Nitrogen Permease Regulator-Like-2 Exhibited Anti-Tumor Effects And Enhanced The Sensitivity Of Colorectal Cancer Cells To Oxaliplatin And 5-Fluorouracil

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Aiyun Liu1
Jiutao Qiao2
Liyuan He1
Zhangmeng Liu1
Jing Chen1
Fenghua Pei1
Yaju Du1

1Department of Gastroenterology, The Second Affiliated Hospital of Harbin Medical University, Harbin, People’s Republic of China; 2Department of Orthopedics, The Second Affiliated Hospital of Harbin Medical University, Harbin, People’s Republic of China

Background: Colorectal cancer (CRC) is one of the most common malignant tumors in the world. Our previous study revealed that nitrogen permease regulator-like-2 (NPRL2), a promising anti-tumor gene, was downregulated at both the blood and tissue levels in CRC patients compared with that in healthy individuals.

Purpose: This study aims to explore the role of NPRL2 in CRC.

Methods: Herein, we constructed NPRL2 overexpression lentivirus vectors and transfected them into HT29 cells. The transfected cells were inoculated subcutaneously into nude mice. Tumor growth, pathology, apoptosis, and the protein expression of caspase-3, caspase-7, Bax, Bcl-2, and phosphorylated protein kinase B (p-Akt) were evaluated. To further explore whether NPRL2 could reduce drug resistance of CRC cells against oxaliplatin (L-OHP) and 5-fluorouracil (5-FU), we constructed a tumor model using HT29 cells. The tumor model was treated with lentiviral particles assembled with vectors encoding NPRL2 and exposed to L-OHP and 5-FU. Tumor growth, pathology, apoptosis, and the protein expression of caspase-3, caspase-7, Bax, Bcl-2, p-Akt, P-glycoprotein (P-gp), and multidrug resistance protein 1 (MRP1) were evaluated.

Results: The results indicated that in the in vivo CRC xenograft model, NPRL2 reduced the tumor volume and weight and enhanced apoptosis. Our results also confirmed that NPRL2 enhanced the sensitivity of CRC cells to L-OHP and 5-FU. Our studies further demonstrated that NPRL2 exerted anti-tumor and anti-drug resistance effects through the caspase-3, caspase-7, Bax, Bcl-2, Akt, P-gp, and MRP1 pathways.

Conclusion: Our present work demonstrated that NPRL2 exhibited anti-tumor and anti-drug resistance effects in CRC cells to L-OHP and 5-FU through the P-gp and MRP1 pathways.

Keywords: NPRL2, colorectal cancer, drug resistance, apoptosis, P-gp, MRP1

Introduction

Colorectal cancer (CRC) is one of the most common digestive cancers worldwide and is a severe threat to patient health.1 In 2010, approximately 275,000 new cases of CRC were diagnosed in China with an incidence rate of around 2/10,000.2 From 1991 to 2011, the mortality rate of CRC in China increased continuously and was relatively high in the elderly compared to that in individuals less than 60 years of age.3 The resulting public health burden has prompted an urgent need for medical and scientific researchers to develop effective treatments for CRC.
Previous studies have identified several factors involved in the occurrence and development of CRC, including changes in oncogenes and tumor suppressor genes, chromosomal instability, and microsatellite instability. The nitrogen permease regulator like-2 gene (NPRL2) is located on human chromosome 3p21.3, a region that contains many genomic abnormalities. NPRL2 generally functions as an anti-tumor gene in many cancers, including renal cancer, glioma, and hepatocellular carcinoma. However, NPRL2 reportedly played pro-tumor roles in prostate cancer. Our previous studies have revealed that NPRL2 acted as a functional tumor suppressor in the CRC cell lines HCT116 and HT29, enhanced the sensitivity of HCT116 cells to oxaliplatin and 5-fluorouracil, and was downregulated at both the blood and tissue levels in CRC patients compared with that in healthy individuals.

Resistance to chemotherapeutic drugs, such as 5-fluorouracil (5-FU) and oxaliplatin (L-OHP), significantly limits the successful treatment of patients with CRC. Since in vitro experiments have not fully evaluated the feasibility and effectiveness of CRC treatment, it is necessary and important to establish a suitable CRC xenograft model to investigate the pathological state of CRC and evaluate treatment effects. Our study aims to confirm whether NPRL2 plays anti-tumor roles in CRC using a nude mouse xenograft model and explore the effects of NPRL2 on drug sensitivity toward 5-FU and L-OHP.

**Materials And Methods**

**Ethical Statement**

Our study was approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University. All nude mouse experiments were approved by the Animal Care and Use Committee and conducted in accordance with the official recommendations of the Care and Use Laboratory Animals of the Second Affiliated Hospital of Harbin Medical University.

**Cell Lines, Construction Of Recombinant NPRL2 Lentiviral Vectors, And Transfection**

HT29 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum at 5% CO₂ at 37°C in 95% humidity. Recombinant NPRL2 lentiviral vectors were constructed and transfected into HT29 cells as previously described. The protein expression of NPRL2 was measured using Western blot 72 h after vector transfection.

**Animal**

BALB/c nude mice (nu/nu, male, 5–6 weeks of age, 17–20 g) were used to establish the CRC subcutaneous xenograft model. Mice were maintained in a specific pathogen-free environment in a 12 hr light/dark cycle at 40–60% humidity and 22–24°C with sterilized food and tap water ad libitum.

**Tumor Xenograft Model With Transfected HT29 Cells**

A total volume of 150 μl of HT29 cells (2 × 10⁶ cells), non-transfected (denoted as Control) or transfected with enhanced green fluorescent protein (eGFP)-tagged mock vector (denoted as Mock vector) or NPRL2-eGFP (denoted as NPRL2), were inoculated subcutaneously into nude mice. The mice were monitored daily and tumor nodules were measured with a caliper. Tumor volume was evaluated with the following formula: volume = width² × length/2.

**In Vivo Tail Vein Metastatic Assay**

The experimental mice were first inoculated subcutaneously with normal HT29 cells (2 × 10⁶ cells) to form the xenograft model. When the tumors reached a volume of approximately 150 mm³ at around two weeks post-cell injection, the mice received tail vein injection of 4 × 10⁸ lentiviral particles assembled with (termed NPRL2) or without (termed Control) vector encoding NPRL2 in 0.2 mL of phosphate-buffered saline (PBS). Other mice received tail vein injection of either 10 mg/kg 5-FU or 10 mg/kg L-OHP with (termed NPRL2 + 5-FU or NPRL2 + L-OHP) or without (termed 5-FU or L-OHP) 4 × 10⁸ lentiviral particles assembled with vectors encoding NPRL2. After seven days, tumor tissues were collected for HE staining, TUNEL assay, immunohistochemistry, and Western blot.

**Western Blot**

Tissues (20 mg) were crushed and lysed in 200 μl of radio-immunoprecipitation assay buffer (Beyotime Biotechnology, Shanghai, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Sigma-Aldrich, St Louis, MO, USA)
fluoride for 30 min and protein concentration was quantified by a bicinchoninic acid protein assay kit (Beyotime Biotechnology). Proteins (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline/Tween 20 (TBST) and then incubated at 4°C overnight with primary antibodies against p-protein kinase B (p-Akt, rabbit mAb; 1:500; cat no. ab89333), caspase-3 (rabbit mAb; 1:500; cat no. ab4051), Bax (rabbit mAb; 1:1,000; cat no. ab53154), Bcl-2 (rabbit mAb; 1:2,000; cat no. ab196495), caspase-7 (rabbit mAb; 1:2,000; cat no. ab181579), multidrug resistance protein 1 (MRP1, mouse mAb; 1:500; ab32574), and NPRL2 (rabbit mAb; 1:1,000; cat no. ab192331) and P-glycoprotein (P-gp, rabbit mAb; 1:500; cat no. ab103477) from abcam (Cambridge, MA, USA), GAPDH (rabbit mAb; 1:1,000; cat no. 2118) from Cell Signaling technology (Danvers, MA, USA). The membranes were then washed, incubated with goat anti-rabbit IgG (1:10,000; cat no. PAB160011) or goat anti-mouse IgG (1:10,000; cat no. PAB160009) secondary antibodies (Bioswamp, Myhalic Biotechnology Co., Ltd., Wuhan, China). Signals were detected by an enhanced chemiluminescence system (GE Health, USA) according to the manufacturer’s protocol.

HE Staining
Harvested tumor tissues were fixed in 10% buffered formalin for 24 h, paraffin-embedded, sectioned, and subjected to HE staining. Results were observed under a light microscope (Nikon, Japan).

TUNEL
TUNEL assay was performed to detect apoptotic cells using the in situ Cell Death Detection Kit, POD (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s protocol. Briefly, 4-μm tissue sections were deparaffinized, rehydrated, and treated with 20 μg/mL proteinase K for 15 min at room temperature. The TUNEL reaction mixture was added, and the sections were washed, counterstained with hematoxylin, and observed under a light microscope (Nikon, Japan).

Immunohistochemistry
Immunohistochemistry was performed to detect the expression of NPRL2 in tumor tissues. Briefly, 4-μm paraffin-embedded sections were heated for 1 h at 68°C. After deparaffinization and rehydration, antigen retrieval was performed by microwave in 10 mmol/L citrate buffer (pH 6.0) twice for 10 min each. Endogenous peroxidase was inhibited with 3% H2O2 for 10 min. Sections were blocked with 2% bovine serum albumin in PBS for 30 min. Sections were incubated with mouse NPRL2 antibody (1:200 dilution; cat no. sc-376986, Santa Cruz) and subsequently with horseradish peroxidase-linked goat anti-mouse secondary antibodies (1:1000; cat no. A0216, Beyotime). The nuclei were counterstained with hematoxylin and the samples were observed using a Nikon microscope (Nikon, Japan).

Statistical Analysis
All data are presented as the mean ± standard error of the mean. Differences between three or more groups were analyzed using one-way analysis of variance. All statistical analyses were performed using the SPSS 16.0 software. All figures were prepared using GraphPad Prism 5 and Adobe Photoshop CS6 software. A two-tailed value of P < 0.05 was considered to be statistically significant.

Results

Lentivirus Transfection
Transfection was evaluated with eGFP expression. Cells transfected with NPRL2 lentiviral vectors showed higher protein level of NPRL2 than that in control and in cells transfected with mock vectors (Figure 1).

Overexpression Of NPRL2 Suppressed Tumor Growth
The anti-tumor function of NPRL2 in vivo was analyzed by examining the tumor weight and volume, which were indicative of tumor inhibition, in the CRC-xenografted mice. From Figure 2, we observed that NPRL2 significantly reduced final tumor volume and weight, thus promoting tumor inhibition compared to the control group (P < 0.05).

NPRL2 Promoted Tumor Apoptosis And Inhibited Akt Activation
We observed the histological changes in the CRC-xenografted tumors with HE staining. In the control group, the tissues were densely packed with tumor cells, but NPRL2 transfection resulted in sparse tumor tissues containing some necrotic and inflammatory cells (Figure 3). TUNEL assay showed that NPRL2 transfection induced tumor apoptosis (the brown) to a higher degree than that in the control group.
Immunohistochemistry was performed to assess the expression of NPRL2, which was the highest in NPRL2-transfected tumor tissues. To further understand the mechanism underlying the effect of NPRL2 on CRC, we measured the protein expression of caspase-3, caspase-7, Bax, Bcl-2, and p-Akt by Western blot. We showed that caspase-3 and caspase-7 were activated, Bax was upregulated, and Bcl-2 and p-Akt were downregulated by NPRL2.

NPRL2 Enhanced Therapeutic Response Of Tumors To L-OHP And 5-FU

To explore the effect of NPRL2 in combination with L-OHP or 5-FU, NPRL2-transfected CRC-xenografted mice were treated with L-OHP or 5-FU (Figure 4). The combination of NPRL2 with L-OHP or 5-FU significantly reduced the final tumor volume and weight, resulting in higher tumor inhibition compared to that induced by NPRL2 transfection alone ($P < 0.05$). These observations suggested that NPRL2 effectively enhanced the inhibitory effects of L-OHP and 5-FU on in vivo CRC cell growth.

NPRL2 Enhanced Tumor Apoptosis Induced By L-OHP And 5-FU

HE staining and TUNEL assay revealed changes in tumor histology, showing that the combination of NPRL2 transfection and the two anti-tumor drugs increased tumor cell apoptosis (Figure 5). To evaluate the expression of NPRL2 in tumors treated with L-OHP or 5-FU, immunohistochemistry was performed (Figure 5). The combination of NPRL2 transfection with L-OHP or 5-FU treatment resulted in higher NPRL2 expression than that induced by single L-OHP or 5-FU treatment.

NPRL2 Sensitized Tumors To L-OHP And 5-FU Through P-gp And MRPI

To further explore the mechanism underlying the anti-tumor activity of NPRL2 in combination with L-OHP or 5-FU, we performed Western blot to examine the protein levels of Bel-2, Bax, caspase-3, caspase-7, p-Akt, P-gp, and MRPI in CRC-xenografted tumors (Figure 6). The combination of NPRL2 transfection with either L-OHP
Figure 3 NPRL2 promoted tumor apoptosis. Tumor sections were subjected to HE staining, TUNEL assay, and immunohistochemistry for NPRL2. Histopathological changes were observed under a light microscope (200×). The protein expression of caspase-3, caspase-7, Bax, Bcl-2, and p-Akt was determined by Western blot.

Figure 4 NPRL2 enhanced inhibitory effects of L-OHP and 5-FU on tumor growth. Final tumor volume, weight, and tumor inhibition were measured after NPRL2 transfection, L-OHP or 5-FU treatment, or combination of NPRL2 transfection with L-OHP or 5-FU. *P < 0.05 vs control, n = 3.
or 5-FU significantly reduced the protein expression of the anti-apoptosis protein Bcl-2, the drug resistance-related proteins P-gp and MRP1, and the PI3K-related factor p-Akt, whereas that of the pro-apoptosis proteins caspase-3, caspase-7, and Bax was significantly increased.

**Discussion**

CRC is the fourth most deadly cancer in the world and its incidence is increasing, thus it is urgent for medical and scientific researchers to develop effective treatments for CRC. NPRL2, a tumor suppressor candidate, is expressed in many normal tissues. Previous research has demonstrated that the reduction of NPRL2 led to a lack of anti-tumor activity in several cancers, such as hepatocellular carcinoma, lung cancer, and renal cancer, and glioma. Yogurteu et al have found that decreased expression of NPRL2 mRNA might lead to the progression of CRC. Our previous clinical study has shown that NPRL2 mRNA was decreased in both the blood and colorectal carcinomas of CRC patients. However, the specific mechanisms of NPRL2 in CRC is not clear. An abundance of evidence has confirmed that the restoration of NPRL2 expression...
was mainly beneficial in inhibiting tumorigenicity and enhancing autophagy in several malignant tumor cells.\textsuperscript{18,19} Our previous studies demonstrated the anti-tumor functions of NPRL2 in HT29 and HCT116 cells, as NPRL2 promoted cell apoptosis, inhibited cell proliferation and migration, and induced cell cycle arrest.\textsuperscript{10} In this study, we further explored the influence of NPRL2 on CRC in vivo. The protein expression of p-Akt decreased significantly in NPRL2-transfected tumors. Constitutively active Akt has been found in a variety of cancers in humans.\textsuperscript{20} In addition, the anti-apoptosis factor Bcl-2 was reduced while the pro-apoptosis factors Bax, caspase-3, and caspase-7 were increased in tumors transfected with NPRL2 and treated with L-OHP or 5-FU. In addition, the expression of p-Akt protein was decreased in these tumors. Uncontrolled activation of Akt results in cancer development and thus, inhibition of Akt activation is a dramatic strategy for cancer treatment.\textsuperscript{27} Multidrug resistance (MDR) is a major cause of failure in cancer chemotherapy. P-gp is the most characterized MDR transporter and has been recognized as a target to overcome MDR in cancer therapy.\textsuperscript{28,29} The MRPs, especially MRP1, promote resistance against chemotherapeutics.\textsuperscript{30} Our in vivo study showed that NPRL2 decreased the expression of P-gp and MRP1.

In conclusion, the present work indicated that NPRL2 exerts anti-tumor effects through regulating apoptotic factors and Akt activation. Moreover, NPRL2 enhanced the sensitivities of CRC cells toward L-OHP and 5-FU through inhibiting P-gp and MRP1 activation. These mechanisms are likely active in other cancers and might provide a promising strategy for cancer therapies.

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**Disclosure**

The authors report no conflicts of interest in this work.
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