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The impact of NudCD1 on renal carcinoma cell proliferation, migration, and invasion

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Abstract. – OBJECTIVE: Renal cell carcinoma (RCC) is the most common malignant tumor in the urogenital system. Its easily metastatic characteristics greatly reduce the postoperative survival rate. NudCD1, as a proto-oncogene, may be involved in the proliferation, migration, and invasion of renal cell carcinoma cell. This study intends to explore the expression of NudCD1 in renal cancer tissue and its effect on renal cell behavior.

PATIENTS AND METHODS: NudCD1 expression in RCC tissue was tested Western blot. The cellular localization of NudCD1 was detected by immunohistochemistry (IHC). NudCD1 highly expressed RCC cell line was selected. NudCD1 knockdown or overexpression was performed through cell transfection. Cell proliferation, migration, and invasion were assessed by MTT assay, wound scratch assay, and transwell assay, respectively.

RESULTS: NudCD1 mainly located in the cytoplasm and significantly upregulated in RCC tissue compared with adjacent normal control ($p < 0.05$). NudCD1 expressed highest in A498 cell line among several RCC cell lines. NudCD1 expression was positively correlated with cell proliferation, migration, and invasion in A498. NudCD1 may be treated as a key factor in regulating cell behavior.

CONCLUSIONS: NudCD1 significantly increased in RCC and was positively correlated with cell proliferation, migration, and invasion. It could be used as an indicator for the early screening and potential treatment target for RCC.

Key Words:
Renal cell carcinoma, NudCD1, Proliferation, Migration, Invasion.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors in urogenital system with an incidence accounting for about 3% of the whole body malignant tumor[1]. Surgery is the main treatment method. However, the easily metastatic characteristics greatly reduce the survival rate after surgery[2, 3]. Therefore, investigating the molecular related to cancer cell metastasis and invasion is of great significance for the diagnosis and treatment of RCC. It was found that NudC domain containing 1 (NudCD1), as a new member of NudC family distributed in nucleus, is a kind of broad-spectrum tumor antigen or cancer gene[4]. It plays an important role in the process of cell mitosis and affects cell movement through acting on tubulin[5]. It was showed that NudCD1 not only highly expressed in chronic myelogenous leukemia cells[6, 7], but also expressed in lung cancer, melanoma, and prostate cancer tissue and cell lines[8-10]. Moreover, it is likely to be involved in cell mitosis, invasion, and migration. Previous works indicated that NudCD1 may have immunogenicity in a variety of malignancies, thus to be a new target for antigen-specific immunotherapy[11]. However, the role and impact of NudCD1 in RCC have not been clarified.

Therefore, this study aimed to detect the expression and cellular localization of NudCD1 protein in RCC tissues and adjacent normal tissues by IHC. Then, we tested NudCD1 expression in RCC cell lines by Western blot to select the cell line with highest abundance. Next, we applied siRNA or DNA overexpression plasmid to regulate NudCD1 expression level to observe its influence on cell behavior. This paper may provide theoretical basis for the early screening, diagnosis, and treatment of RCC.

Patients and Methods

Patients

A total of 45 cases of human RCC tissues and adjacent normal tissues at least 3 cm away from the tumor margin were collected from the surgery

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between Jan 2016 and Jan 2017. The sample was stored at -80°C. All the subjects had signed informed consent.

Main Materials and Reagents

Total protein extraction kit was purchased from Keygen (Nanjing, China). Western blot RIPA and bicinchoninic acid (BCA) protein quantification kits were obtained from Beyotime (Suzhou, China). NudCD1 antibody was bought from Proteintech (Wuhan, China). Horseradish peroxidase (HRP) labeled goat anti-mouse IgG (H+L) and HRP labeled goat anti-rabbit IgG (H+L) were obtained from ZSbio (Beijing, China). Fetal bovine serum (FBS) and cell medium were provided by Gibco (Rockville, MD, USA). Other reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Main Instruments

Benchtop was obtained from Boxun (Shanghai, China). Gel imaging system was provided by UVP Multispectral Imaging System (Upland, CA, USA). PS-9 semi-dry electrophoresis apparatus was bought from Jingmai Company (Dalian, China). BD FACSCalibur flow cytometry was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Carbon dioxide incubator and Thermo-354 microplate reader were got from Thermo Scientific (Waltham, MA, USA).

Western Blot

The lysis buffer was added with phenylmethylsulfonyl fluoride (PMSF) to make the concentration at 1 mM. A total of 100 mg tissue were lysed in the buffer at 4°C for 15 min and centrifuged at 14000 rpm for 15 min. The protein was moved to a new Eppendorf (EP) tube and quantified by bicinchoninic acid (BCA) method. After boiled for 5 min, the protein was stored at -20°C. The protein was separated by 8-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 1 h. Next, the membrane was incubated in NudCD1 primary antibody (1:1000) at 4°C overnight and washed by Tris-buffered saline-Tween (TBST) for three times. The membrane was further incubated in goat anti-rabbit secondary antibody (1:1000) at 37°C for 2 h. At last, the membrane was developed by chemiluminescence and captured by Bio-Rad system containing Chemi HR camera. The image was analyzed by Gel-Pro Analyzer Version 4.0 software.

Immunohistochemistry

The tissue was embedded by wax and cut into slices. After dewaxing, the sample was incubated in 3% hydrogen peroxide for 20 min to eliminate endogenous peroxidase activity. Next, the sample was incubated in citrate buffer at 95°C for 10 min to repair the antigen and further blocked in goat serum for 20 min. Next, the sample was incubated in NudCD1 primary antibody (1:100) at 4°C overnight and further incubated in HRP labeled secondary antibody. The sample was further developed by diaminobenzidine (DAB) and reduced by hematoxylin. The positive result was scored according to the cell number and staining intensity. Score 0, positive cell number < 5%; score 1, positive cell number between 5% and 25%; score 2, positive cell number between 26% and 50%; score 3, positive cell number between 51% and 75%; and score 4, positive cell number between 76% and 100%. For staining intensity evaluation, score 0, colorless; score 1, faint yellow; score 2, claybank; score 3, sepia. The positive degree was assessed by the product of the two scores. Negative (-), score 0; weak positive (+), score 1-4; positive (++), score 5-8; strong positive (+++), score 9-12.

Cell Culture

RCC cell lines A498, ACHN, 786-0, Caki-1, caki-2, and normal renal cell line RCS were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All the cells were maintained at Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37°C and 5% CO2.

Cell Transfection

NudCD1 siRNA and overexpression vector were designed and synthetized by Genepharma. The cells were seeded in 24-well plate on the previous day before transfection. A total of 1.25 μl siRNA or overexpression vector at 20 μM was solved in 100 μl Opti-MEM medium, while 1 μl Lipofectamine 2000 or LipofectamineTM RNAiMAX was solved in Opti-MEM medium. After 5 min incubation, the two types of solutions were mixed for 20 min. After 4 h incubation, the fluid was changed back to DMEM medium containing 10% fetal bovine serum (FBS).

MTT Assay

MTT assay was performed according to the reference[12]. A498 cells in logarithmic phase were digested by 0.25% trypsin and seeded in 96-well plate at 10 μl. The cells were transfected by siR-

NA or vector. After 42 h incubation, the cells were added with 10 µl MTT solution and further incubated for 4 h. The crystal violet was observed under the microscope. Next, the cells were added with 150 µl dimethyl sulfoxide (DMSO) to dissolve the crystal for 12-15 h. At last, the plate was tested at 490 nm to calculate cell survival rate.

Cell survival rate (%) = (OD value in experimental group – OD value in blank group)/(OD value in control – OD value in blank group)

Scratch Assay

The scratch assay was applied according to the reference[13]. The cells were seeded in 6-well plate and transfected to dysregulate NudCD1 expression. When the cell fusion reached 90%, a 200 µl pipette tip was used to make a scratch at the bottom of the plate. After washing off the removed cells, the plate was observed and captured under the inverted microscope. After 24 h, the plate was captured again to observe cell migration.

Cell Invasion Assay

The Transwell chamber was precooled at 4°C refrigerator, while ECM gum was put into 4°C refrigerator for unfreezing. Then, 50 µl ECM gum were added to the transwell chamber and put into 37°C for 4 h. The cells in logarithmic phase were digested and seeded in the upper chamber at 10000 cells/ml. The medium containing 10% fetal bovine serum (FBS) was added to the lower chamber. At last, the chamber was stained by 0.1% crystal violet and observed under the microscope.

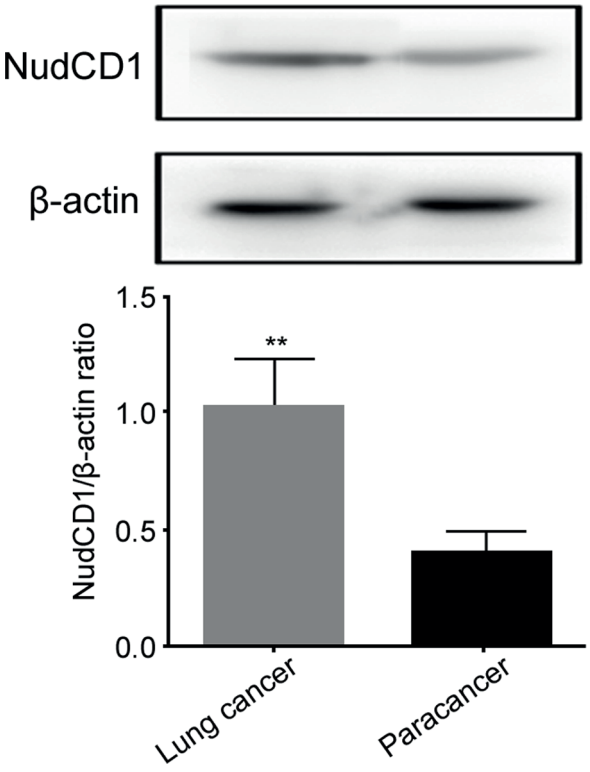

Figure 1. NudCD1 protein expression in RCC tissue and adjacent normal tissue. $^{**}p < 0.05$, compared with adjacent normal tissue.

Statistical Analysis

All data analyses were performed on SPSS 19.0 software (SPSS Inc., Armonk, NY, USA). The measurement data were presented as mean ± standard deviation and compared by t-test or one-way ANOVA. LSD was performed for post-hoc test. $p < 0.05$ was depicted as statistical significance.

Results

NudCD1 Protein Expression in RCC Tissue and Adjacent Normal Tissue

Western blot was adopted to test NudCD1 protein expression in RCC tissue and adjacent normal tissue. NudCD1 expression significantly upregulated in RCC tissue compared with adjacent normal control ($p < 0.05$) (Figure 1).

NudCD1 Expression Changes and Cellular Localization in RCC Tissue and Adjacent Normal Tissue

IHC was applied to observe NudCD1 localization in the RCCC tissue (Figure 2). NudCD1 presented weak positive in adjacent tissue, while it exhibited strong positive in RCC tissue. NudCD1 mainly located in the cytoplasm of renal parenchymal cells, including renal tubule and collecting tube, but not glomerulus. It suggested that NudCD1 level significantly enhanced in RCC tissue compared with normal control, indicating that NudCD1 may be involved in the process of RCC.

NudCD1 Expression in RCC Cell Lines

To further investigate the role of NudCD1 in RCC, we selected RCC cell lines A498, ACHN, 786-0, Caki-1, and Caki-2, and normal renal cell line RCS to test NudCD1 protein expression (Figure 3). Compared with RCS, NudCD1 level markedly increased in multiple RCC cell lines. NudCD1 expressed highest in A498 cell line among several RCC cell lines. Thus, we selected A498 cell line for the following experiments.

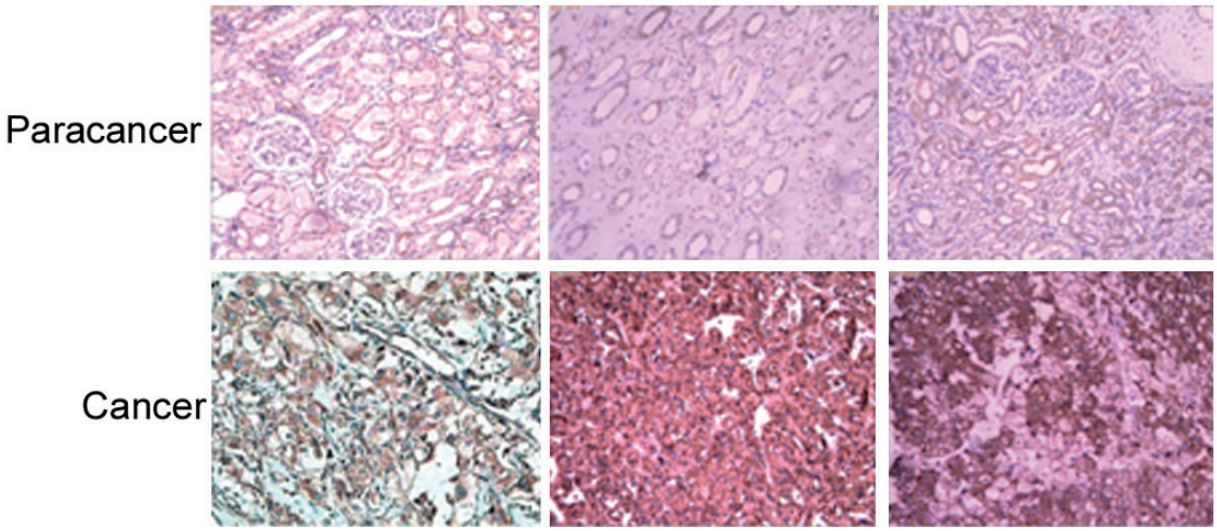

Figure 2. NudCD1 expression changes and cellular localization in RCC tissue and adjacent normal tissue.

The Impact of NudCD1 on Cell Proliferation

To explore the impact of NudCD1 on cell proliferation, we transfected NudCD1 siRNA or overexpression plasmid to change NudCD1 expression in A498 cells. As shown in Figure 4A, NudCD1 level markedly decreased or upregulated in A498 cells after siRNA or plasmid transfection. Moreover, cell proliferation significantly enhanced after NudCD1 overexpression, where restrained after NudCD1 knockdown compared with control (Figure 4B). It indicated that NudCD1 participated in the regulation of cell proliferation.

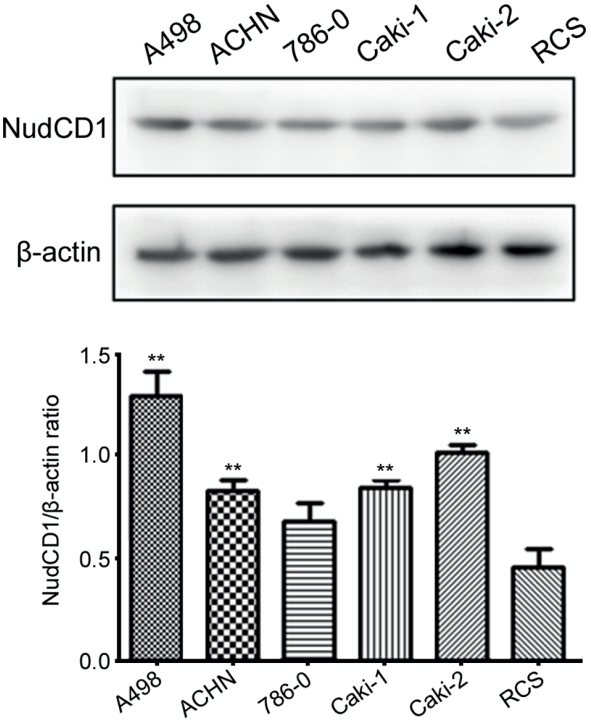

Figure 3. NudCD1 expression in RCC cell lines. $^{**}p < 0.05$, compared with RCS cells.

The Influence of NudCD1 on Cell Migration

Scratch assay was performed to evaluate the influence of NudCD1 on cell migration. As shown in Figure 5, cell migration markedly increased after NudCD1 overexpression, whereas it apparently weakened after NudCD1 knockdown. It showed that NudCD1 level was positively correlated with cell ability level.

The Effect of NudCD1 on Cell Invasion

Transwell assay was selected to test cell invasion[14]. The cell number was counted at 4, 8, 12, and 24 h. As shown in Figure 6, cell invasion was significantly restrained after NudCD1 knockdown, while it was significantly elevated after NuDC1 overexpression, revealing that NudCD1 may affect cell invasion.

Discussion

RCC is one of the most common malignant tumors of the genitourinary system with an incidence accounting for about 3% of the whole body malignant tumor[1]. There are 200,000 cases of new cases and more than 100,000 deaths each year.

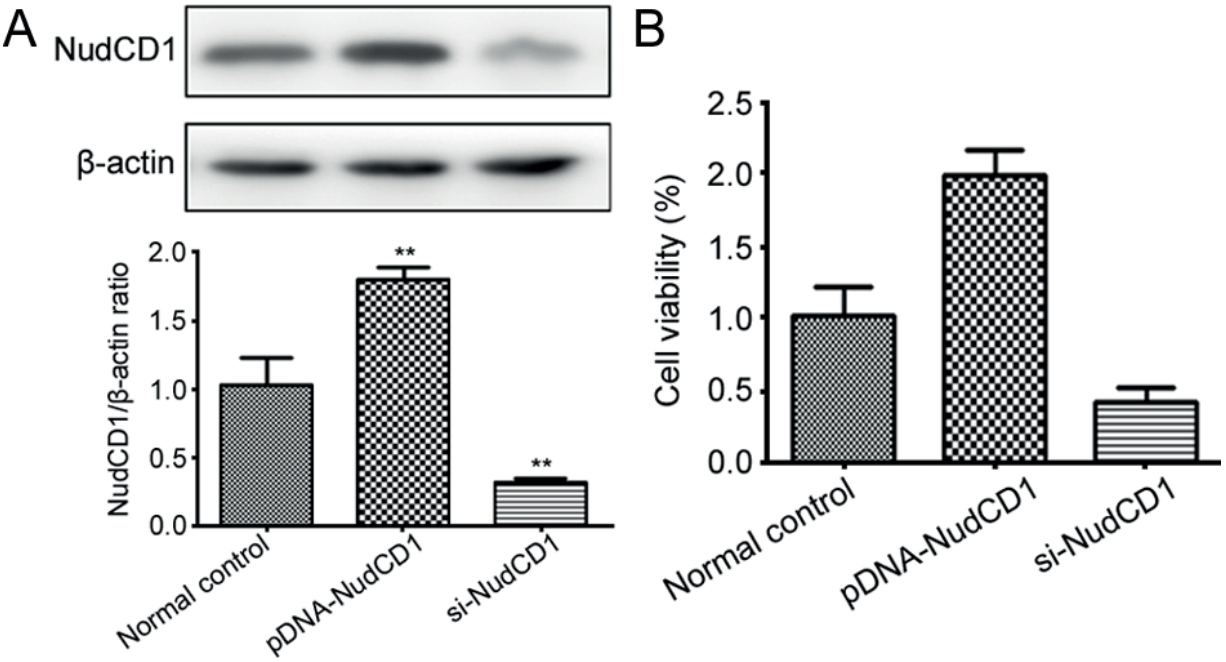

Figure 4. The impact of NudCD1 on cell proliferation. ***A,*** NudCD1 protein expression detected by Western blot. ***B,*** cell proliferation tested by MTT assay. $^{**}p < 0.05$, compared with control.

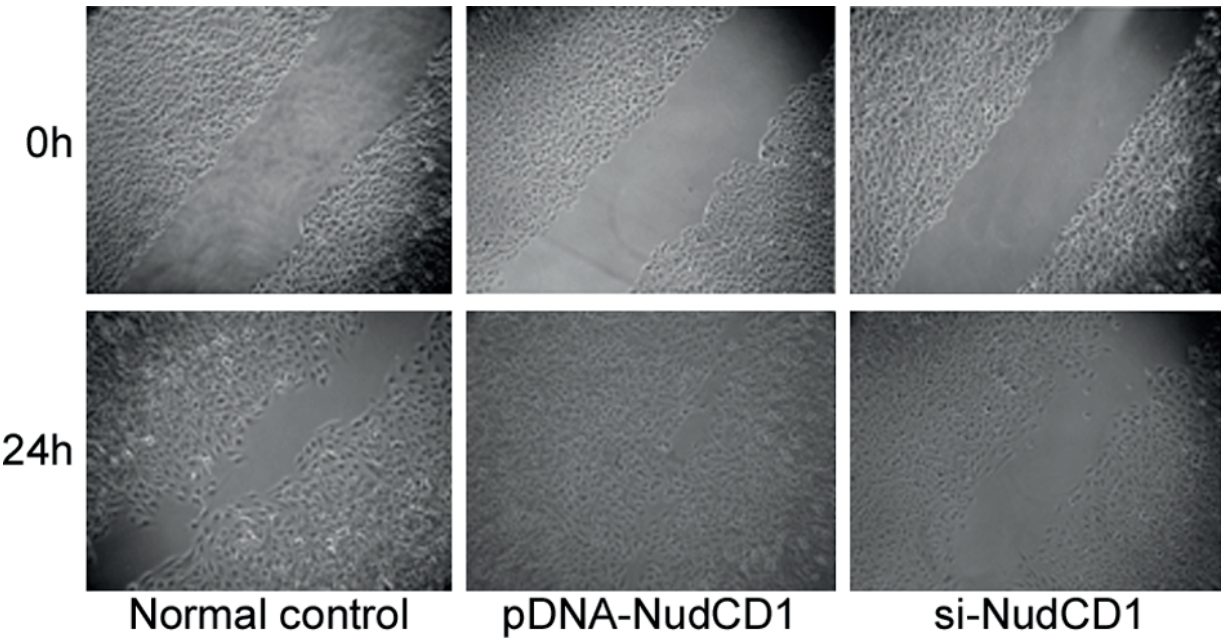

Figure 5. The influence of NudCD1 on cell migration.

RCC patients exhibiting clinical symptoms often indicate advanced stage[15]. About 40% patients were in advanced stage or presented metastatic symptom when diagnosed[16]. Surgical treatment can be used in localized RCC, whereas nearly 20% patients with localized RCC appeared metastasis after primary tumor resection. Moreover, RCC is insensitive to chemotherapy and radiotherapy. Once the lymph node appears metastasis, the survival period is rarely more than 5 years even after radical lymphadenectomy. The prognosis is worse when the patient exhibits liver and lung metastasis or adjacent organ infiltration[17]. Therefore, it is necessary to understand the molecular and

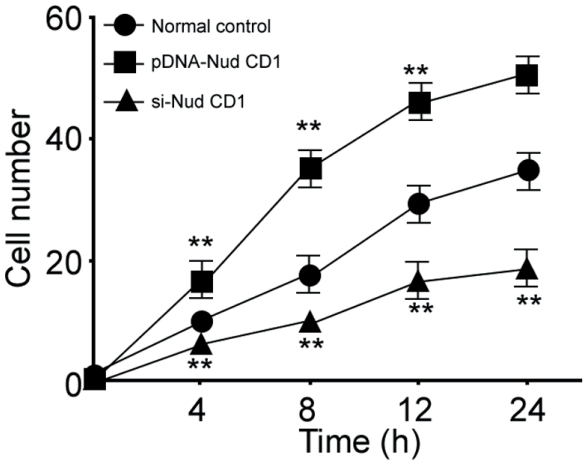

Figure 6. The effect of NudCD1 on cell invasion. $^{**}p < 0.05$, compared with control.

cellular mechanisms of RCC. Screening the key molecules in the regulation of RCC, searching for new markers with high sensitivity and specificity, developing early screening methods and target intervention strategies are of great significance to early detection, diagnosis, targeted therapy, and prognosis evaluation to RCC.

It is considered that dysregulation of cell mitosis, intracellular transport, cell cycle, and cell migration are the earliest cytologic changes in tumor-acquired malignant biological behavior[18,19]. NudC domain containing 1 (NudCD1), also known as chronic myeloid leukemia tumor antigen 66 (CML66), is a broad spectrum of tumor antigens or oncogene, thus to be thought to regulate cell cycle and tubulin related genes[4]. NudCD1 is one of the most widely immunized tumor antigens selected from the sera of patients with chronic myeloid leukemia by recombinant cDNA expression library serum analysis techniques[20]. It locates in chromosome 8q23.3 with a relative molecular mass at 66 kDa[21]. In addition to chronic myeloid leukemia cells[6,7], NudCD1 also highly expresses in lung cancer, melanoma, and prostate cancer tissue and tumor cell lines[8-10]. In this work, we found NudCD1 expression significantly upregulated in RCC tissues and cell lines, which was similar with previous results. It suggested that NudCD1 may participate in the regulation of RCC occurrence and development.

We detected the expression and cellular localization of NudCD1 protein in RCC tissues and adjacent normal tissues by IHC. Then, we tested NudCD1 expression in RCC cell lines by Western blot to select the cell line with highest abundance. Next, we applied siRNA or DNA overexpression plasmid to regulate NudCD1 expression level to observe its influence on cell proliferation, invasion, and migration. It was showed that NudCD1 expression was positively correlated with cell behaviors, indicating that NudCD1 was involved in RCC cell proliferation, migration, and invasion. Moreover, it indicated that NudCD1 may be treated as a new biomarker for the diagnosis of RCC.

In this study, we investigated the expression of NudCD1 in clinical specimens of RCC and the effect on tumor cell behavior in cell lines. However, NudCD1 produced these regulatory effects through signaling pathways, while whether its downregulation can protect cells from RCC still needs further in exploration. It may provide the theoretical basis of the treatment and prevention of RCC.

Conclusions

We showed that NudCD1 increased in RCC and was positively correlated with cell proliferation, migration, and invasion. It could be used as an indicator for the early screening and potential treatment target for RCC.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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