Cancer Letters 444 (2019) 9–19

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

NLRC5 mediates cell proliferation, migration, and invasion by regulating the Wnt/β-catenin signalling pathway in clear cell renal cell carcinoma

Qin Wang[a,b,c], Handong Ding[d,e,f], Yinghua He[a,b,c], Xiaofeng Li[a,b,c], Yahui Cheng[a,b,c], Qingqing Xu[a,b,c], Yue Yang[a,b,c], Guiyi Liao[d,e,f], Xiaoming Meng[a,b,c], Cheng Huang[a,b,c,**], Jun Li[a,b,c,*]

[a] Anhui Province Key Laboratory of Major Autoimmune Diseases, Anhui Institute of Innovative Drugs, School of Pharmacy, Anhui Medical University, Hefei, 230032, China
[b] The Key Laboratory of Anti-inflammatory and Immune Medicines, Ministry of Education, Hefei, 230032, China
[c] Institute for Liver Diseases of Anhui Medical University, Hefei, 230032, China
[d] Department of Urology, The First Affiliated Hospital of Anhui Medical University, Hefei, 230032, China
[e] Institute of Urology, Anhui Medical University, Hefei, 230032, China
[f] Anhui Province Key Laboratory of Genitourinary Diseases, Anhui Medical University, China

ARTICLE INFO

Keywords:
NLRC5
ccRCC
Wnt/β-catenin pathway

ABSTRACT

NLRC5, a newly discovered member of the NLR family, has been reported to regulate immune responses and promote cell proliferation, migration, and invasion in hepatocellular carcinoma. However, to date, the potential regulatory roles and molecular mechanisms by which NLRC5 affects the development and progression of clear cell renal cell carcinoma (ccRCC) remain largely unknown. In this study, human clinical data from The Cancer Genome Atlas database revealed that increased NLRC5 expression was associated with advanced stage and poor prognosis in ccRCC patients. Moreover, experimental results showed that NLRC5 is aberrantly overexpressed in human ccRCC tissues and cell lines. Depletion of NLRC5 attenuated ccRCC cell proliferation, migration, and invasion and suppressed ccRCC growth in a nude mouse model. By contrast, overexpression of NLRC5 promoted the proliferation, migration, and invasion of ccRCC cells *in vitro*. Additionally, NLRC5 expression is not only positively correlated with β-catenin but also coordinates the activation of the downstream Wnt/β-catenin signalling pathway. Together, our data suggest that NLRC5 may be a potential therapeutic target for ccRCC therapy.

1. Introduction

Renal cell carcinoma (RCC) is the second most common cancer in urological system, representing approximately 2–3% of all adult malignancies [1]. RCC is also the most abundant type of kidney cancer, and more than 30% of RCC patients present with locally advanced and metastatic disease at the time of diagnosis [2]. Clear cell renal cell carcinoma (ccRCC) is the most common histologic subtype of RCC, constituting around 75% of cases [3]. ccRCC is characterized by loss of function of the von Hippel Lindau (*VHL*) gene [4]. The five-year survival rate of advanced ccRCC is poor and may result in metastasis or recurrence [5,6]. Hence, it is important to gain a better understanding of the underlying molecular mechanisms of malignant ccRCC and identify new efficacious therapeutic strategies.

Nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) are a family of cytoplasmic pattern-recognition receptors with important roles in both innate and adaptive immunity [7,8]. There are 22 NLR proteins in humans [9]. NOD-like receptor family caspase recruitment domain family domain-containing 5 (NLRC5) is the most abundant NLR protein and contains three structural domains, including the N-terminal atypical caspase activation and recruitment domain (CARD), the centrally located NACHT (named after the NAIP, CIITA, HET-E, and TP-1 proteins) domain, and 27 leucine-rich repeats (LRRs) at the C-terminus [10–13]. Recent studies have revealed that NLRC5 is a key transcriptional coactivator of MHC class I genes, the loss of which has been described as a major immune evasion strategy in many cancers [14]. Cell-specific analysis indicates that NLRC5 is strongly expressed in immune cells and immune-related tissues, such as B cells, bone marrow, and the spleen, lung, thymus, and liver, suggesting that the function of the protein is biologically

* Corresponding author. School of Pharmacy, Anhui Medical University, Hefei, Anhui, China.
** Corresponding author. School of Pharmacy, Anhui Medical University, Hefei, Anhui, China.
E-mail addresses: huangcheng@ahmu.edu.cn (C. Huang), lj@ahmu.edu.cn (J. Li).

https://doi.org/10.1016/j.canlet.2018.11.024
Received 8 September 2018; Received in revised form 3 November 2018; Accepted 21 November 2018

conserved in these tissues [7,11]. However, whether NLRC5 plays a positive or negative role in tumour development remains controversial [15]. Previous evidence has shown that NLRC5 regulates cell proliferation, migration, and invasion in hepatocellular carcinoma (HCC) [16,17] and that NLRC5 and MHC class I may be negative prognostic indicators in stage III non-small-cell lung cancer (NSCLC) [18]. Furthermore, Wang et al. reported that knockdown of NLRC5 inhibits the proliferation of renal fibroblasts [19]. However, the functions of NLRC5 in ccRCC remain to be elucidated.

In this study, we investigated NLRC5 expression in human ccRCC tissues and cell lines and the correlation between NLRC5 expression and human ccRCC progression. We also performed a series of *in vitro* and *in vivo* experiments involving the knockdown of NLRC5 expression in ccRCC cells to investigate effects on proliferation, migration, and invasion. Finally, we assessed the role of the Wnt/β-catenin signalling pathway in the oncogenic function of NLRC5.

2. Materials and methods

2.1. RCC tissue samples and ethics statement

Patients with ccRCC were recruited from the First Affiliated Hospital of Anhui Medical University. There were no age, gender, ethnicity, or cancer stage restrictions on recruitment. After surgical removal, tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C. The current study was approved by the Ethics Committee of Anhui Medical University.

2.2. Cell culture

The human normal cell line HK-2 (an immortalised proximal tubule epithelial cell line) and the ccRCC cell lines Caki-1, 786-O, and 769-P were purchased from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). HK-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) at 37 °C in humidified 5% CO_2. Caki-1, 786-O, and 769-P cells were cultured in RPMI-1640 (HyCone) with 10% FBS in humidified 5% CO_2 at 37 °C.

2.3. Histopathological analysis

Tissues were fixed with 4% paraformaldehyde for 24 h and embedded in paraffin before being were sliced and baked for 1 h at 60 °C. Sections were xylene-dewaxed and dehydrated with a gradient of ethanol. Antigen retrieval was performed by immersing sections in 0.01 M citrate buffer and heating to boiling (98–100 °C) by microwave for 15 min. After antigen retrieval, the sections were blocked with 5% bovine serum albumin (BSA) at 37 °C for 20 min and incubated with primary antibody against NLRC5 (1:100, Abcam, Cambridge, UK) overnight at 4 °C and with secondary antibody for 1 h at 37 °C. The expression of NLRC5 was visualized by staining with diaminobenzidine tetrahydrochloride solution (DAB) for 5 min. Then, the sections were counterstained with haematoxylin for 5 min, mounted with gum, and subjected to microscopic examination.

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted using TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total mRNA was used for reverse transcription with the Takara RT-PCR synthesis kit (Takara, Dalian, China), according to the manufacturer's instructions. cDNA synthesis was performed using SYBR Premix Ex Taq II (Takara) on the PikoReal 96 qPCR system (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used were as follows:

β-actin forward, 5′-GCCAACACAGTGCTG TCTGG-3′;

β-actin reverse, 5′-CTCAGGAGGAGCAATGATCTTG-3′;

NLRC5 forward, 5′-CTATCAACTGCCCTTCCACAAT-3′;

NLRC5 reverse, 5′-TCTCTATC TGCCCACAGCCTAC-3′.

β-actin was used to normalize the expression values of the other genes. All experiments were repeated at least three times.

2.5. RNA interference (RNAi)

Small interfering RNA (siRNA) oligonucleotides against NLRC5 and negative control (NC) scrambled siRNA were designed and synthesized by the Gema Pharma Corporation (Shanghai, China). The siRNA sequences were as follows:

siRNA-NLRC5 (human), 5′-GGAGAGGGCUGCAUUUCUUTT-3′ (sense) and 3′-AAGAAAUGCAGCCCUCUCCTT-5′ (antisense);

scrambled siRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 3′-ACGUGACACGUUCGGAGAATT-5′ (antisense).

Cells (5×10^5 cells) were seeded in 6-well plates for 24 h and transfected with siRNA in opti-MEM (Gibco, Gaithersburg, MD, USA) culture medium. RNAi was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. The opti-MEM was replaced after 6 h with RPMI-1640. Knockdown efficiency was determined by real-time PCR and western blotting analysis.

2.6. Western blotting analysis

Tissues and cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) with 1% phenyl methyl sulfonyl fluoride (PMSF; Beyotime). Proteins (20 μg) were separated on 8–12% sodium dodecyl sulphate-polyacrylamide gels and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking membranes with 5% skim milk, they were incubated overnight at 4 °C with appropriate dilutions of the following specific primary antibodies: anti-NLRC5, anti-PCNA, anti-MMP2 (rabbit, 1:800; Abcam); anti-c-Myc, anti-cyclin D1, anti-β-catenin, anti-MMP9 (rabbit, 1:800; Cell Signaling Technology, Danvers, MA, USA); and anti-β-actin (mouse, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes in TBS/Tween-20, membranes were incubated for 1 h in goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP, Santa Cruz Biotechnology)-conjugated antibody at 1:10000 dilutions in TBS/Tween-20 containing 5% skim milk. After washing in TBS/Tween-20, the membranes were processed with distilled water for antigen detection using the enhanced chemiluminescence system. Proteins were visualized with an ECL-chemiluminescence kit (ECL-plus, Thermo Fisher Scientific). β-actin was used as an internal control. All experiments were repeated three times.

2.7. Transwell migration assay

Two hundred microlitres of a cell suspension (5×10^4 cells) in serum-free medium was added to the upper chamber of a Transwell system (8-μm pore size, #3422, Corning Inc., Corning, NY, USA). The bottom chamber was filled with 0.5 ml medium containing 20% FBS. After a 24-h incubation period, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The numbers of cells that penetrated the membrane in five random fields were counted. All experiments were repeated three times.

2.8. Transwell invasion assay

Transwell invasion assay was performed in 24-well plates with chamber inserts with an 8-μm pore size (#3422, Corning Inc.), according to the manufacturer's protocol. Briefly, Transwell inserts were coated with 50 μl of Matrigel (Corning Inc.) diluted 1:5 for 4 h at 37 °C. Then, cells were seeded at 2×10^5 cells/well with serum-free medium into the upper chamber. The bottom chamber was filled with 0.5 ml medium containing 20% FBS. After incubation for 48 h at 37 °C in a 5% (v/v) CO_2 incubator, the non-invading cells and Matrigel in the upper

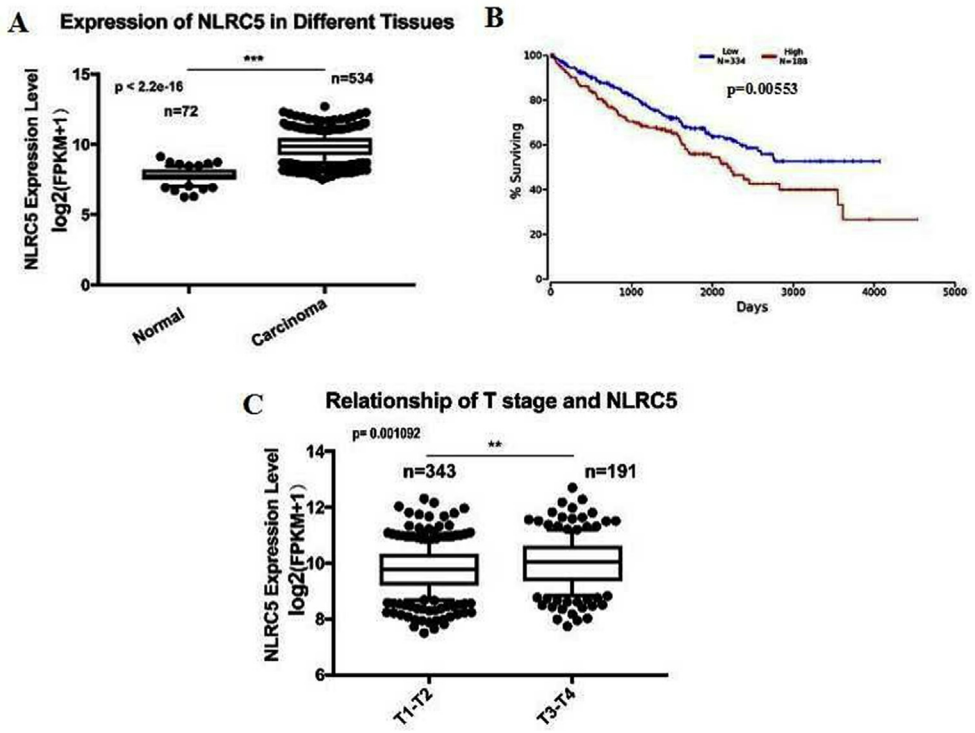

Fig. 1. High expression of NLRC5 is associated with advanced stage and poor prognosis in clear cell renal cell carcinoma patients. (A) Box and whisker plots for NLRC5 gene expression in ccRCC and normal kidney tissues from TCGA. **(B)** Kaplan–Meier survival plot of overall survival of ccRCC patients in TCGA, categorized according to NLRC5 gene expression (high vs. low, based on mean expression). **(C)** T stage analysis of TCGA data. Higher expression of NLRC5 in ccRCC tissues was associated with advanced stage. T3-T4 vs. T1-T2. *P < 0.05, **P < 0.01, ***P < 0.001.

chamber were removed gently by cotton swab, and the cells on the lower surface were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Invaded cells were counted in five randomly chosen microscopic fields. All experiments were repeated three times.

2.9. Cell counter kit-8 (CCK8) cell proliferation analysis

Cells were grown in 96-well plates at a density of 5000 cells per well and were incubated for 24 h. Then, 10 μl of CCK8 (Bestbio, Shanghai, China) solution was added to each well on days 1, 2, and 3. After 2 h, the absorbance of each well was measured at 450 nm using a microplate reader (Thermo Fisher Scientific). Results are shown as the average of at least three independent experiments.

2.10. Cell cycle analysis

After transfection for 48 h, cells were trypsinized, washed with phosphate-buffered saline (PBS), and then fixed in 70% cold ethanol at 4 °C overnight. Cells were centrifuged at 1000 ×*g* for 5 min and re-suspended in PBS. After that, cells were incubated with a 0.5-ml mixture of RNase and PI (Beyotime) at 37 °C for 30 min in the dark. The cell cycle was analysed by flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). DNA content was assessed using ModFit software (Verity Software House, USA). All experiments were repeated three times.

2.11. Stable cell line establishment assay

To generate stable NLRC5 knockdown cell populations, 786-O cells were infected with pLKD-CMV-EGFp-2A-Puro-U6-shNLRC5 or pLKD-CMV-EGFp-2A-Puro-U6-Scramble. Puromycin (8 mg/ml) was used to select for stably transfected cells for 2 weeks. The human NLRC5 shRNA sequence was designed as follows:

5′-GATAGAGAATCTCAGCTTT-3′.

The control shRNA sequence was as follows:

5′-TTCTCCGA ACGTGTCACGT-3′. NLRC5 knockdown in the stable cell line was verified by western blotting assay. Stable knockdown cells were maintained in complete medium containing 4 mg/ml puromycin.

2.12. Xenograft tumour model in nude mice

Six-week-old male BALB/c nude mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and housed under pathogen-free conditions in the animal experiment centre of Anhui Medical University (Hefei, China). The nude mice were assigned to the following two groups: NLRC5-shRNA and NC. Then, 200 μl of a 786-O (NLRC5-shRNA/NC) cell suspension containing 5 X 10^6 cells was subcutaneously injected into the right flank of each mouse. Tumour sizes were measured weekly. Eight weeks later, the mice were killed, and the tumours were removed for further assessment. All protocols involving live mice were approved by the Animal Care and Use Committee of Anhui Medical University.

2.13. The cancer genome atlas (TCGA) data analysis

Published mRNA expression data from 72 normal kidney tissues and 534 kidney cancer (ccRCC/KIRC) cases were downloaded from TCGA (http://xenabrowser.net/). We used the Student's *t*-test to assess the difference between tumour and normal samples. Clinical details from each patient, including TNM stage, survival status, and time to follow-up were also extracted from TCGA database (http://www.oncolnc.org/ and http://xenabrowser.net/, data downloaded in June 2018). We selected high and low target gene expression groups as defined by the mean value, and survival curves were constructed using the Kaplan–Meier method and compared by log-rank test.

2.14. Statistical analysis

All values are presented as mean ± SD of at least three independent experiments unless otherwise stated. The statistical significance of differences was determined by the Student's *t*-test for comparisons between means. $P < 0.05$ was considered statistically significant.

3. Results

3.1. High NLRC5 expression is associated with advanced stage and poor prognosis in ccRCC patients

To investigate the role of NLRC5 in human ccRCC, we first analysed available human datasets of ccRCC patients in TCGA database. We found that mRNA levels of NLRC5 are significantly higher in ccRCC tissues compared to those in normal tissues (Fig. 1A) (http://xenabrowser.net/). TCGA human clinical data revealed that patients with higher NLRC5 mRNA expression exhibited significantly worse overall survival (Fig. 1B) (http://www.oncolnc.org/), suggesting that NLRC5 may play a role in promoting ccRCC progression. In addition, our analysis showed that NLRC5 mRNA levels were significantly higher in T3/T4-stage tumours than in T1/T2-stage tumours (Fig. 1C) (http://xenabrowser.net/).

In summary, results from TCGA human clinical sample surveys suggest that increased expression of NLRC5 is associated with advanced stage and poor prognosis in ccRCC patients.

3.2. NLRC5 is aberrantly overexpressed in ccRCC tissue samples and ccRCC cell lines

To confirm the above findings, NLRC5 expression was first analysed in ccRCC and adjacent noncancer tissues (ANTs). Western blotting and real-time PCR results confirmed that NLRC5 was overexpressed in carcinoma tissues compared to levels in ANTs (Fig. 2A and B). Furthermore, representative haematoxylin and eosin (H&E) staining in ccRCC tissues and immunohistochemistry (IHC) for NLRC5 confirmed these results (Fig. 2C). Next, the expression of NLRC5 was assessed in a panel of ccRCC cell lines (Caki-1, 786-O, and 769-P), with HK-2 cells serving as a control. Results indicated that NLRC5 protein was significantly elevated in ccRCC cell lines in comparison to HK-2 cell levels (Fig. 2E), and this was consistent with the results of real-time PCR (Fig. 2D). Taken together, these results suggest that NLRC5 is aberrantly upregulated in human ccRCC tissues and ccRCC cell lines.

3.3. Lipidosome-mediated transduction resulted in NLRC5 knockdown or overexpression in ccRCC cell lines

To confirm the above data derived from human ccRCC tissues and to corroborate the function of NLRC5 in ccRCC cells, we knocked down NLRC5 expression using targeted siRNA in 786-O and 769-P cells. NLRC5-siRNA induced a clear decrease in NLRC5 protein and mRNA levels in 786-O and 769-P cells in comparison with levels induced by NC-siRNA (Fig. 3A and C). In contrast, NLRC5 protein and mRNA levels were increased by pEGFP-C3-NLRC5 introduction in 786-O and 769-P cells (Fig. 3B and D).

3.4. NLRC5 promotes proliferation of ccRCC cells in vitro

Upon establishment of the NLRC5 knockdown and overexpression lines, CCK8 assay was performed to assess the effect of NLRC5 on 786-O and 769-P cell proliferation. Results showed that depletion of NLRC5 with siRNA dramatically attenuated cell proliferation compared with that in cells treated with NC-siRNA (Fig. 4A and B). Furthermore, we found that expression of the cell proliferation marker PCNA was significantly reduced following NLRC5 depletion (Fig. 4C and E). Conversely, enforced overexpression of NLRC5 in 786-O and 769-P cells resulted in higher PCNA protein levels compared with that in the control group (Fig. 4D and F). Next, a fluorescence-activated cell sorting assay was employed to investigate whether the NLRC5 knockdown-induced inhibition of ccRCC cell proliferation is associated with cell cycle arrest. Flow cytometry results indicated that NLRC5-depleted ccRCC cells exhibited a larger G0/G1 population compared with that in 786-O and 769-P NC cells (Fig. 5A and C), indicating that NLRC5 knockdown blocked cell cycle progression. Conversely, the percentage of cells in G0/G1 phase was reduced in 786-O and 769-P cells overexpressing NLRC5 compared with that in the control group (Fig. 5B and D). These results demonstrate that NLRC5 expression levels are positively correlated with ccRCC cell proliferation.

3.5. NLRC5 mediates migration and invasion of ccRCC cells in vitro

Having observed a change in cell proliferation following the knockdown of NLRC5, we utilised a series of assays to detect whether knockdown of NLRC5 affected ccRCC cell migration and invasion. Transwell migration assays revealed that NLRC5-depleted 786-O and 769-P cells migrated significantly less than cells transfected with NC-siRNA (Fig. 6A and E). Next, using Matrigel-coated Transwell invasion assays, we found that NLRC5 depletion reduced the ability of cells to migrate across the gel matrix into the adjacent chamber compared to that in the control group (Fig. 6C and G). In contrast, overexpression of NLRC5 in 786-O and 769-P cells increased cell migration and invasion compared to that in the control group (Fig. 6B, D, F, and H).

A previous study showed that degradation of the extracellular matrix (ECM) by matrix metalloproteinases potentially contributes to cancer progression, invasion, and metastasis [20]. MMP2 and MMP9 play a critical role in the invasion and metastasis of ccRCC [21–24]. We then examined MMP2/9 protein expression in HK-2, Caki-1, 786-O, and 769-P cells. Results showed a significant increase in MMP2/9 protein levels in ccRCC cell lines compared to that in the HK-2 cells (Fig. 2G). As early studies indicated that HGF/Met signalling may promote cancer cell migration and invasion by elevating MMP2/MMP9 expression [25], we also assayed these potential downstream signals. Results revealed that knocking down NLRC5 with NLRC5-siRNA suppressed the expression of MMP2/9 proteins in 786-O and 769-P cells (Fig. 7A and C), while overexpression of NLRC5 led to increases in the expression of MMP2/9 (Fig. 7B and D).

Collectively, these data reveal that NLRC5 potentially contributes to ccRCC tumour migration and invasion *in vitro*.

3.6. NLRC5 activates Wnt/β-catenin signalling pathway in ccRCC cells

Some oncogenic signalling pathways, such as the Wnt/β-catenin pathway, are associated with tumour cell proliferation, invasion, and migration. The involvement of the Wnt/β-catenin pathway is commonly studied in ccRCC [26]. In this study, western blotting analysis showed that the protein levels of β-catenin, c-Myc, and cyclin D1 in ccRCC cells were higher than those in HK-2 cells (Fig. 2F). To determine the mechanism underlying NLRC5-induced tumour cell proliferation, invasion, and migration, we further measured the protein levels of β-catenin and its downstream targets c-Myc and cyclin D1 when NLRC5 expression levels were modulated in 786-O and 769-P cells. Knockdown of NLRC5 by siRNA significantly decreased the protein levels of β-catenin, c-Myc, and cyclin D1 compared with those in 786-O and 769-P cells transfected with NC-siRNA (Fig. 8A and C). In contrast, the protein expression of β-catenin, c-Myc, and cyclin D1 was dramatically upregulated after transfection with pEGFP-C3-NLRC5 compared with that in the control group (Fig. 8B and D). Taken together, these results demonstrate that NLRC5 depletion suppresses the Wnt/β-catenin pathway.

3.7. Effects of NLRC5 on tumour growth in vivo

In order to confirm the effect of NLRC5 on tumour formation *in vivo*, we first established a stable 786-O cell line using shNLRC5, with control shRNA used as a negative control (Fig. 9A). Treatment with shNLRC5 significantly decreased the expression of NLRC5 compared with that in the control group (Fig. 9B). Then, nude mice received subcutaneous injections of shNLRC5-treated or control 786-O cells to establish the tumour model. After 8 weeks, tumours were completely stripped.

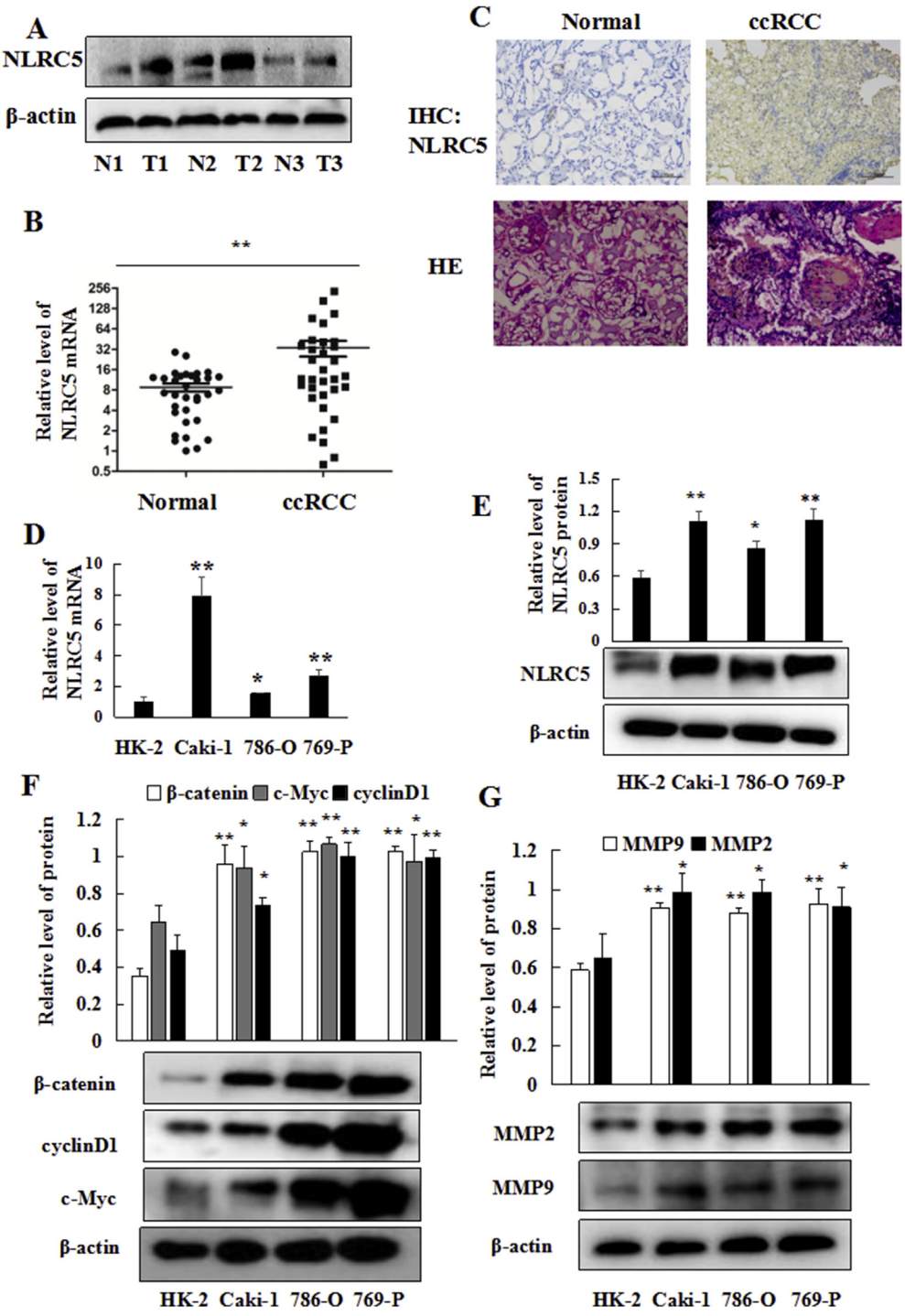

Fig. 2. NLRC5 is overexpressed in ccRCC tissues and cell lines. (A) Western blotting analysis of NLRC5 protein levels in ccRCC and normal tissues. **(B)** Expression of NLRC5 mRNA in ccRCC and normal tissues. (n = 33). **(C)** Expression of NLRC5 as examined by immunohistochemistry (original magnification, ×200). **(D)** Real-time PCR analysis of relative NLRC5 mRNA expression levels in a panel of three human ccRCC cell lines and an immortalised proximal tubule epithelial cell line (HK-2). **(E)** Western blotting analysis of NLRC5 expression in ccRCC cell lines and HK-2 cells. **(F, G)** Western blotting analysis of β-catenin, c-Myc, cyclin D1, MMP2, and MMP9 in ccRCC cell lines and HK-2 cells. β-actin served as a loading control, and the indicated proteins were quantified with ImageJ software. Data are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, compared with normal or HK-2 cells or tissues.

Photographs and measured volumes of the tumours indicated that NLRC5-depleted cells grew much more slowly than control cells (Fig. 9C and E). Moreover, the weights of the tumours from these mice were lower than those from control mice (Fig. 9D). Western blotting showed that NLRC5 knockdown inhibited the expression of MMP9, MMP2, PCNA, β-catenin, c-Myc and cyclin D1 compared with levels in control tumours (Fig. 9F and G). Together, these data demonstrate that NLRC5 knockdown inhibits tumour growth *in vivo* by blocking the Wnt/β-catenin signalling pathway in 786-O cells.

4. Discussion

Pattern recognition receptors (PRRs) are critical sensors in the innate immune system, detecting invading microbes and cellular stress. NLRs are a recently discovered family of cytoplasmic PRRs, the function of which mainly involves inducing inflammation and cell death [27,28]. Additionally, the activation of NLRs have has been implicated in numerous types of cancer, including colon cancer, breast cancer, and glioma [29–31]. NLRC5 is a newly identified member of the NLR family that has been demonstrated to function in both innate and adaptive immune signalling. NLRC5 acts as a transcriptional regulator of MHC class I expression [8]. However, the role of NLRC5 in regulating immune responses is unclear and remains to be further explored [15]. Rodriguez et al. reported that NLRC5 may play a prominent role in antitumour immunity and that its loss may promote tumour immune evasion [32]. However, our previous study showed that NLRC5

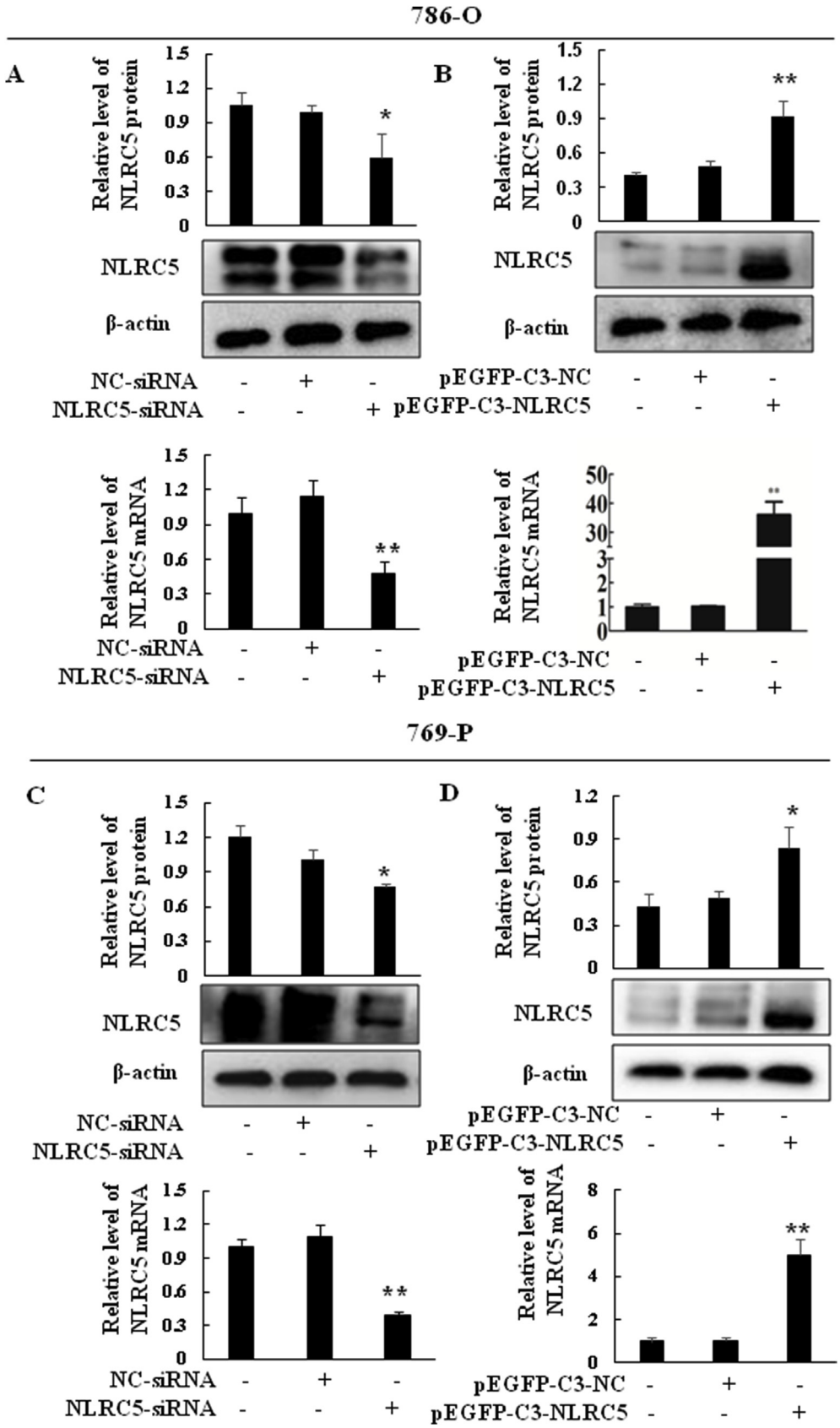

Fig. 3. Downregulation or overexpression of NLRC5 in ccRCC cell lines. (A, C) Protein and mRNA levels of NLRC5 were detected in 786-O and 769-P cells without transfection (lane 1) or transfected with negative control (NC; lane 2) or si-NLRC5 (lane 3). **(B, D)** Protein and mRNA levels of NLRC5 were detected in 786-O and 769-P cells without transfection (lane 1) or transfected with NC (lane 2) or pEGFP-C3-NLRC5 (lane 3). β-actin served as a loading control. Data are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, compared with the NC group.

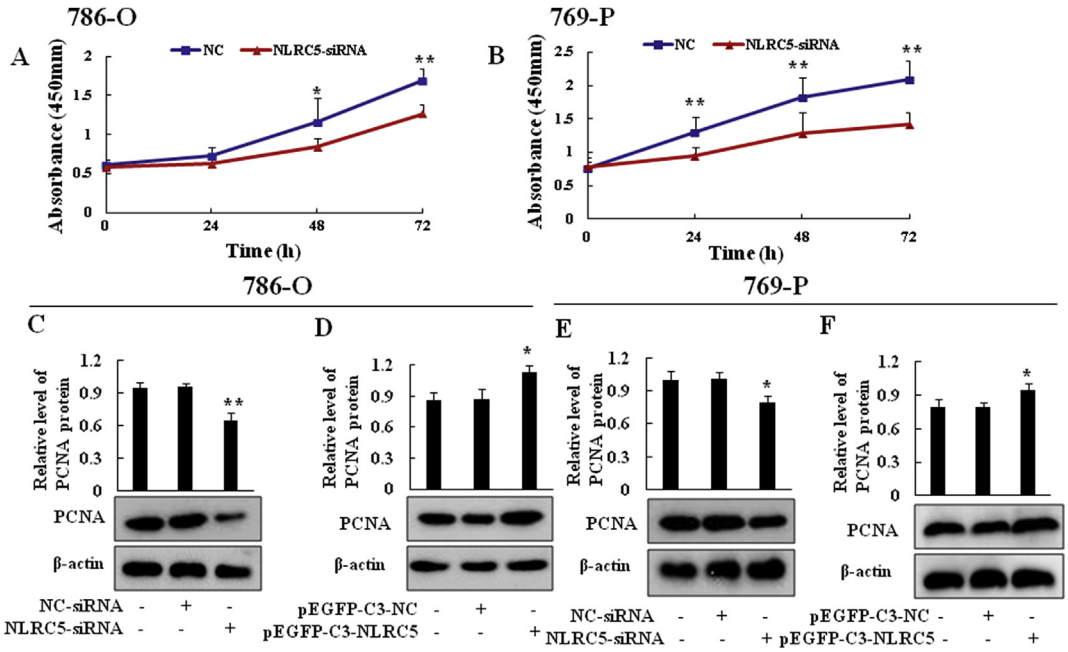

Fig. 4. Effects of NLRC5 on proliferation ability of ccRCC cells. (A, B) Cell growth of 786-O and 769-P cells after transfection with si-NLRC5 as determined by CCK8 assay at different time points. **(C, E)** Western blotting analysis of PCNA in NLRC5-depleted 786-O and 769-P cells. **(D, F)** Western blotting analysis of PCNA in 786-O and 769-P cells overexpressing NLRC5. β-actin served as a loading control. Data are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, compared with the NC group.

promoted cell proliferation, migration, and invasion in HCC [16]. Li et al. reported that NLRC5 may be a negative prognostic indicator in patients with stage III NSCLC [18]. This represents the first report of the effect of NLRC5 on ccRCC.

In the present study, data from TCGA database showed higher expression of NLRC5 mRNA in ccRCC tumour tissues than in normal tissues. Furthermore, clinical data from TCGA database confirmed that increased NLRC5 expression is associated with shorter overall survival and higher T stages in ccRCC patients. This provides powerful clinical evidence that NLRC5 plays a positive role in ccRCC progression. In our experimental analyses, we demonstrated the roles of NLRC5 in human ccRCC and investigated the possible underlying mechanism. We showed that NLRC5 was aberrantly overexpressed in human ccRCC tissues and ccRCC cell lines. These results demonstrate that NLRC5 may function as an oncogene, playing an important role in the tumorigenesis and progression of ccRCC.

The proliferation, migration, and invasion abilities of cells are the three most important features of malignant cell behaviour. We found

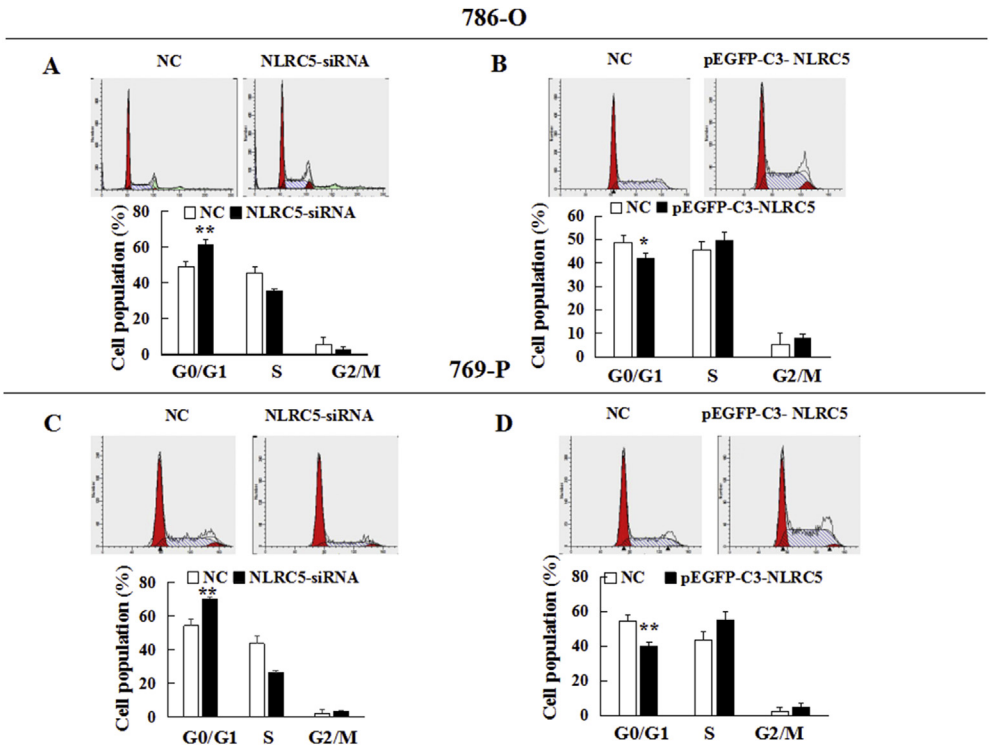

Fig. 5. Effects of NLRC5 on cell cycle distribution of ccRCC cells. (A, C) Knockdown of NLRC5 induced G0/G1 phase arrest in 786-O and 769-P cells according to flow cytometric analysis. **(B, D)** Cell arrest in G0/G1 phase was decreased in 786-O and 769-P cells transfected with pEGFP-C3-NLRC5. Data are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, compared with the NC group.

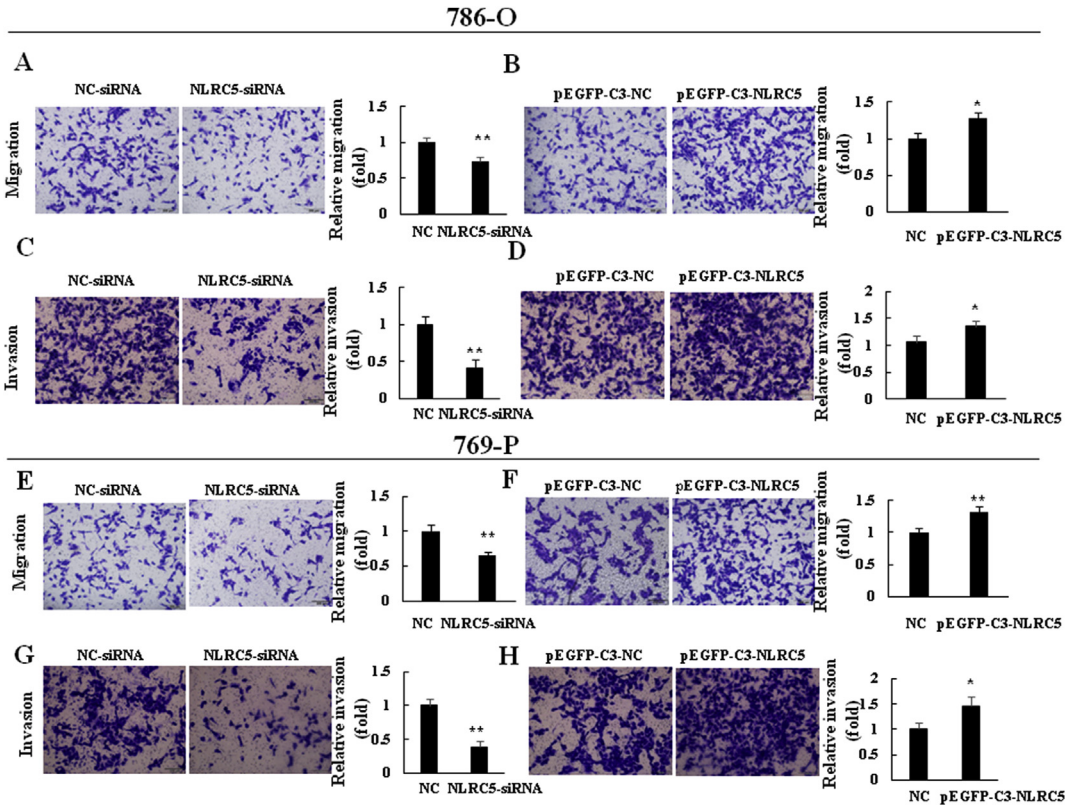

Fig. 6. Effects of NLRC5 on cell migration and invasion of ccRCC cells *in vitro*. (A, B) Transwell migration assay of migratory capacity of 786-O cells after NLRC5 depletion or overexpression. **(C, D)** Matrigel Transwell assay of invasive potential of 786-O cells after NLRC5 depletion or overexpression. **(E, F)** Transwell migration assay of migratory capacity of 769-P cells after NLRC5 depletion or overexpression. **(G, H)** Matrigel Transwell assay of invasive potential of 769-P cells after NLRC5 depletion or overexpression. The optical density of the NC invaded or migrated cells was set at 1. Data are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, compared with the NC group.

that depletion of NLRC5 suppressed the proliferation, migration, and invasion of ccRCC cells. Moreover, our findings indicated that the overexpression of NLRC5 promoted these behaviours in ccRCC cells. The most frequent form of ccRCC presents with inactivating mutations or epigenetic silencing of *VHL* [33]. However, *VHL* inactivation alone may not be sufficient to drive tumour formation, as evidenced in genetically engineered $VHL^{-/-}$ animal models [34]. In addition to *VHL* inactivation, recent studies in mice have identified mutations in other genes, including cell cycle regulatory genes, in the development of ccRCC [35]. We further showed in this study that knockdown of NLRC5 induced cell cycle arrest in G0/G1 phase. However, the effects of NLRC5 on apoptosis and drug resistance remain to be elucidated in future studies.

The inhibition of MMP2/9 expression and secretion is important for preventing cell migration, invasion, and metastasis [23,34,36]. A polymorphism in MMP9, while not associated with increased risk of RCC, is correlated with malignancy potential [37]. In RCCs, Kugler et al. [38] reported strong correlations between increased MMP2 and MMP9 mRNA expression and tumour stage. By contrast, Lein et al. [39] reported that MMP9 expression was elevated in both RCC tissues and sera of patients with RCC, but it was not correlated with tumour type, grade, stage, or prognosis. In the present study, western blotting results indicated that MMP2 and MMP9 were upregulated in ccRCC cell lines, in comparison to levels in HK-2 cells. Our results showed that NLRC5 depletion attenuated MMP2 and MMP9 protein expression *in vitro* and *in vivo*, thereby leading to a less aggressive tumour phenotype. These results further indicate that NLRC5 may be a cancer-promoting protein in ccRCC.

It has been shown that the dysregulation of Wnt signalling contributes to the development of human cancers, including colorectal, ovarian, and breast cancer and ccRCC [26,42,44]. Various cellular functions, such as apoptosis, proliferation, migration, and invasion, are involved in Wnt-dependent carcinogenesis. Besides the PI3K/Akt/mTOR, HGF/Met, and *VHL*/hypoxia cellular signalling pathways, the involvement of the Wnt/β-catenin pathway is commonly studied in ccRCC [26]. Ectopic regulation of the Wnt/β-catenin signalling pathway by DNA methylation [40] or mutation [41–43] can induce changes in the expression of Wnt/β-catenin signalling molecules that are linked to renal malignancy. These findings highlight the potential prognostic and therapeutic value of the Wnt/β-catenin signalling pathway in ccRCC.

The present study indicated that NLRC5 promotes tumour cell proliferation, migration, and invasion by stimulating β-catenin transcription and translation through the Wnt/β-catenin signalling pathway. This was demonstrated by the fact that β-catenin protein levels in ccRCC cells were much higher than those in control HK-2 cells. Moreover, the silencing of NLRC5 significantly suppressed β-catenin expression, while its overexpression had the opposite effect. The oncogene c-Myc and cell cycle regulator cyclin D1 have been identified as target genes in the Wnt/β-catenin signalling pathway [45–47]. We observed increased protein levels of c-Myc and cyclin D1 in Caki-1, 786-O, and 769-P cells. Furthermore, knockdown of NLRC5 attenuated the expression of c-Myc and cyclin D1 proteins, suggesting that NLRC5 may influence cell proliferation, migration, and invasion via the Wnt/β-catenin signalling pathway.

In conclusion, our study showed that NLRC5 is a crucial modulator of human ccRCC progression, proliferation, migration, and invasion via the Wnt/β-catenin signalling pathway. These data suggest that NLRC5 may represent a new therapeutic target for ccRCC therapy.

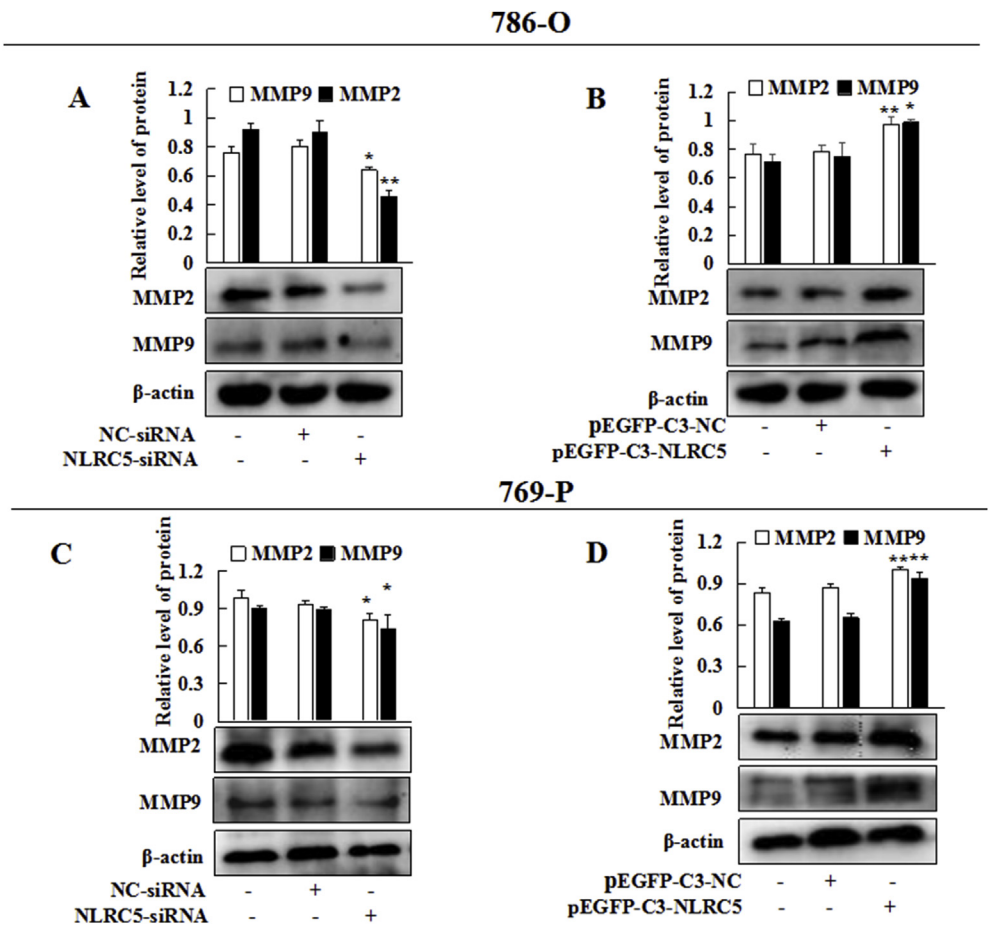

Fig. 7. Effects of NLRC5 on MMP2 and MMP9 in ccRCC cells. (A, B) Western blotting analysis of MMP2 and MMP9 protein levels in 786-O cells after NLRC5 depletion or overexpression. **(C, D)** Western blotting analysis of MMP2 and MMP9 protein levels in 769-P cells after NLRC5 depletion or overexpression. β-actin was used as the loading control. Data are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, compared with the NC group.

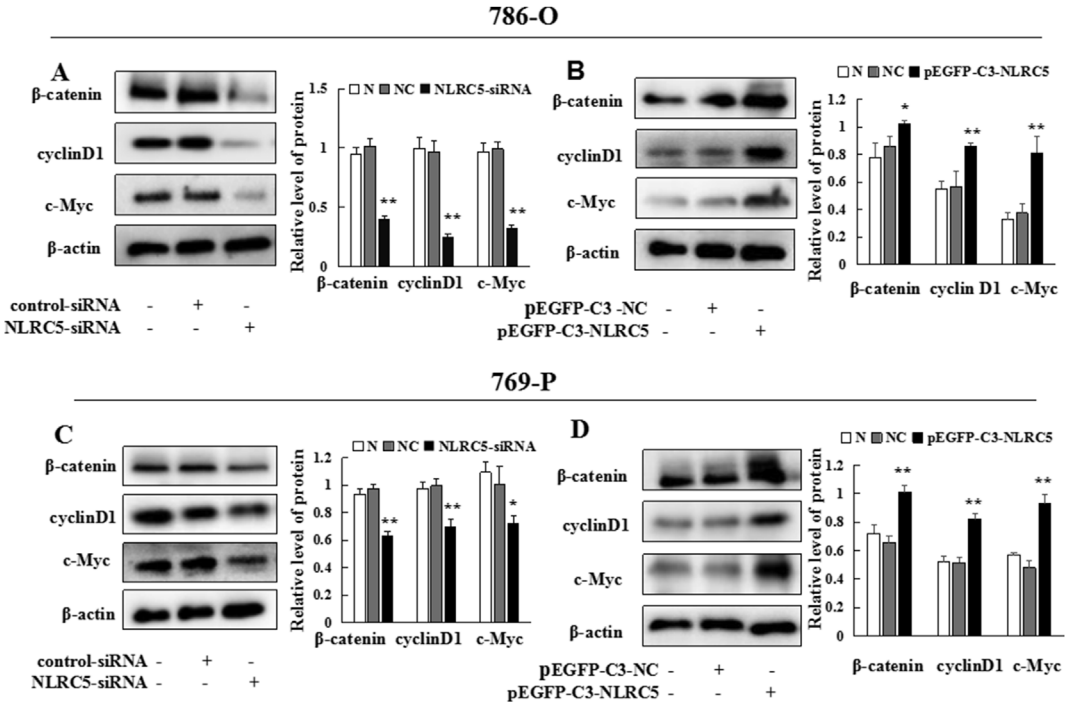

Fig. 8. NLRC5 is associated with the Wnt/β-catenin signalling pathway in ccRCC cells. (A, C) Western blotting analysis of β-catenin, c-Myc, and cyclin D1 in 786-O and 769-P cells with transient silencing of NLRC5 (si-NLRC5) or in NC cells without transfection. **(B, D)** Overexpression of NLRC5 increased the protein expression of β-catenin, c-Myc, and cyclin D1 in 786-O and 769-P cells. Data are presented as the mean ± SD of three independent experiments and were normalized to levels of β-actin. *P < 0.05, **P < 0.01, compared with the NC group.

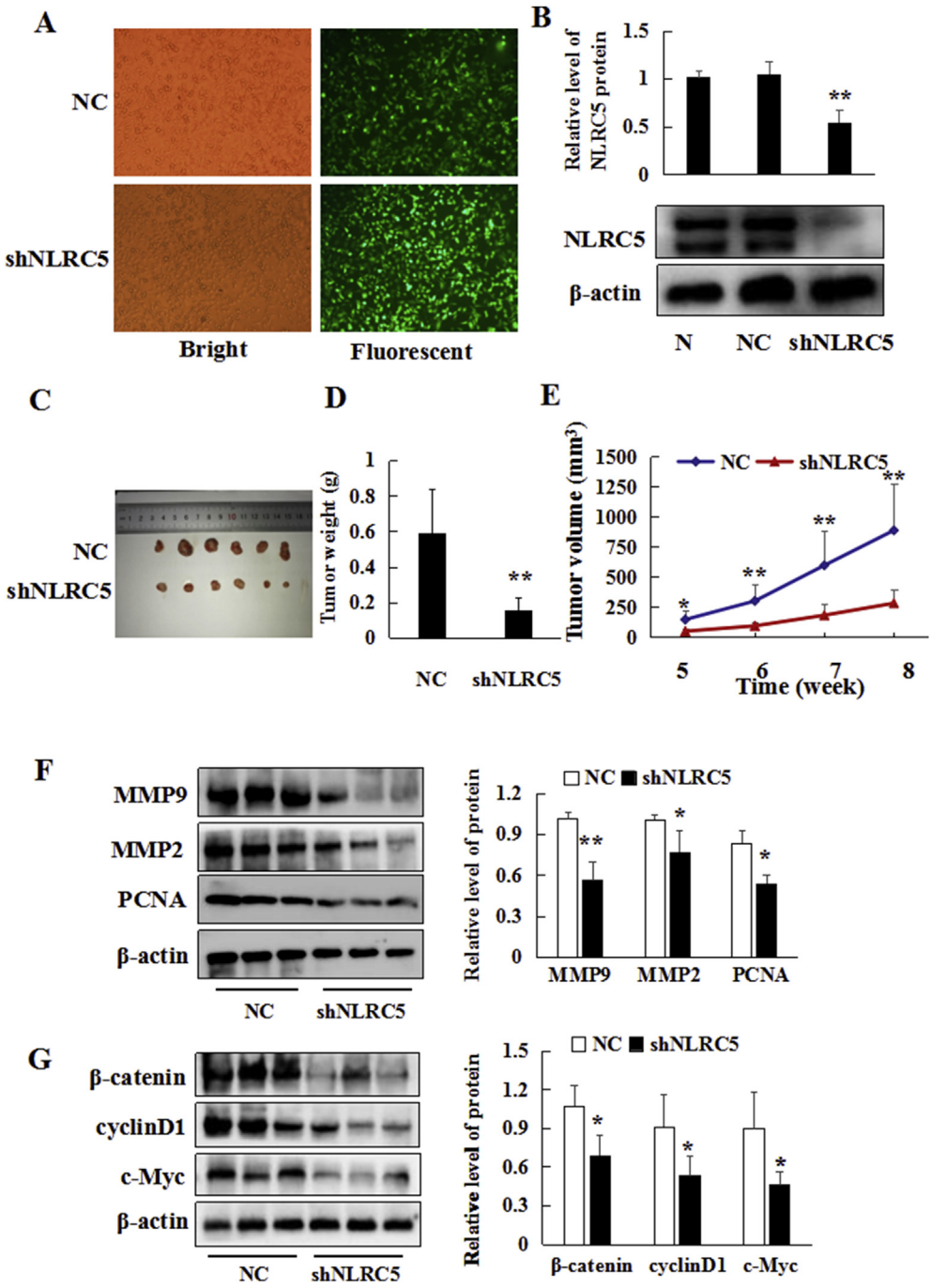

Fig. 9. Effects of NLRC5 on tumour growth *in vivo*. **(A)** Stable NC and shNLRC5-transfected 786-O cell lines were observed by inversed fluorescent microscope. **(B)** Protein expression of NLRC5 in the normal group, NC group, and shNLRC5 group. **(C, D)** Tumour images and weights at experimental endpoints in NC and shNLRC5 786-O xenografts (n = 6 for each group). **(E)** Tumour volumes, measured weekly. **(F, G)** Western blotting analysis of MMP9, MMP2, PCNA, β-catenin, c-Myc and cyclin D1 in tumour tissues of NC and shNLRC5 groups. β-actin was used as an internal control. Bar graphs (mean ± SD) and representative images are shown. *P < 0.05, **P < 0.01, compared with the NC group.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

Funding: This work was supported by the National Natural Science Foundation of China (grant number 81473268).

References

[1] S. Turajlic, J. Larkin, C. Swanton, SnapShot: renal cell carcinoma, Cell 163 (2015) 1556-1556 e1551.
[2] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, Ca - Cancer J. Clin. 67 (2017) 7–30.
[3] H.T. Cohen, F.J. McGovern, Renal-cell carcinoma, N. Engl. J. Med. 353 (2005) 2477–2490.
[4] L. Gossage, T. Eisen, Alterations in *VHL* as potential biomarkers in renal-cell carcinoma, Nat. Rev. Clin. Oncol. 7 (2010) 277–288.
[5] P.X. Li, Y.N. Wong, K. Armstrong, N. Haas, P. Subedi, M. Davis-Cerone, J.A. Doshi, Survival among patients with advanced renal cell carcinoma in the pretargeted versus targeted therapy eras, Cancer Med. 5 (2016) 169–181.
[6] W.M. Stadler, Targeted agents for the treatment of advanced renal cell carcinoma, Cancer 104 (2005) 2323–2333.
[7] J. Cui, L. Zhu, X. Xia, H.Y. Wang, X. Legras, J. Hong, J. Ji, et al., NLRC5 negatively regulates the NF-kappaB and type I interferon signaling pathways, Cell 141 (2010) 483–496.
[8] K.S. Kobayashi, P.J. van den Elsen, NLRC5: a key regulator of MHC class I-dependent immune responses, Nat. Rev. Immunol. 12 (2012) 813–820.
[9] J.P.Y. Ting, B.K. Davis, Caterpiller: a novel gene family important in immunity, cell death, and diseases, Annu. Rev. Immunol. 23 (2005) 387–414.
[10] T.B. Meissner, A. Li, Y.J. Liu, E. Gagnon, K.S. Kobayashi, The nucleotide-binding domain of NLRC5 is critical for nuclear import and transactivation activity, Biochem. Biophys. Res. Commun. 418 (2012) 786–791.
[11] S. Benko, J.G. Magalhaes, D.J. Philpott, S.E. Girardin, NLRC5 limits the activation

of inflammatory pathways, J. Immunol. 185 (2010) 1681–1691.
[12] P.G.M. Gutte, S. Jurt, M.G. Grutter, O. Zerbe, Unusual structural features revealed by the solution NMR structure of the NLRC5 caspase recruitment domain, Biochemistry 53 (2014) 3106–3117.
[13] F. Staehli, K. Ludigs, L.X. Heinz, Q. Seguin-Estevez, I. Ferrero, M. Braun, K. Schroder, et al., NLRC5 deficiency selectively impairs MHC class I-dependent lymphocyte killing by cytotoxic T cells, J. Immunol. 188 (2012) 3820–3828.
[14] S. Yoshihama, S. Vijayan, T. Sidiq, K.S. Kobayashi, NLRC5/CITA: a key player in cancer immune surveillance, Trend. Canc. 3 (2017) 28–38.
[15] Y.K. Yao, Y.C. Qian, Expression regulation and function of NLRC5, Protein Cell 4 (2013) 168–175.
[16] Y.Y. Peng, Y.H. He, C. Chen, T. Xu, L. Li, M.M. Ni, X.M. Meng, et al., NLRC5 regulates cell proliferation, migration and invasion in hepatocellular carcinoma by targeting the Wnt/beta-catenin signaling pathway, Cancer Lett. 376 (2016) 10–21.
[17] Y.H. He, M.F. Li, X.Y. Zhang, X.M. Meng, C. Huang, J. Li, NLRC5 promotes cell proliferation via regulating the AKT/VEGF-A signaling pathway in hepatocellular carcinoma, Toxicology 359 (2016) 47–57.
[18] X. Li, F. Guo, Y. Liu, H.J. Chen, F. Wen, B. Zou, D. Li, et al., NLRC5 expression in tumors and its role as a negative prognostic indicator in stage III non-small-cell lung cancer patients, Oncol. Lett. 10 (2015) 1533–1540.
[19] S. Wang, X. Zhao, S. Yang, B. Chen, J. Shi, Knockdown of NLRC5 inhibits renal fibroblast activation via modulating TGF-beta1/Smad signaling pathway, Eur. J. Pharmacol. 829 (2018) 38–43.
[20] M. Cohen, C. Wuillemin, O. Irion, P. Bischof, Regulation of MMP-9 by p53 in first trimester cytotrophoblastic cells, Hum. Reprod. 23 (2008) 2273–2281.
[21] M.C. Wang, Y. Sun, J.J. Xu, J.Y. Lu, K.F. Wang, D.R. Yang, G.S. Yang, et al., Preclinical studies using miR-32-5p to suppress clear cell renal cell carcinoma metastasis via altering the miR-32-5p/TR4/HGF/Met signaling, Int. J. Canc. 143 (2018) 100–112.
[22] L.S. Yuan, G. Zeng, L. Chen, G. Wang, X.L. Wang, X.Y. Cao, M.X. Lu, et al., Identification of key genes and pathways in human clear cell renal cell carcinoma (ccRCC) by co-expression analysis, Int. J. Biol. Sci. 14 (2018) 266–279.
[23] Y.W. Lin, L.M. Lee, W.J. Lee, C.Y. Chu, P. Tan, Y.C. Yang, W.Y. Chen, et al., Melatonin inhibits MMP-9 transactivation and renal cell carcinoma metastasis by suppressing Akt-MAPKs pathway and NF-B DNA-binding activity, J. Pineal Res. 60 (2016) 277–290.
[24] S. Mikami, M. Oya, R. Mizuno, T. Kosaka, K. Katsube, Y. Okada, Invasion and metastasis of renal cell carcinoma, Med. Mol. Morphol. 47 (2014) 63–67.
[25] J.H. Baek, C. Birchmeier, M. Zenke, T. Hieronymus, The HGF receptor/Met tyrosine kinase is a key regulator of dendritic cell migration in skin immunity, J. Immunol. 189 (2012) 1699–1707.
[26] Q. Xu, M. Krause, A. Samoylenko, S. Vainio, Wnt signaling in renal cell carcinoma, Cancers 8 (2016) E57.
[27] J.P.Y. Ting, S.B. Willingham, D.T. Bergstralh, NLRs at the intersection of cell death and immunity, Nat. Rev. Immunol. 8 (2008) 372–379.
[28] J.M. Wilmanski, T. Petnicki-Ocwieja, K.S. Kobayashi, NLR proteins: integral members of innate immunity and mediators of inflammatory diseases, J. Leukoc. Biol. 83 (2008) 13–30.
[29] L. Jin, R.Q. Yuan, A. Fuchs, Y. Yao, A. Joseph, R. Schwall, S.J. Schnitt, et al., Expression of interleukin-1beta in human breast carcinoma, Cancer 80 (1997) 421–434.
[30] I.C. Allen, J.E. Wilson, M. Schneider, J.D. Lich, R.A. Roberts, J.C. Arthur, R.M.T. Woodford, et al., NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappa B signaling, Immunity 36 (2012) 742–754.
[31] L. Tarassishin, D. Casper, S.C. Lee, Aberrant expression of interleukin-1 beta and inflammasome activation in human malignant gliomas, PLoS One 9 (2014) e103432.
[32] G.M. Rodriguez, D. Bobbala, D. Serrano, M. Mayhue, A. Champagne, C. Saucier, V. Steimle, et al., NLRC5 elicits antitumor immunity by enhancing processing and presentation of tumor antigens to CD8(+) T lymphocytes, OncoImmunology 5 (2016) e1151593.
[33] M.L. Nickerson, E. Jaeger, Y. Shi, J.A. Durocher, S. Mahurkar, D. Zaridze, V. Matveev, et al., Improved identification of von Hippel-Lindau gene alterations in clear cell renal tumors, Clin. Canc. Res. 14 (2008) 4726–4734.
[34] S.J. Mandriota, K.J. Turner, D.R. Davies, P.G. Murray, N.V. Morgan, H.M. Sowter, C.C. Wykoff, et al., HIF activation identifies early lesions in *VHL* kidneys: evidence for site-specific tumor suppressor function in the nephron, Cancer Cell 1 (2002) 459–468.
[35] Y. Sato, T. Yoshizato, Y. Shiraishi, S. Maekawa, Y. Okuno, T. Kamura, T. Shimamura, et al., Integrated molecular analysis of clear-cell renal cell carcinoma, Nat. Genet. 45 (2013) 860-U191.
[36] S.W. Tang, T.C. Yang, W.C. Lin, W.H. Chang, C.C. Wang, M.K. Lai, J.Y. Lin, Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells, Carcinogenesis 32 (2011) 138–145.
[37] Y. Awakura, N. Ito, E. Nakamura, T. Takahashi, H. Kotani, Y. Mikami, T. Manabe, et al., Matrix metalloproteinase-9 polymorphisms and renal cell carcinoma in a Japanese population, Cancer Lett. 241 (2006) 59–63.
[38] A. Kugler, B. Hemmerlein, P. Thelen, M. Kallerhoff, H.-J. Radzun, R.-H. Ringert, Expression of metalloproteinase 2 and 9 and their inhibitors in renal cell carcinoma, J. Urol. 160 (1998) 1914–1918.
[39] M.J. Lein, K. Jung, C. Laube, T. Hübner, B. Winkelmann, C. Stephan, S. Hauptmann, B. Rudolph, D. Schnorr, S.A. Loening, Matrix-metalloproteinases and their inhibitors in plasma and tumor tissue of patients with renal cell carcinoma, Int. J. Canc. 85 (2000) 801–804.
[40] N. Shenoy, N. Vallumsetla, Y.Y. Zou, J.N. Galeas, M. Shrivastava, C. Hu, K. Susztak, et al., Role of DNA methylation in renal cell carcinoma, J. Hematol. Oncol. 8 (2015).
[41] G. Banumathy, P. Cairns, Signaling pathways in renal cell carcinoma, Cancer Biol. Ther. 10 (2010) 658–664.
[42] E. Cojocaru, L. Lozneanu, S.E. Giusca, I.D. Caruntu, M. Danciu, Renal carcinogenesis – insights into signaling pathways, Rom. J. Morphol. Embryol. 56 (2015) 15–19.
[43] S. Majid, S. Saini, R. Dahiya, Wnt signaling pathways in urological cancers: past decades and still growing, Mol. Canc. 11 (2012) 7.
[44] A.H.N. Kamdje, P.F.S. Etet, L. Vecchio, J.M. Muller, M. Krampera, K.E. Lukong, Signaling pathways in breast cancer: therapeutic targeting of the microenvironment, Cell. Signal. 26 (2014) 2843–2856.
[45] N. Janssens, L. Andries, M. Janicot, T. Perera, A. Bakker, Alteration of frizzled expression in renal cell carcinoma, Tumor Biol. 25 (2004) 161–171.
[46] K.A. Furge, J. Chen, J. Koeman, P. Swiatek, K. Dykema, K. Lucin, R. Kahnoski, et al., Detection of DNA copy number changes and oncogenic signaling abnormalities from gene expression data reveals MYC activation in high-grade papillary renal cell carcinoma, Cancer Res. 67 (2007) 3171–3176.
[47] M.L. Gumz, H. Zou, P.A. Kreinest, A.C. Childs, L.S. Belmonte, S.N. LeGrand, K.J. Wu, et al., Secreted frizzled-related protein 1 loss contributes to tumor phenotype of clear cell renal cell carcinoma, Clin. Canc. Res. 13 (2007) 4740–4749.