Original Article
Expression and functional role of miR-29b in renal cell carcinoma

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Abstract: Objectives: microRNAs (miRNAs) play essential roles in many tumors, including renal cell carcinoma (RCC). The aim of the present study was to investigate the expression and functional role of miR-29b in RCC and to identify its target genes. Methods: We determined the expression of miR-29b in clear cell RCC (ccRCC) tissues and RCC cell lines (786-O, A498, and SN12-PM6) using quantitative real-time PCR (qRT-PCR). The associations between miR-29b expression and clinical pathological parameters and prognosis were explored. Besides, the role of miR-29b in the SN12-PM6 cells proliferation, apoptosis, cycle, and invasion were investigated after transduction with lentivirus vectors. The kines in family member 1B (KIF1B), possible miR-29b target genes, were predicted using bioinformatics approaches, as well as the role in the pathogenesis of RCC. Results: Elevated expression of miR-29b was found in both tumor tissues and cell lines. High expression of miR-29b was significantly associated with tumor-node-metastasis (TNM) stage (P = 0.026) and the overall survival (P = 0.009) in the ccRCC. Inhibition of miR-29b expression could promote apoptosis, and inhibit proliferation and invasion ability in SN12-PM6 cells. Also, we confirmed that miR-29b could directly regulate the expression of KIF1B at the post transcriptional level. Conclusion: These data suggest that miR-29b acts as an oncomiR, promoting proliferation and invasion ability through KIF1B suppression, and it might be a potential marker for prognosis of RCC.

Keywords: Renal cell carcinoma, miR-29b, kinesin family member 1B

Introduction

It has been reported that renal cell cancer (RCC) accounts for approximately 2-3% of all human tumors, and the incidence is still increasing at a rate of 2% per year [1, 2]. Among the genitourinary cancers, RCC has the highest mortality rate [3]. It mainly affects older subjects (at the age of 50-70 years), and men have higher incidence. Most infections are asymptomatic and not clinically evident in early stages. But RCC presents longitudinal clinical effectiveness by surgery if detected early. However, more than 50% RCC are found incidentally, and approximately 25-30% patients who are diagnosed as RCC develop a metastatic recurrence [4]. Although the 5-year disease-specific survival rate is more than 60%, patients with metastatic recurrence still have a poor prognosis [5, 6]. Therefore, it is important to search new molecular markers for early detection and prognosis.

Recently, microRNAs (miRNAs), endogenous small non-coding RNAs, have been gained a great deal of attention in many pathophysiology processes. Accumulated evidence has shown that miRNA servers as gene expression regulators in tumorigenesis by regulating many cell functions and biological processes like proliferation, differentiation, migration, invasion and apoptosis [7, 8]. Not only are miRNA expression profiles responsible for distinguishing malignant and non-malignant tissues, but also for distinguishing different tumor entities [9]. The researches respect to the association between miRNA and RCC have been well demonstrated [10-13]. Previous studies have confirmed various of miRNAs are involved in RCC, such as microRNA-1285 [14], microRNA-200c [15], miR-1233 [16], miR-29a [10], miR-26a-2, miR-191, miR-337-3p and miR-378 [17]. These miRNAs affect the growth, invasion and metastasis of RCC by regulation of downstream target genes. However, rare studies are available concerning the expression and functional role of miR-29b in RCC. A previous study has confirmed that miR-29b acts as an oncomiR in oral squamous cell carcinoma (OSCC) and maybe a potential therapeutic target for preventing progression of

OSCC [18]. Therefore, we speculated that miR-29b might also play a significant role in RCC.

This study was focused on the expression and functional role of miR-29b in RCC. The correlation between the expression of miR-29b and clinical pathological parameters was explored. Besides, we investigated the role of miR-29b in the development of RCC including cell proliferation, apoptosis, cycle, and invasion. Using bioinformatics approaches, we predicted related target genes of miR-29b and explored their roles in the pathogenesis of RCC. Our study may provide a theoretical basis for further study on the pathogenesis and new treatment for RCC.

Materials and methods

Patients and samples

The study was approved by the local Ethics Committee and informed consents were obtained from all patients. Between April 2008 and May 2012, a total of 45 patients with ccRCC who underwent partial or radical nephrectomy at our hospital were enrolled into the retrospective study. Of the patients, 30 were men and 15 were women. The ages of the patients ranged from 50 to 75 years (mean, 62.4 years). The diagnosis was based on the clinical pathological examination. Patients received non-preoperative special treatment. The original tumors were staged according to the clinical tumor-node-metastasis (TNM) classification [19] and graded according to Fuhrman. The carcinomas tissues and matched adjacent normal tissues (located 3 cm away from the tumor margin) were harvested from surgical specimens. All specimens were immediately snap-frozen at -80°C after operation. A fully complete follow up for survival was obtained from all patients.

Cell lines and cultures

The clear cell renal cell carcinoma (ccRCC) cell lines 786-O and A498 were purchased from Shanghai cell biochemical institute, China Academy of Science (Shanghai, China). SN12-PM6 cell line was supplied by MD Anderson Cancer Center (Houston, TX). Additionally, human 293T cell line was purchased from American Type Culture Collection. The 786-O and A498 cells were maintained in RPMI (GIBCO Invitrogen GmbH, Karlsruhe, Germany).

The 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen). The SN12-PM6 cells and were maintained in DMEM with 1% minimum essential medium (MEM) Vitamin Solution (Invitrogen) and 1% MEM non-essential amino acid solution (Invitrogen). Each culture medium was supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc.), 100 U/mL penicillin (Invitrogen), and 100 U/mL streptomycin (Invitrogen). All cell lines were maintained at 37°C in humidified atmosphere of 5% CO_2.

Lentivirus production and transduction

The 3rd generation of lentivirus vector systems containing enhanced green fluorescent protein (EGFP) (GeneCopoeia Inc. Guangzhou, China) was used to construct the hsa-miR-29b lentivirus expression vector pEGFP-antagomiR-29b as well as the lentivirus vector pEGFP-negative as a negative control. The plasmid and vector were transfected into cells using lipofectamine 2000. Briefly, 293T cells were seeded in 24-well plates. When the cells density reached 70%, they were transduced with recombinant lentivirus vectors at a multiplicity of infection (MOI) of 30. The cell line stably expressing miR-29b was termed SN12-PM6-AmiR-29b, and while the negative control cell line was termed SN12-PM6-ctr. After 48 h post-transfection, luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was normalized to firefly luciferase activity. Thereafter, the cells were then collected for further analysis including quantitative real-time PCR (qRT-PCR), western blotting analysis, cell proliferation analysis, cell cycle, cell apoptosis, and Transwell invasion assays.

Analysis of miRNAs

Total RNA was extracted both from the three cell lines and the specimens using the mirVana miRNA Isolation kit (Ambion, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) templates were synthesized by Multiscribe TM Reverse Transcriptase (Applied Biosystems; Grand Island, NY). The products of PCR were identified by an Applied Biosystems 7500 Detection system (Applied Biosystems) using miScript SYBR Green PCR Kit (Qiagen SA, Hilden, Germany). The expres-

sion levels of miRNAs were measured using TaqMan® MicroRNA assays (Applied Biosystems) and then calculated by the comparative $2^{-\Delta\Delta CT}$ methods. U6 snRNA was used as a loading control. The primers used in RT-PCR were listed as below: miRNA29b, forward 5'-ACAC-TCCAGCTGGGUAGCACCAUUUGAAAUC-3', Reverse 5'-TGGTGTCGTGGAGTCG-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-CTCGCTTCGGCAGCACA-3'.

Cell proliferation assay

After transient transfection with pEGFP-miRNA, cell viability was measured by 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. In brief, SN12-PM6 cells were placed in 96-well plate at a final concentration of 1×10^4 per mL in an assay medium, and incubated at 37°C in a 5% CO_2 incubator. At different time points (24 h, 48 h, 72 h, 96 h, and 120 h), 10 μl MTT was added to each well, and then incubated at 37°C for another 4 h. The absorbance at 570 nm was read on a BioTek synergy 4 multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA).

Cell cycle and apoptosis

Cell cycle status and cell apoptosis were detected by flow cytometry (FCM) and analyzed by CellQuest software (Becton Dickinson, Bedford, MA). The transfected cells (1×10^6 to 2×10^6 cells) were collected, washed three times with phosphate buffered saline (PBS), and then re-suspended in PBS. Thereafter, the cells were fixed in ice-cold 70% ethanol at 4°C for 24 h, washed and re-suspended with PBS, stained with 0.25% propidium iodide (PI, Sigma-Aldrich) for 30 min and digested with RNase (500 U/mL, Sigma-Aldrich) in a 37°C water bath for 30 minutes. The cells were then analyzed by FCM with a FACS LSR II (BD Biosciences). For the cell apoptosis, 1×10^6 cells were processed for labeling with Annexin V/7AAD according to the PE Annexin V apoptosis detection kit, BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed by CellQuest software.

Transwell invasion assay

Cell invasion ability was analyzed by Transwell chamber assay (Costar, Cambridge, MA). Briefly, the transfected cells (1×10^5 cells/mL) were harvested and suspended in serum-free 100 μL DMEM media on 24-Transwell membranes (8 μm pores). The upper chamber was coated with 50 μL Matrigel, and the lower chamber was filled with 10% FBS. Cell suspensions were added to the upper side, and then were disassembled after incubation at 37°C for 48 h. The membranes were then fixed with 75% ethyl alcohol, stained with 0.5% crystal violet reagent, and calculated in eight random microscopic fields.

Prediction of miRNA-targeting genes

We used TargetScan 6.2, miRanda, and Pictarto search for putative miR-29b targets. A 3'-Untranslated region (UTR) of wild-type (WT) kinesin family member 1 B (KIF1B) containing the miR-29b-binding sites were cloned into the psi-CHECK-2 vector (Promega, Madison, WI, USA) downstream of the Renilla luciferase gene. Besides, the mutant-type (MT) 3' UTR plasmids were also constructed as a control. The regulatory effect of miR29b on KIF1B was verified by luciferase reporter system.

Real-time PCR

We extracted total RNA from SN12-PM6 cells with Trizol RNA isolation kit (Life Technologies) according to the manufacturer's instructions. RNA samples (1 μg) were reverse transcribed into cDNA, amplified, and quantified using qRT-PCR with SYBR green PCR master mix (Invitrogen) on LightCycler 480 (Roche Applied Science). Primers for KIF1B were designed by Invitrogen. GAPDH gene was used as a reference.

Western blotting

Cell protein concentration was identified by Bio-Rad DC protein Assay kit (Bio-Rad Laboratories, Hercules, CA). The protein samples were resolved with 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and sealed in 5% fresh nonfat dry milk in PBS for 2 h. Thereafter, the membranes were incubated either with the primary anti-KIF1B antibody or anti-β-actin (Santa Cruz Biotechnology) overnight at 4°C followed by incubation with a secondary antibody. Enhanced chemiluminescence and densitometric analysis were finally performed.
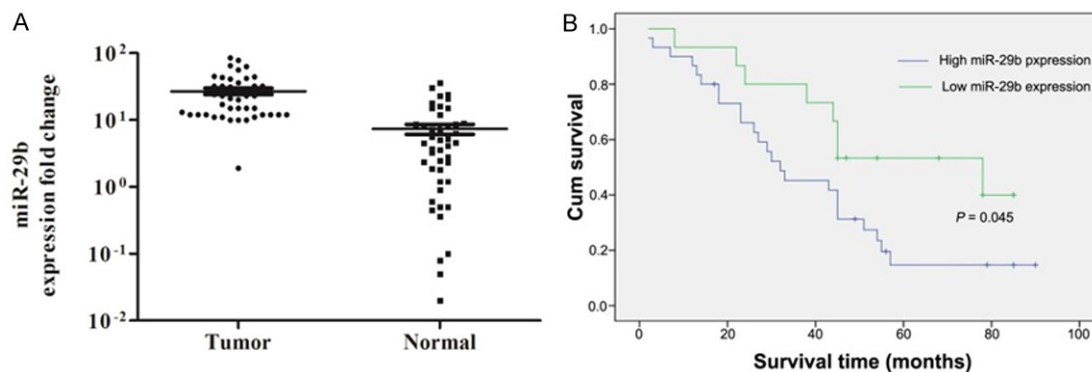
Figure 1. Expression of miR-29b in ccRCC tumor tissues and high expression correlates with a lower cancer patient survival rate. A. Expression of miR-29b in ccRCC tumor tissues; B. Survival curve of cancer patient. ccRCC, clear cell renal cell carcinoma.

Table 1. Clinical characteristics and the correlation between the expression of miR-29b and clinical pathological parameters and prognosis in RCC

Characteristics	Number	High level (n) %	Low level (n) %	*P* value
Gender				
Male	30 (66.7%)	23 (76.7)	7 (23.3)	0.368
Female	15 (33.3%)	11 (73.3)	4 (26.7)	
Ages (years)				
< 60	26 (57.8%)	15 (57.7)	11 (42.3)	0.569
≥ 60	19 (42.2%)	11 (57.9)	8 (42.1)	
Recurrence				
Yes	29 (64.4)	16 (55.2)	13 (44.8)	0.654
No	16 (35.6%)	9 (56.25)	7 (43.75)	
TNM stage				
Stage I	7 (15.6%)	1 (14.3)	6 (85.7)	0.026
Stage II	23 (51.1%)	19 (82.6%)	4 (17.3)	
Stage III	15 (33.3%)	11 (73.3)	4 (26.7)	
Lymph node metastasis				0.387
Yes	28 (62.2%)	19 (67.9)	9 (32.1)	
No	17 (37.8%)	11 (64.7)	6 (35.3)	
Tumor size (cm)				
D < 3	12 (26.7%)	6 (50)	6 (50)	0.698
D > 3	33 (73.3%)	16 (48.5)	17 (51.5)	
Overall survival				
Yes	20 (44.4%)	4 (20)	16 (80)	0.009
No	25 (55.6%)	21 (84)	4 (16)	

TNM, Tumor node metastasis; D, diameter, RCC, renal cell carcinoma.

Statistical analysis

The experiment was repeated three times. All data were showed as mean ± standard deviation (SD). The intergroup differences were com-pared by using a Student's t test or Chi-squared test. Kaplan-Meier method was used to determine univariate survival analysis, and log-rank test was performed to analyze the differences between the survival curves. All the statistical analysis was analyzed by the SPSS software version 19.0 (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Association between miR-29b expression and clinical pathological parameters and prognosis

To confirm the expression of miR-29bin ccRCC, we harvested the tumor tissues and matched adjacent normal tissues. The expression of miR-29b was determined by qRT-PCR. As shown in **Figure 1A**, the expression of miR-29b in ccRCC was significantly increased in ccRCC compared with the matched adjacent normal tissues ($P < 0.05$). Furthermore, we explored the association between the expression levels of miR-29b and clinical pathological parameters and prognosis (**Table 1**). Our analysis demonstrated that high expression of miR-29b was significantly associated with TNM stage ($P = 0.026$) and the overall survival ($P = 0.009$) in the ccRCC, whereas no statistically
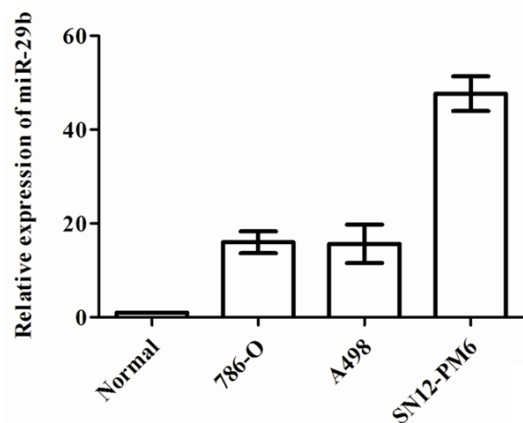
Figure 2. Relative expression of miR-29b in RCC cell lines. RCC, renal cell carcinoma.

significant correlation was found between miR-29b expression and gender ($P = 0.368$), ages ($P = 0.569$), recurrence ($P = 0.654$), lymph node metastasis ($P = 0.387$), and tumor size ($P = 0.698$). In addition, the survival curves were obtained from Kaplan-Meier method (**Figure 1B**). The results demonstrated that high miR-29b expression in ccRCC cells could be used as a potential marker for prognosis.

Upregulation of miR-29b in RCC cell lines

We performed qRT-PCR to confirm the expression of miR-29b in RCC cell lines (786-O, A498 and SN12-PM6). As shown in **Figure 2**, in contrast to the levels of miR-29b in the matched adjacent normal tissues, the relative levels of miR-29b were all significantly increased in the three cell lines, especially in the SN12-PM6 cells ($P < 0.05$).

Establishment of stably transduced in SN12-PM6 cells

To investigate the effect of miR-29b on tumor cells, SN12-PM6 cells were transduced with pEGFP-hsa-miR-29b to generate cell lines stably expressing miR-29b. The transduction efficiency was assessed by fluorescence microscope at 8 random fields for each condition. The transduction efficiency was more than 85% in SN12-PM6-ctr and SN12-PM6-AmiR-29b (**Figure 3A**). The results of qRT-PCR showed that compared to the SN12-PM6-ctr, SN12-PM6-AmiR-29b cells had lower miR-29b expression ($P < 0.05$), indicating that the expression

of miR-29b was successfully inhibited (**Figure 3B**).

Effect of miR-29b dysregulation on SN12-PM6 cells

To validate the contribution of miR-29b dysregulation to proliferation, invasion, and apoptosis, functional analysis was performed to confirm the effects of miR-29b. In the proliferation test, the results showed that inhibition of miR-29b expression could significantly reduce the cell viability compared with SN12-PM6-ctr group ($P < 0.05$) (**Figure 4A**). In the invasion test, the mean number of invaded cells expressing miR-29b per field was 40, which was significantly higher than that in the control group (15 per mm^2, $P < 0.05$) (**Figure 4B** and **4C**). In the apoptosis test, apoptosis rate was significantly higher by inhibition of miR-29b expression compared with the control group ($P < 0.05$) (**Figure 4D**). Furthermore, we examined the effect of miR-29b on cell cycle regulation by FCM. As shown in **Figure 4E**, knockdown of miR-29b promoted cell cycle arrest in the G0-G1 phase. These results indicated that inhibition of miR-29b expression could promote apoptosis, and inhibit proliferation and invasion ability in SN12-PM6 cells.

Regulation of miR-29b on KIF1B

To predict that KIF1B gene mRNA 3'-UTR might contain a hsa-miR-29b binding site, the TargetScan 6.2, miRanda, and Pictar was used to confirm the prediction. We constructed a WT psiCHECK3' UTR KIF1B plasmid (WT 3'UTR), which could bind with miR-29b and degrade fluorescence activity. Also, we constructed a mutant plasmid (MT3'UTR) as a control, which could not be combined with miR-29b, and the fluorescence activity was unchanged. As shown in **Figure 5A**, the fluorescence activity was significantly increased by dysregulation of miR-29b ($P < 0.05$), suggesting that KIF1B could be directly regulated by miR-29b. To further confirm how hsa-miR-29b regulates the expression of KIF1B, we determined the protein and mRNA levels of KIF1B in SN12-PM6 cells after dysregulation of miR-29b. We found that there were no significant differences in the mRNA levels of KIF1B, but the protein levels were markedly higher in the SN12-PM6-AmiR-29b group than that in the SN12-PM6-ctr group (**Figure 5B** and **5C**). These results indicated that miR-29b could regulate the expression of KIF1B at the post transcriptional level.
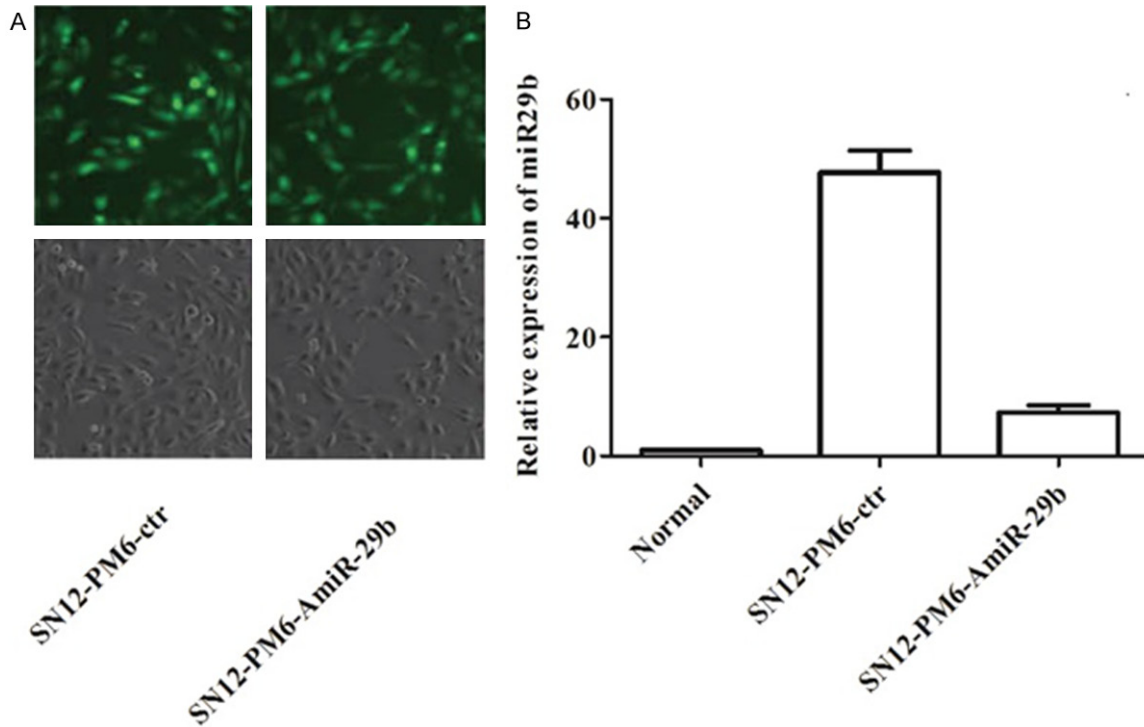
Figure 3. The transduction efficiency and the expression of miR-29b after transduction. A. The transduction efficiency; B. The relative expression of miR-29b after transduction.
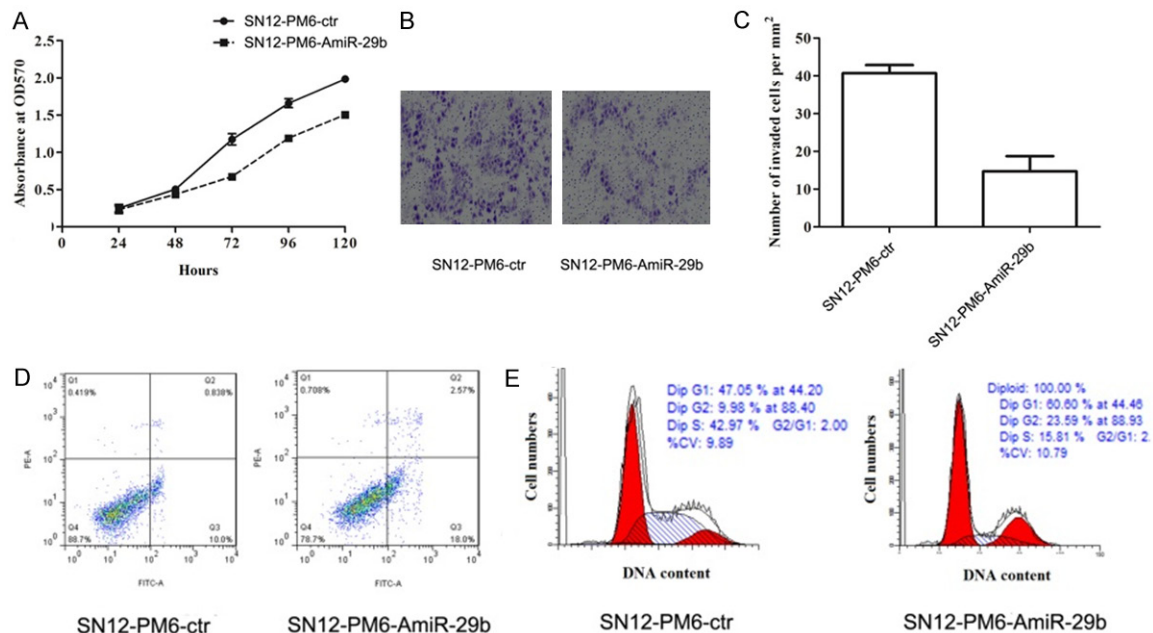

Figure 4. Effect of miR-29b dysregulation on SN12-PM6 cells. A. Effect of miR-29b dysregulation on SN12-PM6 cells proliferation; B. Effect of miR-29b dysregulation on SN12-PM6 cells invasion ability; C. Quantitative of invasion ability; D. Effect of miR-29b dysregulation on SN12-PM6 cells apoptosis; E. Effect of miR-29b dysregulation on cell cycle.

Discussion

Prognosis for ccRCC patients with metastatic recurrence is still extremely worse. Thus, there is a strong need to develop new strategies that can provide robust biomarkers to predict short- and long-term survival in ccRCC. In this present study, we show that miR-29b expression is
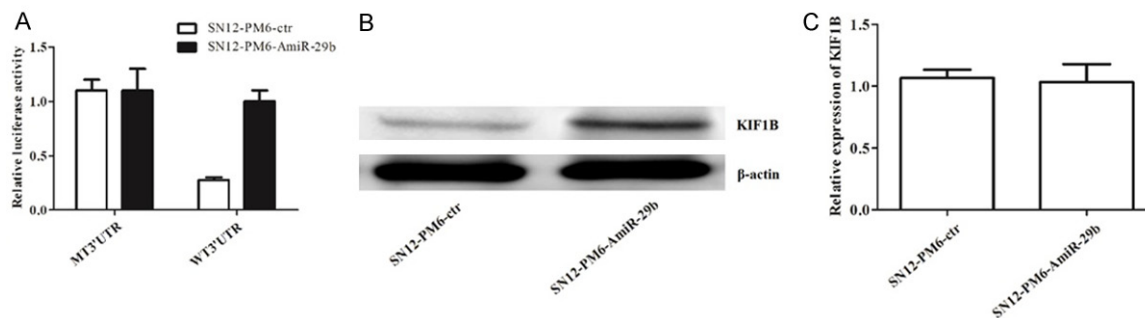
Figure 5. Regulation of miR-29b on KIF1B. A. Relative luciferase activity; B. Expression of KIF1B protein; C. Expression of KIF1B mRNA. KIF1B, kinesin family member 1B; WT, wild-type; MT, mutant-type; UTR, untranslated region.

increased in RCC cell lines and tissues compared to matched normal tissues. Moreover, we found that miR-29b promotes cell proliferation, invasion, and apoptosis resistance by repression of KIF1B expression. Additionally, miR-29b is strongly associated with survival rate and might be a potential molecular prognostic marker for RCC and disease progression.

MiRNAs are an abundant class of 20-22-nt non-coding single-strand RNA and play significant roles in various physiological and pathological processes, such as organogenesis, cell proliferation, apoptosis, and differentiation [20-22]. A great deal of miRNAs has been shown the association with tumorigenesis [23, 24]. They show their functional abilities by directly binding to target genes, leading to translational repression of messenger RNAs. To date, a number of studies have identified several miRNAs that exhibit aberrant expression patterns in RCC tumors compared to normal tissue [25-27]. In spite of a role of miRNAs in RCC has been proposed, the possible molecular mechanisms involved in modulation tumor metastases are still covered. In our study, we focused on the expression and functional role of miR-29b in RCC.

MiR-29b is one of members of miR-29b family. Previous studies have shown the expression and functional role of miR-29b in many tumors. However, there are some contradictions. Some researchers suggested that miR-29 family has a potential tumor suppressive function. For example, Wang *et al.* suggested that miR-29a/b/c significantly repressed lung cancer [28], and Fabbri *et al.* showed that miR-29 family had suppressed rhabdomyosarcoma cell lines to

develop tumor *in vivo* [29]. Xiong *et al.* found that miR-29 was downregulated in hepatocellular carcinoma (HCC) tissues, and miR-29 might be an independent prognosis predictor for HCC patients [30]. On the contrary, other studies have identified that miR-29b is an oncomiR. A previous study has showed that miR-29b is up-regulated in highly metastatic human breast cancer, and miR-29b contributes to cancer cells growth, migration, invasion, and anti-apoptosis. It may also acts as a molecular prognostic marker for breast cancer and disease progression [31]. Another study conducted by Yang *et al.* has suggested that miR-29b servers as an oncomir, and might be a potential therapeutic target for preventing OSCC progression [18]. To clarify the possible function and effect of miR-29b on RCC, we first collected ccRCC tumor tissues and matched normal tissues. Both qRT-PCR and Western blotting showed that miR-29b was increased in the tumor tissues compared to the matched normal tissues, indicating that miR-29b might have an inhibitory effect on cancer. Thereafter, we analyzed the association between expression levels of miR-29b and clinical pathological parameters. The results demonstrated that the expression of miR-29b was strongly related with TNM stages and survival rate. High expression of miR-29b suggests a worse prognosis. We further performed functional analysis in SN12-PM6 cells stably low-expressing miR-29b. In our proliferation and invasion test, blockade of expression of miR-29b in SN12-PM6 cells inhibited cell viability and invasion activity. In addition, dysregulation of miR-29b in SN12-PM6 cells increased apoptotic rate. The cell cycle test suggested that dysregulation of miR-29b promoted cell cycle arrest in the

G0-G1 phase. Therefore, we demonstrated that regulation of miR-29b expression could alter the proliferation and invasion abilities of SN12-PM6 cells, indicating that miR-29b plays significant roles in RCC cells.

We then identified miR-29b as a promoter of RCC through direct targeting of KIF1B, which is a well-known tumor suppressor gene. KIF1B is one of the important microtubule-dependent monomeric motors and functions as a regulator of transporting membranous organelles, such as mitochondria and synaptic vesicle [32]. Recent studies have showed that abnormal expression of KIF1B is involved in some malignant diseases such as neuroblastoma and gastrointestinal stromal tumors [33-35]. Moreover, KIF1B is involved in tumor development by regulating cell apoptosis [36]. In our study, we confirmed that KIF1B is one of miR-29b targeted genes by qRT-PCR, Western blotting and luciferase report assay system. Our results showed that the fluorescence activity was significantly increased by dysregulation of miR-29b, indicating that miR-29b could directly regulate KIF1B. The mRNA and protein levels of KIF1B indicated that miR-29b could regulate the expression of KIF1B at the post transcriptional level, not at the transcriptional level.

In conclusion, we provide evidence for the concept that miR-29b acts as an oncomiR and might be a potential marker for prognosis of RCC. In addition, miR-29b promotes SN12-PM6 cell proliferation and invasion ability, and inhibits the cell apoptosis by directly suppressing the expression of KIF1B. Our research might provide important theoretical support for prognosis evaluation and development of new treatment for RCC. However, further study should be performed to confirm the results.

Disclosure of conflict of interest

None.

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