing active β -catenin accumulation, GSK-3 β phosphorylation and MMP-7 expression ($P < 0.05$). LRP5/6 co-knockdown showed opposite effects.

Conclusion: LRP5/6 promote CRC progression by activating Wnt/ β -catenin signaling and regulating pro-metastatic/stemness genes, serving as potential therapeutic targets.

Keywords: Colorectal Cancer; Cell Proliferation; Transwell; Low-Density Lipoprotein Receptor-Related Proteins 5/6

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality globally, with ~935,000 annual deaths¹. The Wnt/ β -catenin pathway is constitutively activated in over 85% of CRC cases and its activation depends on the formation of a ternary complex consisting of Wnt ligands, Frizzled (Fzd) receptors and LRP5/6 co-receptors². LRP5/6, members of the

low-density lipoprotein receptor superfamily, are essential for Wnt signal transduction: upon Wnt binding, LRP5/6 undergo phosphorylation, recruit Axin-GSK-3 β complex to the cell membrane and inhibit β -catenin degradation, leading to nuclear translocation of β -catenin and transcription of target genes (e.g., MMP-7, c-Myc, CD44) involved in cell invasion, stemness maintenance and angiogenesis^{3,4}. Clinical studies have

shown elevated LRP5/6 expression in CRC tissues, correlating with tumor stage, lymph node metastasis and reduced 5-year survival^{5,6}. However, the combined functional role of LRP5/6 in CRC cell behaviors and their synergistic regulation of Wnt/ β -catenin activation remain to be fully clarified. This study uses CRC cell lines to verify the effect of LRP5/6 on tumor progression and their association with Wnt 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) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO₂ incubator. For Wnt signaling stimulation, cells were treated with 200 ng/mL Wnt3a (R&D Systems, Minneapolis, MN, USA) for 24h.

Transfection

LRP5/6 overexpression plasmids (pcDNA3.1-LRP5, pcDNA3.1-LRP6) and empty vector were obtained from Addgene (Cambridge, MA, USA). LRP5/6 siRNAs (si-LRP5, si-LRP6) 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/siRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at 60-70% confluency. LRP5/6 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). LRP5 primers: Forward 5'-ATGACCGAGTACGAGAAGCC-3', Reverse 5'-TCAGCTGCTTCTCGTTGCTC-3'; LRP6 primers: Forward 5'-ATGGACTACAGGGACGACCT-3', Reverse 5'-TCAGCTGGGGTTTCTGTTTC-3'; target genes (MMP-7, CD44) and GAPDH (internal control) primers were designed based on NCBI sequences. Relative expression was calculated via the $2^{-\Delta\Delta C_t}$ method.

Western Blot: Total and membrane proteins were extracted using Membrane Protein Extraction Kit (Beyotime, Shanghai, China). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA) and probed with primary antibodies against LRP5, LRP6 (total/membrane), active β -catenin, p-GSK-3 β (Ser9), MMP-7 (Cell Signaling Technology, Danvers, MA, USA), Na⁺/K⁺-ATPase (membrane loading control) and GAPDH (total protein 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 and 72h after adding 10 μ L CCK-8 solution (Dojindo, Kumamoto, Japan).
- **Scratch Assay:** Confluent cells were scratched with a 200 μ L pipette tip. Migration rate was calculated as (wound width at 0h - wound width at 24h)/wound width at 0h \times 100%.

- **Transwell Invasion Assay:** Matrigel-coated Transwell chambers (8 μ m 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.
- **Sphere Formation Assay:** Cells (1×10^3 cells/well) were seeded in ultra-low attachment 6-well plates with stem cell medium (DMEM/F12 + 20 ng/mL EGF + 20 ng/mL bFGF + $1 \times$ B27). Spheres ($>50 \mu$ m) were counted after 7 days.

Statistical analysis

Data were presented as mean \pm standard deviation (SD, n=3). 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

LRP5/6 are upregulated in CRC cell lines

qRT-PCR showed LRP5 mRNA expression in HCT116/SW480 was $4.65 \pm 0.44/5.52 \pm 0.51$ folds of NCM460 (P<0.01) and LRP6 mRNA was $4.48 \pm 0.42/5.35 \pm 0.49$ folds (P<0.01). Western blot revealed total LRP5 protein in HCT116/SW480 was $3.32 \pm 0.30/4.25 \pm 0.38$ folds of NCM460 (P<0.01), total LRP6 was $3.25 \pm 0.29/4.18 \pm 0.37$ folds (P<0.01); membrane-bound LRP5/6 and active β -catenin levels were further elevated in SW480 ($2.45 \pm 0.23/2.38 \pm 0.22$ and 2.32 ± 0.21 folds of HCT116, P<0.05).

LRP5/6 promote CRC cell proliferation

Co-overexpression of LRP5/6 increased HCT116 cell OD450 at 48h (1.28 ± 0.12 vs. 0.83 ± 0.08 , P<0.05) and 72h (1.52 ± 0.15 vs. 0.99 ± 0.10 , P<0.05). Co-knockdown of LRP5/6 reduced OD450 at 48h (0.70 ± 0.07 vs. 0.96 ± 0.09 , P<0.05) and 72h (0.83 ± 0.08 vs. 1.45 ± 0.14 , P<0.05). Wnt3a stimulation enhanced proliferation in LRP5/6-overexpressing cells (OD450 at 72h: 1.80 ± 0.17 vs. 1.52 ± 0.15 , P<0.05).

LRP5/6 enhance CRC cell migration and invasion

Co-overexpression of LRP5/6 increased HCT116 cell migration rate to $76.8 \pm 6.5\%$ (vs. $48.2 \pm 4.9\%$ in control, P<0.01) and invasive cell number to 145 ± 13 (vs. 65 ± 7 in control, P<0.01). Co-knockdown of LRP5/6 reduced migration rate to $39.2 \pm 4.7\%$ (vs. $74.5 \pm 6.2\%$ in si-NC, P<0.01) and invasive cell number to 57 ± 6 (vs. 130 ± 11 in si-NC, P<0.01).

LRP5/6 maintain CRC cell stemness

Co-overexpression of LRP5/6 increased HCT116 cell sphere formation efficiency to 3.2 ± 0.3 folds of control (P<0.01) and upregulated CD44 (2.08 ± 0.20 vs. 1.00 ± 0.09 , P<0.05). Co-knockdown of LRP5/6 reduced sphere formation efficiency to 0.40 ± 0.10 folds of si-NC (P<0.01) and downregulated CD44 (0.42 ± 0.04 vs. 1.00 ± 0.09 , P<0.05).

LRP5/6 activate Wnt/ β -catenin signaling

Co-overexpression of LRP5/6 increased membrane-bound LRP5 (2.52 ± 0.24 vs. 1.00 ± 0.09 , P<0.05), membrane-bound LRP6 (2.45 ± 0.23 vs. 1.00 ± 0.09 , P<0.05), active β -catenin (2.38 ± 0.22 vs. 1.00 ± 0.08 , P<0.05), p-GSK-3 β (2.25 ± 0.21 vs. 1.00 ± 0.08 , P<0.05) and MMP-7 (2.12 ± 0.20 vs. 1.00 ± 0.08 , P<0.05). Co-knockdown of LRP5/6 showed opposite effects:

membrane-bound LRP5/6, active β -catenin, p-GSK-3 β and MMP-7 decreased ($P<0.05$), while total GSK-3 β increased ($P<0.05$).

Discussion

This study confirms LRP5/6 are upregulated in CRC cells and their co-overexpression promotes proliferation, migration, invasion and stemness by activating Wnt/ β -catenin signaling-consistent with their oncogenic role in gastric and pancreatic cancer^{7,8}. Mechanistically, LRP5/6 localize to the cell membrane, form a complex with Fzd and Wnt ligands, induce GSK-3 β phosphorylation (inhibiting its activity), reduce β -catenin degradation and drive transcription of pro-metastatic genes (e.g., MMP-7) and stemness markers (e.g., CD44)⁴, which enhances CRC's malignant potential. Limitations include lack of in vivo validation; future studies should explore LRP5/6's crosstalk with the Notch pathway in CRC, as both pathways are critical for gastrointestinal tumor progression. Targeting LRP5/6 (e.g., via monoclonal antibodies blocking LRP5/6-Wnt interaction) may be a promising strategy for CRC treatment.

Conclusion

LRP5/6 are upregulated in colorectal cancer cell lines and promote CRC progression by activating Wnt/ β -catenin signaling and regulating pro-metastatic/stemness-associated genes, highlighting their potential as therapeutic targets for CRC.

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