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Original Article

Nucleobindin 2 (NUCB2) in renal cell carcinoma: a novel factor associated with tumor development

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Abstract: This study aimed to examine the expression of NUCB2 in renal cell carcinoma (RCC) tissues and detect its effect on the apoptosis and proliferation of RCC cells both *in vivo* and *vitro*. NUCB2 was detected with higher expression in the RCC tissues compared with the adjacent noncancerous tissues by immunohistochemical analysis ($P < 0.05$). Moreover, the protein levels of NUCB2 was significantly associated with perinephric tissues invasion, cancerous thrombus and distant metastasis. NUCB2 knock-down inhibited cell proliferation by arresting the cell cycle at S phase and increased the cell apoptosis detected by CCK-8 and flow cytometry analysis. Finally, the tumor-bearing mice models were constructed through injection of 786-0 cells transfected with lentivirus carrying shRNAs targeting NUCB2 or ACTB cDNA plasmids. As expected, the mice in the NUCB2-shRNA group demonstrated a reduced tumor volume and growth rate compared with that in negative control group. This study observed the up-regulated expression of NUCB2 in the ccRCC tissue and 786-0 cell line. NUCB2 is involved in the tumor genesis and development in ccRCC both in the research of tumor-bearing mice model and cell lines.

Keywords: NUCB2, renal cell carcinoma, tumor genesis, tumor development

Introduction

Nucleobindin 2 (NUCB2) is a hypothalamic neuropeptide, which is the precursor of nesfatin-1. It is reported as a metabolic factor regulating food intake and energy homeostasis. This protein was first identified by Oh-I as a satiety molecule expressed in the hypothalamus [1]. The peripheral and central administration of NUCB2/nesfatin-1 regulates the glucose and fatty acid metabolism and improves the insulin resistance [2, 3]. NUCB2 is expressed in various human organs and tissues, including stomach mucosa, pancreas, fat tissue, hypothalamus and so on [4, 5]. Recent publications reported that the function of nesfatin-1/NUCB2 was associated with tumor development and metastasis. The high serum level and local expression of NUCB2 were associated with shorter biochemical recurrence-free (BCR) survival time in the patients of prostate cancer as well as breast carcinoma [6, 7]. However, it acted as a tumor suppressor in human adrenocortical carcinoma and ovarian epithelial carcinoma cells [8, 9]. Furthermore, the proliferative and pro-metastatic effect of NUCB2 was reported in breast cancer and colorectal cancer [7, 10]. Recent studies also reported NUCB2 as a potent prognostic factor associated with cell proliferation and migration in endometrial carcinoma [11]. As reported in new studies, NUCB2 was upregulated in tissues of clear cell renal cell carcinoma (ccRCC), which played an important role in regulating tumorigenesis and tumor development [12].

Renal cell carcinoma (RCC), the most common malignant neoplasms of kidney [13], accounts for 3~4% of adult solid tumors. About 30% of the RCC patients have metastases when diagnosed at the first time. The 5-year survival rate of patients with metastatic RCC was reported less than 10% [14]. Targeted therapy become the main therapy due to the helpless of surgery. As is widely accepted, metabolic syndrome contributes to RCC genesis and development. Many mutation genes, such as VHL, MET, FLCN, FH, SDH, TSC1, and TSC2, play fundamental roles in the regulation of metabolism [15]. The further studies of the metabolic hor-

mones provide opportunities for the discovery of novel therapeutic strategies.

As mentioned previously, NUCB2 is a newly reported biological marker for the renal malignant carcinoma. In this study, we further explored the effect of NUCB2 on the cell growth of RCC *in vivo* and *in vitro*. To the best of our knowledge, no *in-vivo* studies about the role of NUCB2 on RCC development were reported. In addition, we did a mini review about the NUCB2's role on cancer based on some researches up to now.

Materials and methods

Histological analysis

The renal tissues (60 cases) were collected from the tissue bank and stored in 10% formal saline and embedded in paraffin blocks. Sections (3-5 mm) were prepared from paraffin blocks for further staining. In the immunohistochemical analysis, sections were hydrated in gradient alcohol dilutions. After the antigen being repaired, the sections were blocked by goat serum for 60 min. Primary antibody, anti-NUCB2 (1:50, santa cruz, sc-133853) was incubated overnight at 4°C. Secondary antibody, goat anti-rabbit IgG (1:200, santa cruz, sc-2040) was incubated for an hour. Hematoxylin was further used for the staining of nucleus. Three to five views for each chip were collected and analyzed. The result was quantified with Image J (1,46r; National Institutes of Health).

Cell culture

786-0 cells were incubated in the cell culture medium consisted with RPMI 1640 + 10% FBS + 1% P/S + 1% Gln at 37°C in an atmosphere of 5% CO_2 under saturating humidity. The growth condition was recorded every 24 and 48 hours. Cells were divided into three groups according to the different transfection: (1) non-transfected control cells (NT group), (2) ACTB transfected cells (lenti-NC cells), and (3) NUCB2 knock-down cells (lenti-NUCB2-KD cells). The cells were stored at -80°C for further research.

Lentivirus transfection

Lentivirus carrying shRNAs targeting NUCB2 or ACTB cDNA plasmids were transfected into 786-0 cells according to the manufacturer's protocol. Three sense strands, including 5-GAGGACCACTGCTACAGTA-3, 5-TTTAGTAACACCATGTGAG-3 and 5-GCTCAGAATGGA ATATCAT-3, were selected to be knocked down in our preliminary experiment, of which the third one was the most remarkable strand. Thus, the lentivirus was structured using the third sense strand. Western blotting was performed for the analysis of gene expression in cells.

Western blotting analysis

The protein concentration of cell lysis was determined by a bicinchoninic protein assay kit. 50 μg total protein was loaded in each lane and separated by 10% SDS-PAGE gels. Then proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Primary antibody for NUCB2 (Santa cruz, sc-133853) and GAPDH (Santa cruz, sc-25778) were used in the research. The secondary antibody was peroxidase-conjugated IgG (Beijing Zhongshan Jinqiao Biotec) diluted at 1:8000. The intensity of bands was analyzed using Quantity One software (Bio-Rad).

CCK-8 analysis

Tumor cell proliferation was analyzed by CCK-8 assay (Beyotime, Shanghai, China, C0037) according to the manufacture's instruction. Different types of cells were first seeded into 96-well flat-bottom plates for 8 h to adhere. After 24 h, 10 μl of solution from CCK-8 was added to each well. These plates were incubated for 45 minutes in a humidified CO_2 incubator at 37°C. Finally, the absorbance of sample taken from each well was measured at 450 nm, on the basis of which the percentage of surviving cells in each treatment group was plotted relative to the untreated.

Apoptosis analysis

Cells and cell culture supernatant were collected with 1000 rpm centrifuge for 5 min. After washing with PBS twice, cells were mixed into 400 ul binding buffer with APC and PI. Cell mixture was incubated for 30 min at 20°C. Flow cytometry was used to detect the final apoptosis rate.

Cell cycle analysis

The cell cycle of 786-0 cells was analyzed by flow cytometry using BD FACSAria Cell Sorter (Becton Dickinson, Franklin Lakes, NJ). 786-0 cells were trypsinized and collected by centrifu-

gation and fixed with 70% ethanol. Samples were treated with 5 μl RNase A (10 μg/mL) for 1 h at room temperature, stained with 10 μl propidium iodide (10 μg/mL) for 30 min at 4°C and analyzed using the FACSCalibur flow cytometer.

Mice and tumor models

Control and transfected 786-O cells were injected subcutaneously (s.c., 5×10^6 cells) into the flank of 18-20 g male nude mice. Three groups of mice were divided: 786-O group (control, 786-O cells injected, n = 5), NC group (negative control, ACTB transfected cells injected, n = 5) and NUCB2-KD group (NUCB2-shRNA, transfected cells injected, n = 5). Fifteen nude mice were maintained under specific pathogen-free conditions and free to food and water in a 12 h light/dark cycle. Three weeks after the cell injection, subcutaneous tumor growth was measured every five days. The mice were sacrificed after 35 days, and the subcutaneous tumors were removed for further research. Total protein was extracted from tumorigenic tissues of NUCB2-KD, 786-O and NC-control nude mice, and the expression of NUCB2 protein was detected by Western blotting.

Statistical analysis

The data in this passage were showed as mean ± S.E.M. Statistically significant differences between groups were assessed using one-way analysis of variance followed by the LSD test. The association between NUCB2 expression and pathologic parameters of RCC was conducted using the chi-square test. All statistical analysis was performed using SPSS 17.0. *P* value < 0.05 denotes statistical significance.

Results

High expression level of NUCB2 in RCC tissues and cells.

To determine whether NUCB2 was up-regulated in RCC, we analyzed NUCB2 protein level in RCC tissue and cells using immunohistochemical staining and Western blotting. NUCB2 was highly expressed in the RCC tissues compared with the adjacent noncancerous tissues, most of which was located in the cytoplasm (**Figure 1A**). The positive NUCB2 expression rate in RCC tissues (60%, 36/60) was significantly higher than that in adjacent noncancerous tissues (33.3%, 20/60) ($P < 0.05$) (**Table 1** and **Figure 1B**). The associations between NUCB2 expression and RCC clinicopathological characteristics were summarized in **Table 2**. NUCB2 protein expression was not significantly associated with age, gender, tumor stage, Fuhrman grade or tumor recurrence ($P > 0.05$). However, NUCB2 protein expression was associated notably with perinephric tissues invasion, cancerous thrombus and distant metastasis ($P = 0.044$ and 0.002, respectively). These data suggested that NUCB2 played an important role in metastasis and invasion. We further confirmed the increased expression of NUCB2 in 786-O cell line compared with 293 control cells and ACHN cells (**Figure 1C**, **1D**). This study indicated that NUCB2 acted as an oncogene in RCC.

Decreased proliferation and increased apoptosis rate in the NUCB2-KD 786-O cells

To assess whether the expression of NUCB2 is important for the proliferation of RCC cells, we first established NUCB2 deleted stable cell lines by transfection of lentiviruses expressing shRNA targeting the human NUCB2 gene or control shRNA and investigate the effects on the cell proliferation rate. As shown in **Figure 2A**, lentivirus carrying shRNAs targeting NUCB2 or ACTB cDNA plasmids effectively transfected into 786-O cells. NUCB2 was detected to have low expression in NUCB2 knock-down 786-O cells using Western blotting analysis (**Figure 2B**, **2C**). Subsequently, it was shown that silence of NUCB2 increased the apoptosis rate and reduced the cell proliferation (**Figure 2D-F**). This observation indicated that NUCB2 is a potential therapeutic target for RCC.

Inhibition of NUCB2 arrests cell cycle at the S phase

To elucidate the molecular mechanism underlying the effect of NUCB2 on RCC cell growth, we transduced 786-O cells with the lentivirus expressing NUCB2 shRNA or a control to establish stable cell lines. We performed a flow-cytometric analysis to determine the impact of silencing of NUCB2 expression on the distribution of cell cycle in 786-O cells. As shown in **Figure 3**, in agreement with its inhibitory function, knockdown of NUCB2 increased the percentage of cells in the S phase, suggesting that the cells were significantly arrested in the S

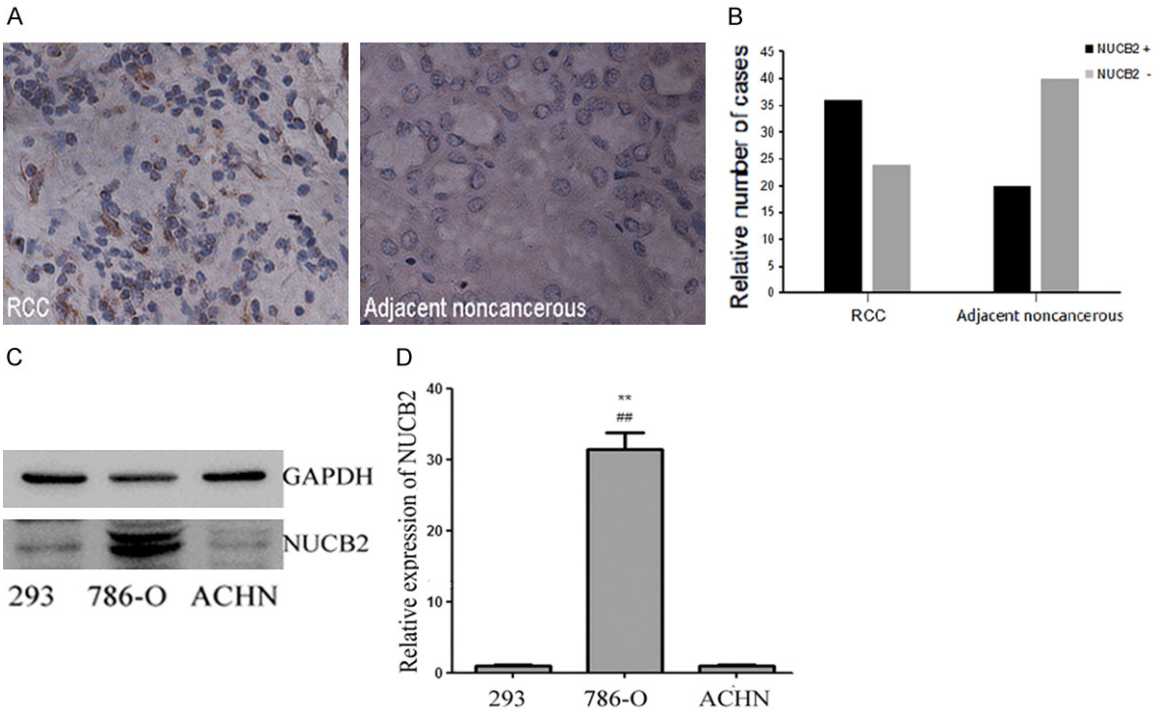

Figure 1. NUCB2 is overexpressed in clinical specimens and RCC cell lines. A. NUCB2 in RCC tissues and the adjacent noncancerous tissues was assessed by histological analysis. The protein level of NUCB2 in RCC tissues was significantly elevated compared with the non-carcinoma tissues (Magnification, × 400). B. Number of the cases of the NUCB2 expression in RCC and adjacent noncancerous tissues respectively. C, D. Western blotting analysis was used to detect NUCB2 expression in the ACHN and 786-O cells. NUCB2 expression was significantly increased in the 786-O cells compared with the 293 and ACHN cell lines (cod).

Table 1. NUCB2 expression in RCC and adjacent noncancerous tissues

Parameter	n	NUCB2 expression		P
		Positive (%)	Negative (%)	
RCC	60	36 (60%)	24 (40%)	0.003*
Adjacent noncancerous	60	20 (33.3%)	40 (66.7%)	

Note: Immunohistochemical staining and Western blotting were used to analyze NUCB2 protein level in RCC tissue and cells. *P < 0.05.

phase (P < 0.01). These data imply that NUCB2 may regulate some of the cell cycle proteins in S phase.

Subcutaneous tumor development in the nude mice

We successfully constructed mice tumor models. At the end of the experiment, the removed tumor tissues from animal models are shown in **Figure 4A**. We detected one tissue randomly from three tumorigenesis tissues by Western blotting. The results showed that the expression of NUCB2 in tumorigenic tissues of 786-O-NUCB2-KD stable transgenic plants was significantly lower than that of 786-O group and NC group (**Figure 4B**). Furthermore, the growth rate of tumorigenic tissues decreased in the NUCB2 knock-down group compared with negative control group (**Figure 4C**).

Discussion

In this study, we first demonstrated that NUCB2, the anorectic peptide, enhanced tumor development in RCC *in vitro*. The distribution of NUCB2 in human renal sections appeared to be more in the RCC tissues compared with the adjacent noncancerous tissues. In the animal study, NUCB2 appeared to function as a tumor promoter, which is consistent with the result of cell research. In the cell study, NUCB2 was observed to stimulate the cell proliferation and reduce the apoptosis in the 786-O cell line.

NUCB2 was first reported in 2006 by Oh-I as a metabolic factor reducing food intake and improving energy homeostasis [1]. The gene is located on chromosome 11, consisting of 14 exons spanning 54,785 nucleotides, which tr-

Table 2. Relationships of NUCB2 and clinicopathological characteristics in 60 patients with RCC

Parameter	n	NUCB2 expression		P
		Positive (%)	Negative (%)	
Age (year)				0.213
< 65	28	15 (53.6%)	13 (46.4%)	
≥ 65	32	21 (65.6%)	11 (34.4%)	
Gender				0.292
Male	37	28 (75.7%)	9 (24.3%)	
Female	23	15 (65.2%)	8 (34.8%)	
Tumor stage				0.766
T1	18	8 (44.4%)	10 (55.6%)	
T2	20	12 (60%)	8 (40%)	
T3	14	10 (71.4%)	4 (28.6%)	
T4	8	6 (75%)	2 (25%)	
Fuhrman grade				0.197
I-II	36	24 (66.7%)	12 (33.3%)	
III-IV	24	12 (50%)	12 (50%)	
Recurrence				0.157
Yes	10	8 (80%)	2 (20%)	
No	50	28 (56%)	22 (44%)	
Invade perinephrictissues/cancerous thrombus				0.044*
Yes	27	20 (74.1%)	7 (25.9%)	
No	33	16 (48.5%)	17 (51.5%)	
Distant metastasis				0.002**
Yes	16	14 (87.5%)	2 (12.5%)	
No	44	22 (50%)	22 (50%)	

Note: *P < 0.05, **P < 0.01.

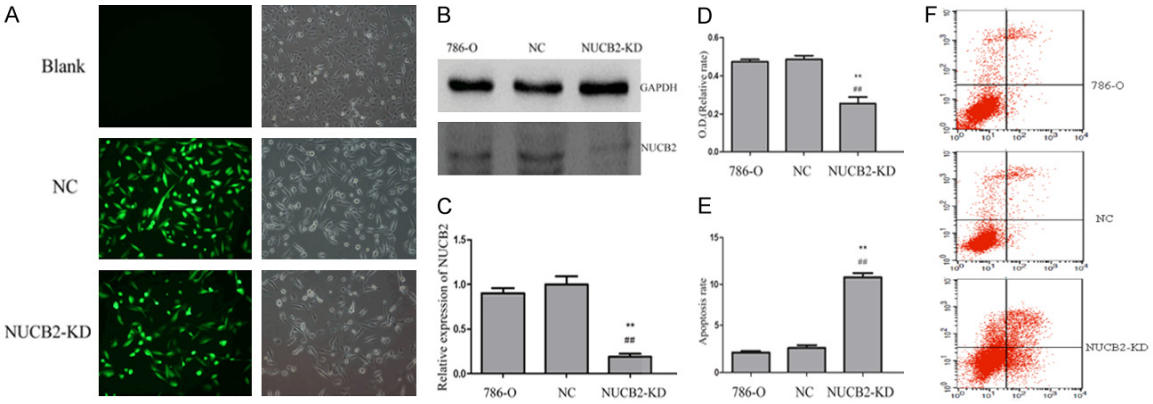

Figure 2. The effect of silencing of NUCB2 expression on 786-O cell proliferation. A. Comparative analysis of the efficiency of lentivirus transfection into 786-O cells. B, C. Western blotting was performed to validate the efficiency of NUCB2 interference in RCC cells. NUCB2 is significantly decreased in the knocked down cells. *p <0.05, **p < 0.01, compared with the 786-O control group, #p < 0.05, ##p < 0.01 compared with the negative control group. D. CCK-8 kit was used to detect the proliferation. Decreased proliferation was shown in the NUCB2-KD cells. E, F. Flow cytometry was used for validating the apoptosis rate. Increased proliferation was shown in the NUCB2-KD cells. In summary, NUCB2 knock-down decreased the proliferation and elevated the apoptosis rate by CCK-8 and flow cytometry analysis. *p < 0.05, **p < 0.01, compared with the 786-O control group, #p < 0.05, ##p < 0.01 compared with the negative control group.

anscripts and translates a precursor 396-amino-acid protein NUCB2 [6, 16]. The location of NUCB2 protein is found to be various. Centrally, it is abundantly expressed in the hypothala-

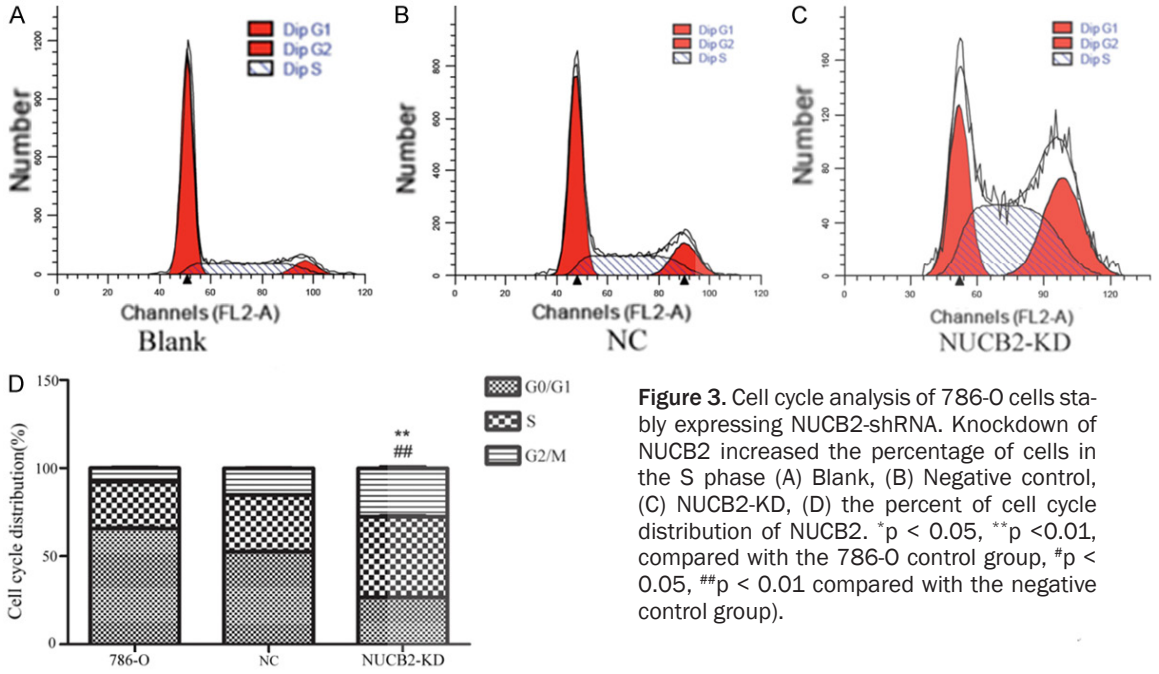

Figure 3. Cell cycle analysis of 786-0 cells stably expressing NUCB2-shRNA. Knockdown of NUCB2 increased the percentage of cells in the S phase (A) Blank, (B) Negative control, (C) NUCB2-KD, (D) the percent of cell cycle distribution of NUCB2. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the 786-0 control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the negative control group).

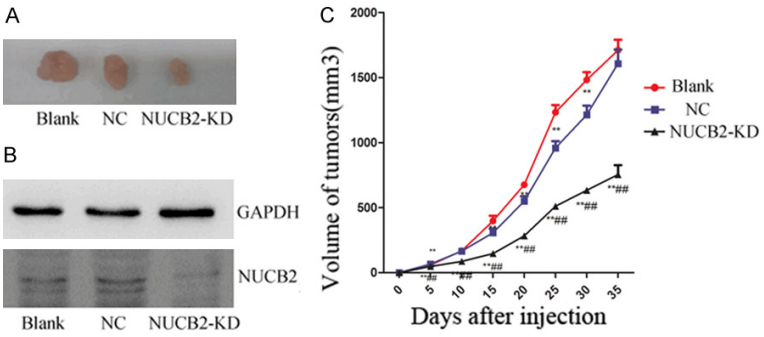

Figure 4. Suppression of NUCB2 inhibits tumor formation in murine tumor model. A. Removed tumor tissues in animal models. B. The expression of NUCB2 in tumorigenic tissues of 786-0-NUCB2 KD stable transgenic plants was significantly lower than that of 786-0 group and NC group. C. The variation curve of the tumor formation analysis from 0 to 35 days after the injection of 5×10^6 cells per mouse. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the 786-0 control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the negative control group.

mus nucleus, such as arcuate nucleus, lateral hypothalamus, paraventricular nucleus and supraoptic nucleus [1, 16]. There are 3 cleavage products of NUCB2: nesfatin-1 (1-82), nesfatin-2 (85-163), and nesfatin-3 (166-396). Among the 3 cleavage products, only nesfatin-1 can decrease food intake and body weight, which is an 82-amino-acid peptide with molecular mass of 9.8 kDa [1]. NUCB2/nesfatin-1 regulates the energy metabolism through hormone secretion and the systematic nerves [4]. Peripherally, some argue that gastric mucosa is the main source of the serum NUCB2 while others consider that the serum NUCB2 is mainly produced by the pancreatic islets or fat tissues [4, 5]. Furthermore, serum NUCB2 is observed to decrease significantly in type 2 diabetes (T2DM) patients compared with healthy or type 1 diabetes (T1DM) subjects [17]. In the peripheral tissues, NUCB2 was shown to regulate adipocyte differentiation and improve the fatty acid oxidation through the AMPK-ACC pathway [2]. Furthermore, the data suggest that NUCB2 participates in the regulation of pancreatic α cell and β cell secretion and increases glucose-induced insulin secretion and improves glucose elimination by promoting Ca^{2+} influx through L-type channels independently of protein kinase A (PKA) and phospholipase A2 (PLA2) in mouse islet β-cells in vitro [18]. In this way, it is an important factor for the metabolism regulation. In the current papers, it was also reported as a biomarker for many pathologic progresses, including the metabolic states, reproduction as well as tumor development (ref).

In this report, we found that NUCB2 was highly expressed in the RCC tissues. It was also shown in **Figures 2** and **3** that NUCB2 knockdown increased the apoptosis rate and reduced the cell proliferation. With the transfected cells being injected, the tumor diameters of the NUCB2 knocked down group were significantly reduced. Furthermore, the growth rate was decreased after the NUCB2 was knocked down compared with negative control (shown in **Figure 4**).

Serum NUCB2 was changed in several types of diseases and the expression may indicate the pathology process of varied disease, including the malignant tumor. NUCB2 shows different function in different types of cancers. Online dataset also indicated that the NUCB2 expression was associated with early metastasis. The effect, however, are diverse in different tissues. In colon carcinoma, it plays a role in the migration, invasion and EMT through LKB1/AMPK/TORC1/ZEB1 pathways *in vitro* and *in vivo* [10]. In breast cancer, NUCB2 is up-regulated by estrogens and participates in the process of metastasis [7]. Recent studies also suggest NUCB2 as a novel peptide in the progression of endometrial carcinomas [11]. In the study of ovarian cancer, however, nesfatin-1 contributed to ovarian cancer prevention and therapy, especially in obese patients through RhoA/ROCK pathway. In the studies of urologic cancers, NUCB2 was proved for a positive role in PCa development and serves as an independent predictor of BCR-free survival [6, 19, 20]. Furthermore, it also stimulates the tumorigenesis and progression in ccRCC and may be a potential molecular bio-marker for the treatment [12]. In our research, *in-vitro* studies present a role of NUCB2 in the proliferation and anti-apoptosis of RCC, which contributes to the cancer genesis and development. The nesfatin-1/NUCB2 signaling pathways are not well understood currently because the receptor has not been fully studied. However, it is a G protein coupled receptor (GPCR) for the NUCB2 [21]. Involved in cancer malignancy, GPCR is important for identifying novel therapies for different cancers. Interestingly, GPCRs are multiple-functioned in varied types of carcinomas [22, 23]. In the area of migration, invasion and metastasis, chemokines in the tumor microenvironment increase the survival and migration of varied cancer cells, and the GPCRs of chemokines are associated with the organ-specific metastasis [24]. Moreover, recent studies have spotted the abundance of mutations in GPCRs. The mutation genome analyses indicate that GPCRs are mutated in about 20% of all cancers. Surprisingly, in certain malignancies, some GPCRs and G proteins may play different roles in the cancer genesis and invasion [22]. Our research reports that NUCB2 acts as the tumor promoter in the genesis and development for RCC, which may be potential for the further study of the receptor of NUCB2.

Conclusion

Our study indicates that NUCB2 is expressed highly in the ccRCC tissue as well as the 786-O cell line and is involved in the tumor genesis and development of renal cancer both in the research of tumor-bearing nude mice model and cell lines. Further research should be done about the detailed mechanism and the characteristic of NUCB2 receptor towards different types of carcinoma.

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Disclosure of conflict of interest

None.

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