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RESEARCH

Decreased Expression of NPRL2 in Renal Cancer Cells is Associated with Unfavourable Pathological, Proliferation and Apoptotic Features

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Abstract The tumor suppressor gene nitrogen permease regulator-like 2(NPRL2) NPRL2 expressed obviously in many normal human tissues, but reduced in expression in many human tumors significantly. In this study, we detected the expression of NPRL2 in 78 clear cell renal cell carcinoma (ccRCC) by immunohistochemistry and correlated it with clinicopathological parameters. Meanwhile, the function of NPRL2 in human ccRCC was further explored after transfected recombinant expressing plasmids pEGFP-N1-NPRL2 into human renal cancer 786-0 cells. NPRL2 protein showed high expression in 67 of 78 cases of adjacent normal tissues (85.9 %), which was significantly higher than that in ccRCC tissues (23/78, 29.5 %). Clinic pathological analysis showed that NPRL2 expression was significantly correlated with histological grade (P=0.044), TNM stage (P=0.025) and lymph node metastasis (P=0.028). MTT assay demonstrated that NPRL2 could obviously inhibit renal cancer cell proliferation. Flow cytometric analysis revealed that NPRL2 could induce renal cancer cells apoptosis and arrest the cell cycle in G0/G1 phase. In conclusion, NPRL2 is closely correlated to unfavourable pathological, proliferation and apoptotic features in ccRCC.

Keywords NPRL2 · Renal cancer carcinoma · Transfection · Proliferation · Apoptosis

Yongyong Tang and Li Jiang contributed equally to this work.

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Background

Renal cell carcinoma (RCC) is the most common kidney cancer and represents approximately 3 % of all adult malignancies [1]. The most common histological subtype is clear cell RCC (ccRCC), comprising 70 % of all RCC cases [2]. The incidence of this type of carcinoma presents a rising tendency in recent years. It has increased from 54,390 new cases estimated for 2008 to 65,150 for 2013 in the United States [1, 3]. With the improvement of diagnostic techniques, more patients with RCC have been diagnosed at an early stage. However, the number of estimated deaths for 2013 is still over 13,000 in the United States [1]. Unfortunately, there are a considerable number of RCC cases were diagnosed as the late stage and more than one third of patients with RCC have evidence of metastases at the time of diagnosis [4]. At present, traditional surgical treatment is still the main therapy for early and local RCC, about 30 % of patients would experience metastasis after surgery [5]. For patients with unresectable and/or metastatic RCC, the therapeutic options are limited, neither chemotherapy nor radiotherapy is very effective. However, with the further research and application on gene therapy, it has become a research hotspot of renal cancer especially in middle-late stage of renal cancer treatment [6, 7].

Tumor suppressor gene nitrogen permease regulator-like 2(NPRL2) is also called tumor suppressor candidate gene 4(TUSC4), widely exists in all kinds of human tissues and is highly conserved in different kinds of biological species. NPRL2 is identified as a tumor suppressor gene located in the 120 kb minimum deletion region on human chromosome 3p21.3 [8]. NPRL2 expressed in many normal tissues including the heart, liver, brain, kidney and pancreas [9], but its expression was significantly reduced in a variety of tumor such as lung cancer, renal cancer, liver cancer, breast cancer, nasopharynx cancer and ovarian cancer, the mechanism

remains unknown [10–14]. New researches suggest that tumor suppressor gene played an importance role in DNA mismatch modification, the regulation of cell cycle signal transduction and apoptosis biology function. And NPRL2 has received considerable attention about interfering with the growth of a variety of tumor cells [15, 16]. Thus, we deduced that the occurrence of renal cancer is related to the inactivation of tumor suppressor gene NPRL2.

NPRL2 is a newly discovered tumor suppressor gene. Numerous studies have showed that it may be related to the occurrence, development and prognosis of tumor. The study by WANG et al. demonstrated that NPRL2 showed strong growth inhibitory activity in small cell lung cancer cell line U2020 and renal carcinoma cell line KRC/Y cells almost 90 % [17]. And in the paper of Senchenko et al., NPRL2 showed reduced expression in primary non-small cell lung cancer [18]. Moreover, the combination of NPRL2 and cisplatin can resensitize cisplatin nonresponders to cisplatin treatment through the activation of the DNA damage checkpoint pathway, leading to cell arrest in the G2/M phase and induction of apoptosis [19]. Furthermore, the mechanistic studies by SATOSHI OTANI et al. suggested that higher NPRL2 mRNA expression could function as an independent prognostic factor for overall survival in hepatocellular carcinoma patients [20]. We can conclude from the former studies that the expression of NPRL2 may play diverse roles in cancer progression and could be a promising therapeutic target.

At present, the studies on NPRL2 are almost focusing on some basic aspects, the expression pattern, clinical relevance, and biological function of NPRL2 in ccRCC have so far not been investigated, the specific mechanism and effect needed further probing. In this study, we detected the protein expression patterns in ccRCC tissues and the relationships between NPRL2 expression and diverse clinicopathological characteristics. Then, to observe the effects of NPRL2 expression on the proliferation of ccRCC cells, we used gene transfection technology to transfect recombinant plasmid pEGFP -N1-NPRL2 into the renal cancer cell line 786-0.

Materials and Methods

Patients and Surgical Specimens

The study was approved by the Institute Research Ethics Committee of Chongqing Medical University and prior patient's consent was obtained. ccRCC tissues and paired adjacent nontumor renal tissues were obtained from 78 primary patients who underwent radical nephrectomy in the Department of Urology, First Affiliated Hospital of Chongqing Medical University between 2010 and 2012. No patient received chemotherapy or radiotherapy before surgery. There were 43 male and 35 female, the median age of the patients was 59 years (range, 28–86 years). The clinic data were abstracted from the medical records. The tumor tissues were confirmed as ccRCC by a pathologist of the hospital. The tumor specimens were classified primarily according to UICC 2009 TNM tumor staging system [21], and nuclear grading was based on the Fuhrman criteria [22].

Construction of Recombinant Expressing Plasmids

The human gene NPRL2 (Shanghai sunbio medical biotechnology co., LTD, shanghai, China) was served as the target gene and amplified by PCR. The sequences of the primers were as follows: forward: 5′-GGACTCAGATATGGGCAG CGGCTGCCG-3′ and reverse: 3′-GGCGACCGGTCTTC CAGCAGATGATGATGTTGG-5′ (amplicon size 1,180 bp). Restriction enzyme cutting site XhoI-F and protective bases was added to forward primer. Restriction enzyme cutting site BamHI-R and protective bases was added to reverse primer. The length of the product was 1,180 bp after amplified. The PCR product and plasmid pEGFP-N1 were double digested with XhoI-F and BamHI-R, respectively. After digestion, the NPRL2 cDNA sequence was purified and then recombined with eukaryotic expression vector pEGFP-N1 (Clinical and diagnostic laboratory of Chongqing medical university). Then we transformed prepared cells and screened positive colonies through the culture medium containing kanamycin, extracted the plasmid by without endotoxin plasmid extraction kit (Omega), named the recombinant plasmid as pEGFP-N1-NPRL2. The recombinant plasmid was sequenced in Shanghai sunbio medical biotechnology co., LTD (Shanghai, China). The DNA sequencing showed that NPRL2 was ligated to the pEGFP-N1 vector correctly.

Transfection and Cell Culture

Human renal cancer 786-0 cells were obtained from Clinical and diagnostic laboratory of Chongqing medical university and cultured in medium RPMI 1640 containing 10 % fetal bovine serum. After 24 h incubation at 37 °C with 5 % CO2, logarithmic growth phase cells in good condition were digest by 0.25 % trypsin + ethylenediaminetetraacetic acid. After calculating the cell number, cells were seeded into 24-well plates at 2.5×10^5 cells per well until reached 70–80 % confluence. The cells were then transfected by using HG_TransGene (Health & Gene, Shanghai, China) according to the manufacturer's instructions. The expression of green fluorescent emission protein was detected by fluorescent microscopy 24 h after transfection. The cells transfected with pEGFP-N1-NPRL2, pEGFP-N1 plasmid were served as the pEGFP-N1-NPRL2 group and the pEGFP-N1 group, respectively. Untreated cells from the same batch were treated as the blank control group.

Immunohistochemistry Staining

Tissues were cut in 4-μm-thick sections and mounted onto glass slides. The slides were deparaffinized with xylene and dehydrated through a graduated alcohol series. Endogenous peroxidase activity was blocked with 3 % H_2O_2 at room temperature for 20 min. To unmask the epitopes, the sections were boiled in 10 mmol/l citrate buffer (pH 6.0). Non-specific antibody binding was performed by incubations with 10 % normal goat serum for 30 min. Sections were incubated at 4 °C overnight with the polyclonal mouse anti-human NPRL2 antibody (Abcam, Cambridge, MA, USA) diluted at 1:200. The negative control was performed by replacing the primary antibody with antibody diluent. After phosphate-buffered saline (PBS) washing, sections were incubated using a anti-mouse Immunohistochemistry kit (Maixin Bio., Fujian, China) at 37 °C for 20 min. After washing with PBS, the tissue sections were stained for 5 min with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Maixin Bio., Fujian, China), counterstained with hematoxylin, followed by dehydration and coverslip mounting. Negative controls were done by omission of the primary antibody and substituting it with phosphate-buffered saline.

The stained sections were reviewed by two independent observers under double-blinded conditions without prior knowledge of the clinicopathological data of the patients. Immunohistochemical staining of NPRL2 was evaluated using a semi-quantitative scoring system for both color intensity and the percentage of positive cells. Intensity was grade as follows: negative scored as 0, mild scored as 1, moderate scored as 2 and strong scored as 3. The percentage of positive cells was evaluated quantitatively and scored as follows: 0 for staining <5 % of total cells counted, 1 for 5–25 %, 2 for 26–50 %, 3 for 51–75 % and 4 for 76–100 %. A score was calculated by multiplying the intensity by percentage of stained cells. Scores of multiplication were graded as follows: −, 0; +, 1–3; ++, 4–8; +++, 9–12. Additionally, for statistical analysis, the − and 1+ cases were pooled into the low-expression group, and the 2+ and 3+ cases were pooled into the high-expression group [23].

Real-Time Quantitative PCR

The cells in the pEGFP-N1-NPRL2 group, the pEGFP-N1 group and the blank control group were respectively seeded in 6-well plate and cultured until reached 70 %~80 % confluence. After culturing for 48 h, total RNA was respectively extracted from the cells of the three groups by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 20 μl on a 7900 Real-Time PCR System (Applied Biosystems). Prime Premier 5.0 was used to design the NPRL2 and β-actin primer. And the primers were synthesized in Shanghai Sunbio Medical Biotechnology Co., LTD (Shanghai, China). The sequences of the primers were as follows: NPRL2 5′ GTAGGAGGGA CTACCCACAGC 3′ (forward), and NPRL2, 5′TACAGGCT GCCACAGCTATG3′ (reverse), amplification fragment was 315 bp; β-actin 5′ATAGCACAGCCTGGATAGCAACGT AC-3′ (forward), and 5′CACCTTCTACAATGAGCTGCGT GTG3′ (reverse), amplification fragment was 678 bp. The PCR initial activation step was at 94 °C for 2 min, followed by 30 s at 94 °C for denaturation, 30 s at 62 °C for annealing, and 1 min at 72 °C for extension. The cycle number was set as 30 cycles.

Western Blot Analysis

Cells were lysed in RIPA cell lysis buffer (Sangon Biotech Co., Ltd., Shanghai, China) containing protease and phosphatase inhibitors. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, USA). Proteins were separated by 10 % SDS-PAGE and were transferred to PVDF membrane (Millipore Company, Shanghai, China). The membrane was blocked with 5 % nonfat milk for 1 h at room temperature with rocking. Polyclonal anti-NPRL2 (1:1000; Abcam, Cambridge, MA, USA) and anti-β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used as primary detection reagents and horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the secondary detection reagent. The blots were visualized with the enhanced chemiluminescence (ECL) blotting reagents (Amersham Biosciences, USA) according to manufacturer's instructions.

MTT Assay for Cell Proliferation

786-0 cells were seeded in 96-well microtitre plates at a density of 5×10^3 cells per well, and cultured for 24 h, 48 h and 72 h at 37 °C with 5 % CO2. At the end of the culture, 20 μl MTT (5 g/l) was added to the cells which were then cultivated for an additional 4 h period at 37 °C. Subsequently, the supernatant fluid was carefully removed and followed by adding 200 μL per well DMSO to the cells which were agitated for 10 min. Absorbance was measured at 490 nm by an ELISA reader. Untreated 786-0 cells served as controls. Each experiment was repeated three times. Cell proliferation curve was drawn based on the D490 value.

Flow Cytometric Analysis of Cell Apoptosis and Cell Cycle

Cells were harvested 48 h after transfection and washed three times with cold PBS. Annexin v-FITC and PE Apoptosis Detection Kit (BD Co., United States) was used to determine cell apoptosis. 10 μL 7-ADD and 5 μL AnnexinV-PI were added to the cells, which were incubated without shaking at

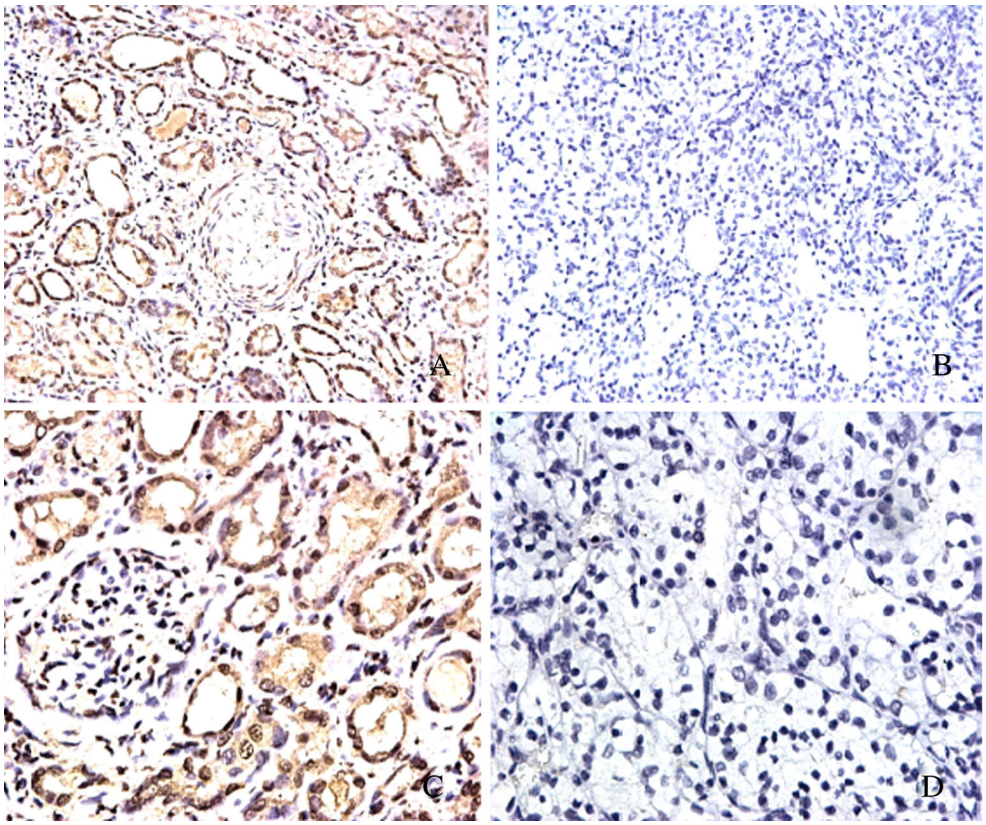

Fig. 1 Immunohistochemical analysis of NPRL2 expression. NPRL2 protein expression was obviously higher in adjacent nontumor tissues (**a** and **c**) than in ccRCC tissues (**b** and **d**). Magnifications ×100 (**a** and **b**) and ×400 (**c** and **d**)

room temperature for 20 min in the dark. After about 400 μL of buffer was added, flow cytometry was used to assay the apoptosis rate of each group.

The transfected cells were harvested and fixed in 70 % ice-cold ethanol at −20 °C overnight. Then cells were washed with PBS and treated with RNase (Sigma) and 50 μg/mL propidium iodide (PI) (Sigma), and incubated at 37 °C for 30 min in the dark. The cell cycle phase distributions were analyzed with flow cytometer (FACS CantoII, BD Bioscience, USA) according to the manufacturer's guidelines. Experiments were performed in triplicate for each sample.

Statistical Analysis

The chi-squared and Fisher's exact tests were used to calculate the relationships between expression of NPRL2 and clinicopathologic variables. For other comparisons, analysis of variance was used to analyze the correlation of data among

Table 1 Immunohistochemical analysis of NPRL2 expression in 78 ccRCC tissues and adjacent nontumor tissues

Renal tissues	N	NPRL2 protein expression		X^2-value	P-value
		Low no. (%)	High no. (%)		
ccRCC tissues	78	55(70.5)	23(29.5)	50.844	<0.001
Adjacent nontumor tissues	78	11(14.1)	67(85.9)		

Table 2 NPRL2 protein expression in 78 ccRCC tissues determined by immunohistochemistry

Variables	N	NPRL2 protein expression		X^2-value	P-value
		Low no. (%)	High no. (%)		
Age				0.018	0.892
<60	45	32(71.1)	13(28.9)		
>=60	33	23(69.7)	10(30.3)		
Gender				0.026	0.873
Female	35	25(71.4)	10(28.6)		
Male	43	30(69.8)	13(30.2)		
Histological grade				6.270	0.044
G1	22	12(54.5)	10(45.5)		
G2	23	15(65.2)	8(34.8)		
G3/4	33	28(84.8)	5(15.2)		
TNM stage				5.015	0.025
I/II	46	28(60.9)	18(39.1)		
III/IV	32	27(84.4)	5(15.6)		
Lymph node metastasis				4.855	0.028
Positive	28	24(85.7)	4(14.3)		
Negative	50	31(62.0)	19(38.0)		

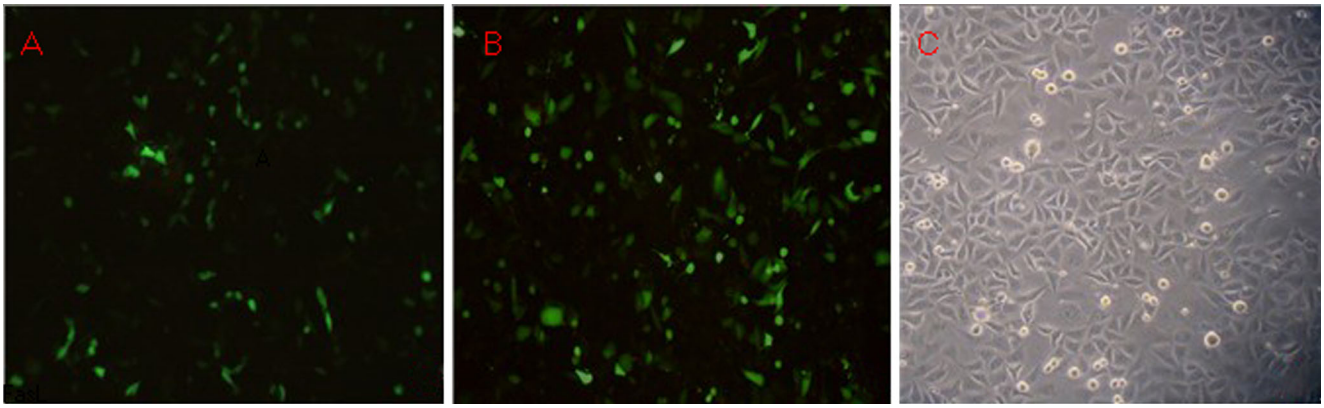

Fig. 2 GFP expression in three groups of 786-0 cells by fluorescence microscope. Magnification is 200. The image of pEGFP-N1-NPRL2 group (**a**), pEGFP-N1 group (**b**) and blank control group (**c**)

three or more groups, and *t* test was used for pairwise comparison between groups. SPSS 17.0 was used for statistical assessment. In all tests, $P<0.05$ was considered to indicate a statistically significant difference and all p-values were two-sided.

Results

Changes in NPRL2 Expression in Cancer Tissues and Adjacent Nontumor Tissues in ccRCC Patients Analyzed by Immunohistochemistry

The expression of NPRL2 was determined by immunohistochemical analysis in 78 paraffin-embedded ccRCC cancer tissues and matched adjacent nontumor tissues. NPRL2 protein contents were obviously higher in adjacent nontumor tissues than in cancer tissues ($P<0.001$, Fig. 1 and Table 1).

Immunohistochemistry Analysis of the Relationships Between NPRL2 Protein Expression and Clinical Features in 78 ccRCC Tumor Samples

Immunohistochemistry analysis was performed in 78 paraffin-embedded ccRCC tumor samples to further assess the correlation between NPRL2 expression and various clinicpathological parameters. As shown in Table 2, patients with higher histological grade ($P=0.044$), higher TNM stage ($P=0.025$) or have lymph node metastasis ($P=0.028$) were more likely to have lower NPRL2 protein expression, but there was no significant association between NPRL2 expression and patients' age ($P=0.892$) and gender ($P=0.873$).

Sequencing Identification

Recombinant plasmid pEGFP - N1 - NPRL2 was forward and reverse sequenced by using universal primers. The homology of recombinant plasmid with the target gene was up to 100 % through NCBI BLAST.

Fluorescence Microscopy Analysis of Cell Transfection

Fluorescence microscopy was used to detect the expression of green fluorescent emission protein after transfection for 24 h. The result showed that the green fluorescent was observed in the pEGFP-N1-NPRL2 group and the pEGFP-N1 group, but not in the blank control group. It suggests that the recombinant plasmid had been successfully transfected into the human renal cancer 786-0 cells (Fig. 2).

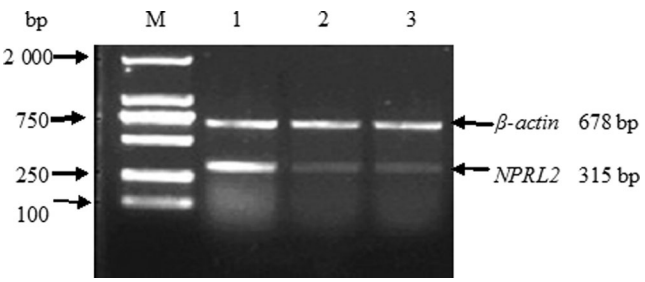

Fig. 3 Detection of expression of NPRL2 RNA of 786-o cells by PCR. The expression of NPRL2 mRNA level was significantly higher in pEGFP-N1-NPRL2 group (1) than that in pEGFP-N1 group (2) and Blank control group (3) ($p<0.05$). The maker is DNA marker (DL 2 000) (M)

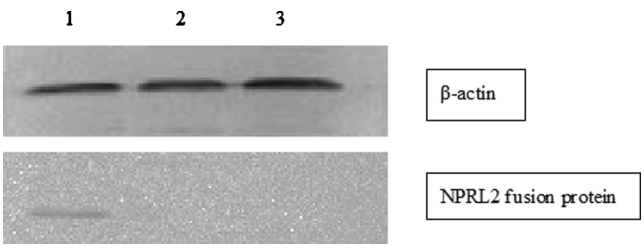

Fig. 4 Expression of NPRL2 in 786-0 cells by western blot. Expression of NPRL2 in pEGFP-N1-NPRL2 group (1), pEGFP-N1 group (2) and blank control group (3)

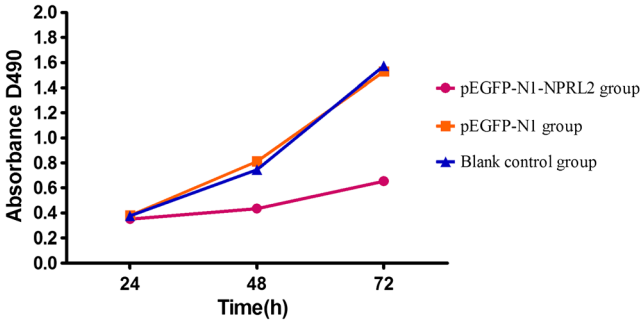

Fig. 5 Cell growth analysis of the transfected cells by MTT. The cell proliferation in pEGFP-N1-NPRL2 group was obviously inhibited compared with pEGFP-N1 group and blank control group, respectively ($P<0.05$)

RT-PCR Analysis of the Expression of NPRL2

Total RNA was extracted from cells of each group after transfection for 48 h and analyzed by RT-PCR, respectively. The result showed that the ratio of the expression of NPRL2 to β-actin grey value in the pEGFP-N1-NPRL2 group, the pEGFP-N1 group and the blank control group were 1.12±0.07, 0.68±0.03, 0.65±0.06, respectively. Compared with the other two groups, pEGFP-N1-NPRL2 group showed a statistically significant difference ($P<0.05$), respectively. There was no significant difference between the pEGFP-N1 group and the blank control group ($P>0.05$). It further suggests that the recombinant plasmid had been successfully transfected into the human renal cancer 786-0 cells and well expressed (Fig. 3).

Western Blot Analysis of the Expression of NPRL2

Total protein was extracted from cells of each group after transfection for 48 h and analyzed by western blot, respectively. The result showed that the ratio of the expression of NPRL2 to GAPDH grey value in the pEGFP-N1-NPRL2 group, the pEGFP-N1 group and the blank control group were 0.30±0.04, 0.10±0.02, 0.09±0.01, respectively. The expression of NPRL2 protein of pEGFP-N1-NPRL2 group was significant increased compared with the other two groups ($P<0.05$), respectively. There was no significant difference between the pEGFP-N1 group and the blank control group ($P>0.05$). This result is consistent with RT-PCR (Fig. 4).

Cell Proliferation Assay

The MTT assay revealed that the cell proliferation in pEGFP-N1-NPRL2 group was obviously inhibited compared with other groups, respectively ($P<0.05$). There was no significant difference between the pEGFP-N1 group and the blank control group ($P>0.05$). The result suggests that tumor suppressor gene NPRL2 could inhibit the proliferation of human renal cancer cell 786-o (Fig. 5).

Apoptosis Analysis of 786-0 Cells

The cell apoptotic rate of the pEGFP-N1-NPRL2 group (18.82±0.40) was significantly higher than that of the pEGFP-N1 group (5.65±0.12) and the blank control group (5.85±0.07) after transfection for 48 h; the difference was statistically significant ($P<0.05$). The difference between the pEGFP-N1 group and the blank control group was not statistically significant ($P>0.05$) (Fig. 6).

Cell Cycle Analysis of 786-0 Cells

The flow cytometric revealed that the G0/G1 phase cells percentage in the pEGFP-N1-NPRL2 group (69.80 %± 1.40 %) was higher than that in the pEGFP-N1 group (46.24 %±1.30 %) and in blank control group (47.03 %± 0.45 %) after transfection for 48 h, while the S phase cells percentage in the pEGFP-N1-NPRL2 group (16.92 %±1.3 %) was lower than that in the pEGFP-N1 group (41.46 %±0.6 %) and in blank control group (40.96 %±1.5 %), respectively. The difference was statistically significant ($P<0.05$). But the

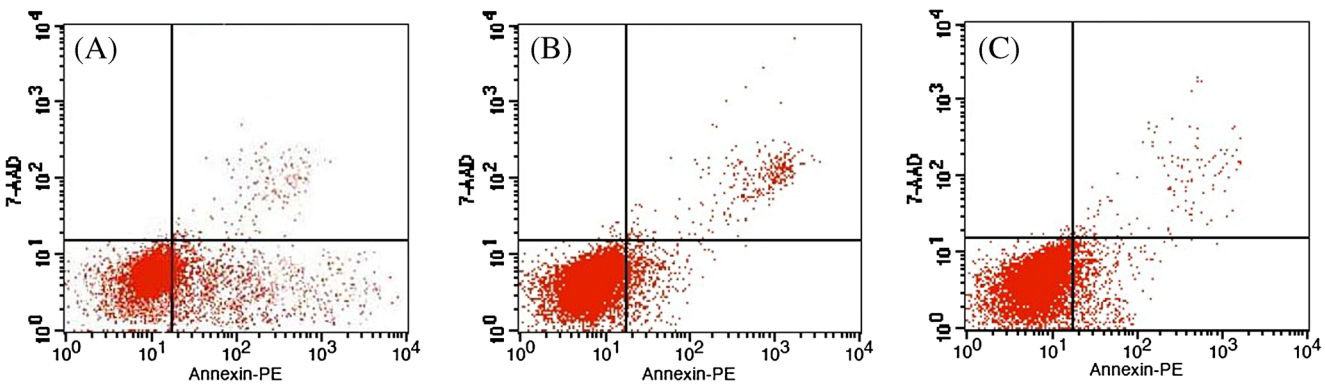

Fig. 6 Apoptosis analysis of 786-0 cells after transfection. The cell apoptotic rate of pEGFP-N1-NPRL2 group (**a**) was significantly higher than that of pEGFP-N1 group (**b**) and blank control group (**c**)

Table 3 The phase distribution of cell cycle by flow cytometric ($\bar{x} \pm s$)

Groups	G_0/G_1	S	G_2/M
pEGFP-N1-NPRL2 group	69.80 %±1.40 %*	16.92 %±1.3 %*	13.28 %±2.08 %*
pEGFP-N1 group	46.24 %±1.30 %	41.46 %±0.6 %	12.30 %±1.92 %
Blank control group	47.03 %±0.45 %	40.96 %±1.5 %	12.01 %±1.87 %

*$P<0.05$, compared with other two groups

difference between the pEGFP-N1 group and the blank control group was not statistically significant ($P>0.05$) (Table 3, Fig. 7).

Discussion

NPRL2 has been reported plays an important role in tumor progression and can be served as an independent prognostic factor in numerous types of human cancers. But we know little about its expression pattern and biological significance in clear cell renal cell carcinoma. In this study, we have observed that NPRL2 protein expression detected by immunohistochemistry staining was significantly lower in ccRCC tissues than that in adjacent nontumor renal tissues. We found that low NPRL2 expression is significantly related to histological grade, TNM stage and lymph node metastasis by analyzing the correlation between the NPRL2 expression levels and the clinicopathological parameters of 78 patients with ccRCC. It suggests that NPRL2 plays an important role in tumorigenesis and progression in ccRCCs. The results is consistent with studies in other human cancers decreased expressing NPRL2 [24–26].

The evidence of NPRL2 decreased expressing in ccRCC cells prompt us to investigate the biological function of NPRL2 in more details through analysis of ccRCC cell lines in vitro. Therefore, we first constructed recombinant plasmid pEGFP - N1 - NPRL2 and then transfected it into renal cancer cells 786-0 by gene subcloning technology. After transfection, we found the expression of NPRL2 in the pEGFP-N1-NPRL2 group was significant increased compared with the pEGFP-N1 group and the blank control group by detecting NPRL2 RNA and protein. And the result of RT-PCR is consistent with western blot. It suggests that recombinant plasmid pEGFP - N1 - NPRL2 was successfully constructed and transfected into cells. Cancers, including ccRCC, have common characteristics: abnormal cell proliferation and growth. Most of the proliferative factors influence cell growth by affecting cell cycle progression. In the current study, we also found tumor suppressor gene NPRL2 could obviously inhibit the proliferation of human renal cancer cell 786-0, which was showed in the cell growth curve of MTT assay. This result is consistent with the research on non-small cell lung cancer (NSCLC) by Ji L [27]. According to the result of flow cytometry, gene NPRL2 significantly altered the proliferation and cell cycle of the 786-0 cells at 48 h after transfection. The G0/G1 phase cells were increased, while S phase cells were decreased. And cell cycle was arrested in G0/G1. So the expression of NPRL2 inhibited G0/G1 to S transition in cell cycle progression, which may explain the mechanism of NPRL2 on ccRCC cell proliferation. These results suggest that that tumor suppressor gene NPRL2 could effectively regulate the checkpoint of renal cancer cell cycle, arrest the cell cycle in G0/G1 phase, inhibit the cell proliferation and induce the cell apoptosis.

The loss of tumor suppressor regulating role because of the inactivation of gene NPRL2 results in the progression of tumor [28]. There may be three kinds of possible mechanisms. Firstly, genomic instability plays an important role in progression. Microsatellite instability (MSI) is a form of genomic instability due to reduced fidelity during the replication of

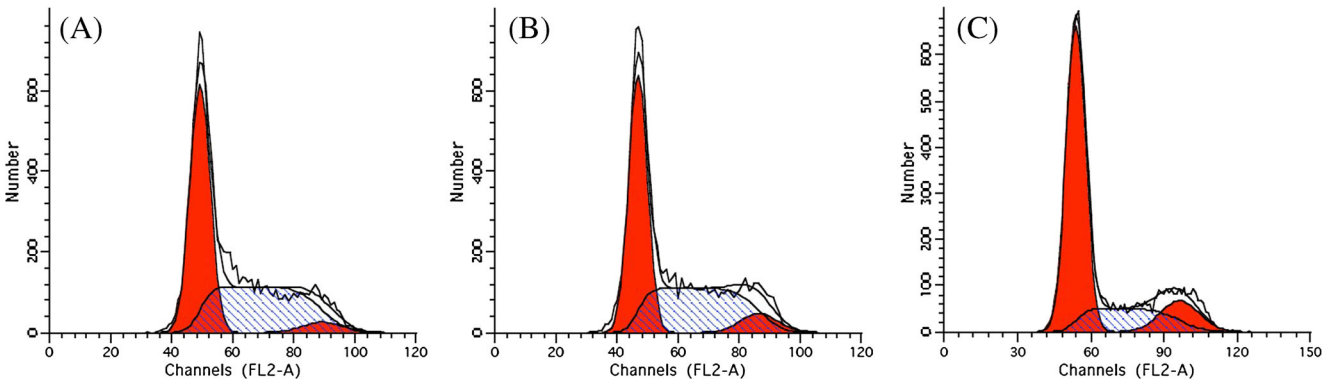

Fig. 7 Cell cycle analysis of 786-0 cells by flow cytometric. The G0/G1 phase cells percentage in pEGFP-N1-NPRL2 group (**c**) was higher than that in pEGFP-N1 group (**b**) and in blank control group (**a**) ($P<0.05$)

repetitive DNA. The occurrence of MSI may be the "strand slippage" phenomenon in the process of DNA replication. Under normal circumstances, the "looped out" region formed in the process of DNA replication can be corrected by mismatch repair system. But when the system goes wrong, such as substring DNA extending continuously, then mutation occurring, then leading to the inactivation of gene NPRL2, resulting in the progression of tumor. To varying degrees, microsatellite instability phenotype occurs in gastric cancer, renal cancer, lung cancer, colorectal cancer, breast cancer, ovarian cancer patients and so on [29, 30]. Secondly, inhibit the activation of 3-Phosphoinositide-dependent protein kinase-1 (PDK1/PDPK1). PDK1 is a key regulator of cell proliferation and survival signal transduction. NPRL2 is a novel PDK1-interacting protein. NH2-terminal 133 amino acid residues of NPRL2 bind to PDK1 to inhibit Src-mediated PDK1 tyrosine phosphorylation, leading to inactivation of Akt and S6K. And NPRL2 expression inactivated Akt and p70 ribosomal protein S6 kinase, causing conduction disorders in the PDK1 downstream signaling pathway, and then inhibiting the cell proliferation [31]. If NPRL2 protein expression decreased, the inhibition mechanism will be broken and the tumor will occur and develop. Thirdly, improve the activity of CHK1 and CHK2 kinase. Both CHK1 and CHK2 are the cell cycle checkpoints. They can arrest the cell cycle and express abnormally in breast cancer, prostate cancer, lung cancer and esophagus cancer [32, 33]. NPRL2 gene can effectively improve the activity of CHK1 and CHK2 kinase to preserve DNA damage repair and the apoptosis of cancer cells caused by cell cycle checkpoint regulation [32]. However, cell cycle regulation disorder caused by the inactivation of gene NPRL2 may result in tumorigenesis.

Conclusions

In summary, our study revealed that NPRL2 protein expression was decreased in the majority of the ccRCC clinical tissue specimens and correlated with histological grade, TNM stage and lymph node metastasis. We also demonstrated that after transfected into non-NPRL2 gene renal cancer cells 786-0, tumor suppressor gene NPRL2 could inhibit the cell proliferation by arresting the cell cycle in G0/G1 phase. These results indicate that tumor suppressor gene NPRL2 is closely correlated to unfavourable pathological, proliferation and apoptotic features in ccRCC, which imply that NPRL2 might be played as a potential therapeutic target for ccRCC.

Conflict of interest The authors declare that they have no conflict of interest.

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