MicroRNA-320a suppresses tumour cell proliferation and invasion of renal cancer cells by targeting FoxM1

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Abstract. An increasing body of evidence has indicated that microRNAs (miRNAs/miRs) may play an important role in tumourigenesis and tumour progression. Recent studies have demonstrated that miR-320a is aberrantly expressed in a variety of different types of human cancer. The results of the present study confirmed that the expression of miR-320a was decreased in clinical specimens and cell lines. Expression of miR-320a inhibited the growth and invasive ability of ACHN and Caki-1 cells. Bioinformatics analysis and a luciferase reporter assay demonstrated that forkhead box protein M1 (FoxM1) was directly regulated by miR-320a. Rescue experiments in vitro revealed that the upregulation of FoxM1 antagonized the miR-320a-mediated malignant phenotype in renal cancer. Furthermore, experiments employing a xenograft mouse model revealed that the upregulation of miR-320a inhibited the proliferation of renal cancer cells in nude mice when FoxM1 protein expression was reduced. Collectively, the present study demonstrated a novel molecular interaction regulated by miR-320a, which may provide a novel insight into the treatments for renal cancer.

Introduction

Renal cell carcinoma (RCC) is the third leading cause of mortality amongst adult genitourinary cancers due to space-occupying lesions, and accounts for ~3% of all malignancies. Worldwide, ~90,000 patients succumb to RCC each year (1). In addition, the mortality rate of RCC is ≤40%. RCC is derived from renal proximal tubule cells, and is comprised of the clear cell, papillary and chromophobe subtypes (2-4). Despite therapeutic advances and new biological insights, current treatments are not expected to have curative effects (5,6). Although surgical tumour resection is the optimal treatment strategy at present, the 5-year survival rate for RCC patients remains poor (5-10%) (7). Therefore, the investigation of RCC progression at the molecular and genetic levels is urgently required as it may provide more effective therapeutic approaches for RCC.

MicroRNAs (miRNAs/miRs) are endogenous, small non-coding molecules (19-22 bp in length) that modulate the expression of target genes post-transcriptionally and promote target mRNA deadenylation and degradation in a sequence-specific manner, including via proliferation, angiogenesis, invasion and apoptosis (8). It has been recognized that miRNAs may regulate numerous biological processes (9). An increasing body of evidence has indicated that miRNA may exert a critical role in early cancer detection and treatment (7,8). Recent studies have shown that miRNAs, including miR-10b/21, miR-1, miR-29, miR-335 and miR-133a, may act as oncogenes or tumour-suppressor genes, which are associated with patient survival (9-13). In addition, it has been revealed that miR-320a serves a tumour-regulatory role in human liver cancer (14,15). However, the underlying mechanism of miR-320a in RCC remains unclear.

Forkhead box protein M1 (FoxM1; previously known as HFH-11, INS-1, WIN, MPP2/MPHOSPH2, or Trident/FKHL16) serves as a regulator in animal development (16). Overexpression of FoxM1 has been reported in many types of cancer, including RCC (17), promoting angiogenesis, invasion and metastasis. Recently, a range of studies have revealed that FoxM1 is a key regulator of chemotherapy sensitivity and resistance (18,19). Matrix metalloproteinase (MMP)-2/9 are members of the MMP family, and play a vital role in the process of extracellular matrix degradation and promote cancer cell migration from the primary tumour to form metastases (20).

In the present study, the downregulation of miR-320a was explored using The Cancer Genome Atlas (TCGA) database and was validated by experiments with RCC tissues. Subsequently, the expression of miR-320a was detected in 4 RCC cell lines. miR-320a overexpression resulted in reduced cell proliferation, migration and invasion. Notably, FoxM1 was confirmed as a direct downstream target of miR-320a in RCC.
Materials and methods

Cells and tissues. Human RCC cell lines (A-498, ACHN, 786-O and Caki-1) were obtained from the Chinese Academy of Sciences (Shanghai, China). Human kidney cells (HK-2) were purchased from the American Type Cell Culture Collection (ATCC; Rockville, MD, USA). These cells were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS); both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at 37°C in an atmosphere containing 5% CO₂ in an incubator in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS.

Human renal cancer tissues and adjacent normal tissues were collected from 40 patients (patient age range, 20-70 years; mean age, 55.45 years; male patients account for 57.5% (23/40) and female 42.5% (17/40) with histologically confirmed renal cancer who underwent radical nephrectomy at The Second Hospital of Jilin University between May 2011 and December 2016. All RCC cases were confirmed by a senior pathologist, and staging was based on the 2011 Union for International Cancer Control TNM classification of malignant tumors. No patients had received any anticancer treatment. All tissues were pathologically confirmed and immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Written informed consent for research purposes was obtained from each patient. All procedures were subjected to the Declaration of Helsinki. The study was approved by the Medical Ethics Review Committee of Jilin University (Changchun, China).

Gene set enrichment analysis with miR-320a expression. The data of 529 RCC and 71 matched normal samples were deposited in the TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/). The expression of miRNAs was quantified by the customized data analysis pipeline that included the steps of quality control, alignment and expression quantification. Gene Ontology (GO) term analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (21) with the following GO terms: Biological processes (GO_BP_FAT), cellular components (GO_CC_FAT) and molecular functions (GO_MF_FAT).

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR). Total RNA was extracted from preserved fresh tissues and cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed in triplicate on an ABI 7500 HT fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) to the manufacturer's protocol. To assess miR-320a, proliferation of RCC cells was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The absorbance was detected at a wavelength of 490 nm and the optical density was calculated. For colony formation assays, transfected cells were seeded onto 6-well plates (200 cells/well) and cultured for a further 14 days; then, cells were combined with formalin and stained with Giemsa (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the colonies (>50 cells) were counted using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Hecules, CA, USA).

Cell migration and invasion assays. ACHN and Caki-1 cells were seeded in 6-well plates. The cell monolayer was wounded using a plastic pipette tip (200 µl) washed with phosphate-buffered saline (PBS), and then cultured with serum-free RPMI-1640 medium for 48 h. The extent of the
wound closure was captured using a light microscope (at x200 magnification) (Olympus Corp., Tokyo, Japan).

The Transwell filters (Corning Inc., Corning, MA, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to quantify in vitro glioma cell invasion. Transfected cells were seeded into the upper chamber (Costar; Corning) in serum-free medium. To the lower chambers, RPMI-1640 medium with 20% FBS was applied for 24 h, and then the upper chamber medium was removed and the non-invading cells were cleaned away. The bottom chamber invading cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted under a light microscope (at x100 magnification).

Western blot analysis. RCC tissues and cells were lysed using Thermo Fisher Scientific RIPA buffer (Pierce; Thermo Fisher Scientific, Inc.). The Micro BCA protein assay kit was used to detect the protein concentration. Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk (w/v) at room temperature for 1 h and subjected to incubation with the corresponding primary antibodies at 4°C overnight: Rabbit anti-human FoxM1 (1:1,000; cat. no. 5436), mouse anti-human MMP-2 (1:1,000; cat. no. 4022) and MMP-9 (1:1,000; cat. no. 3852) and rabbit anti-human PCNA (1:1,000; cat. no. 13110; all from Cell Signaling Technology, Danvers, MA, USA) and mouse anti-human β-actin (1:500; cat. no. sc-8432; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, the PVDF membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. ab150077; Abcam, Cambridge, MA, USA) for 1 h. An enhanced chemiluminescence western blotting detection system (EMD Millipore) was used to detect the protein expression level. Immunodetection was visualized on a Gel Doc 2000 Imaging System (Bio-Rad Laboratories).

Tumour xenograft model. ACHN cells with high expression of miR-320a were transfected with miR-320a mimics. Female nude mice purchased from Vital River Laboratory Animal Inc., (Beijing, China) were kept under standard conditions (4-5 weeks old; 15-20 g; n=4 BALB/c nude mice per group) were inoculated subcutaneously with 2x10⁶ cells expressing NC in the right flank and miR-320a in the left flank. These mice were kept in OptiMice IVC cages (Animal Care Systems) in special clean rooms with HEPA14-filtered incoming air, under regular 14/10-h light/dark cycle (lights on at 02:00 a.m.), constant room temperature of 22±2°C, and relative humidity of 45±15%. All materials including cages, food, bedding and environmental enrichment items were obligatorily autoclaved. Millipore filtration was used to produce water for animals. Tumour volume (V) was calculated in mice on a weekly basis using the following formula: V = 0.5 x length x width². The mice were anesthetized using isoflurane and sacrificed using the carbon dioxide method of euthanasia on day 35 and the tumours were excised. The wet weight of tumours was determined and tumor tissues were stored at ‑80°C for further analysis. All procedures were subjected to the Declaration of Helsinki. All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The mice experiments were approved by the Medical Ethics Review Committee of Jilin University (Changchun, China).

Table I. Correlation between the expression of miR-320a and the clinicopathological features in RCC from a TCGA database.

| Variables    | No. of cases | Low | High | χ²  | P-value |
|--------------|--------------|-----|------|-----|---------|
| Age (years)  |              |     |      |     |         |
| <60          | 245          | 87  | 154  | 0.325 | 0.569   |
| ≥60          | 284          | 109 | 175  |     |         |
| Sex          |              |     |      |     |         |
| Male         | 339          | 120 | 219  | 1.105 | 0.293   |
| Female       | 190          | 76  | 114  |     |         |
| TNM stage    |              |     |      |     |         |
| I            | 202          | 26  | 176  | 8.064 | 0.045   |
| II           | 62           | 10  | 52   |     |         |
| III          | 165          | 23  | 142  |     |         |
| IV           | 100          | 25  | 75   |     |         |
| Grade        |              |     |      |     |         |
| 1            | 26           | 8   | 18   | 13.187 | 0.004   |
| 2            | 173          | 20  | 153  |     |         |
| 3            | 190          | 30  | 160  |     |         |
| 4            | 140          | 35  | 105  |     |         |

*p<0.05 was considered significant (χ² test between two groups).
The researchers optimized the experimental scheme and treated the animals kindly by improving the experimental method and adjusting the observation index of the experiment to ensure the implementation of the animal welfare measures.

Statistical analysis. The data are shown as the mean ± SD of three independent experiments. All statistical data were analyzed using the SPSS GradPack, version 19.0, statistical software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Spearman’s rank correlation analysis was performed to analyze the correlation between miR-320a and FoxM1. Comparisons between groups were analyzed using two-tailed Student’s t-test or one-way ANOVA with post hoc Tukey’s test. All differences were considered to be statistically significant at the level of P<0.05.

Results
miR-320a is downregulated in RCC tissues and cell lines. To explore the expression of miR-320a in RCC, TCGA data was employed to analyse the expression of miRNA in RCC. The results demonstrated that miR-320a was decreased in RCCs (n=529) compared with adjacent normal tissues (n=71; Fig. 1A). In addition, the expression of miR-320a was substantially reduced when comparing the 40 RCC tissues with the pair-matched normal tissues collected from patients admitted to The Second Hospital of Jilin University (Fig. 1B; P<0.001). A decreased level of miR-320a was identified in the RCC cell lines compared with HK-2 cells (Fig. 1C). In addition, low expression of miR-320a was significantly associated with high tumour-node-metastasis (TNM) stage and tumour grade in RCC from (Table I; P<0.05; TCGA database).
Analysis using miR-320a correlation genes was conducted and miR-320a was revealed to be associated with cell adhesion and migration (Fig. 1D). Analysis of the TCGA database indicated that lower miR-320a expression in RCC was correlated with shorter overall survival. The proportion of surviving patients was 142/196 in the high miR-320a group and 211/333 in the low miR-320a group (Fig. 1E; P=0.006). Collectively, these results indicated that miR-320a may serve a role in a series of RCC biological processes.

Effect of miR-320a on RCC cell viability, migration and invasion. To assess the role of miR-320a in the tumorigenesis of RCC, ACHN and Caki-1 cells were transfected with miR-320a mimics and NC, and then cell viability and invasion were determined. miR-320a overexpression was detected in ACHN and Caki-1 cells, as displayed in Fig. 2A. The data revealed that miR-320a reduced RCC cell proliferation (Fig. 2B and C).

Since metastasis of cancer cells has been identified as a pivotal factor in cancer progression, the present study...
investigated the effect of restored miR-320a expression in RCC cells and revealed that it significantly reduced RCC invasion and migration capacity (Fig. 2D and E). Subsequently, specific markers of cell proliferation and invasion (including PCNA, MMP-2 and MMP-9) were assessed at the protein and mRNA levels following miR-320a deregulation (Fig. 2F and G). The results revealed that miR-320a inhibited RCC cell viability and invasion.

FoxM1 is a direct target of miR-320a in RCC cells. Using algorithm prediction, it was observed that miR-320a targets FoxM1 through a conserved binding site in the 3’-UTR of FoxM1 (Fig. 3A). The present study then detected whether the mRNA and protein expression levels of FoxM1 in RCC cells are regulated by miR-320a. The results demonstrated that the overexpression of miR-320a decreased both the mRNA and protein expression of FoxM1 in RCC cells (Fig. 2F and G). These results revealed that FoxM1 may be a direct target of miR-320a in RCC cells.

Anti-RCC effect of miR-320a is mediated by inhibition of the expression of FoxM1. To determine whether FoxM1 was involved in the antitumor effects of miR-320a, the expression of FoxM1 was silenced by siRNA in the ACHN cells (Fig. 4A and B). The inhibition of FoxM1 function in RCC cells transfected with FoxM1-siRNA was confirmed by CCK-8, wound healing and Transwell assays (Fig. 4C-E).

Further experiments were employed to validate FoxM1 as a direct target of miR-320a in RCC. ACHN cells were transfected with NC, FoxM1, miR-320a or miR-320a plus FoxM1 and then the expression of FoxM1 was determined (Fig. 5A). Rescue experiments demonstrated that the restoration of FoxM1 expression was reduced via the overexpression of miR-320a (Fig. 5B). In addition, the overexpression of FoxM1 was observed to reverse the miR-320a-mediated antitumour effect (Fig. 5C and D). These findings confirmed that miR-320a exerted its biological effect in RCC by suppressing FoxM1.

miR-320a inhibits tumour growth in a nude mouse model. To further validate the biological function of miR-320a in vivo, the present study assessed the in vivo therapeutic efficacy of
Figure 4. Downregulation of FoxM1 inhibits cellular growth and invasion. (A and B) Silencing of FoxM1 expression was determined by RT-qPCR and western blot analysis. (C) Ectopic knockdown of FoxM1 inhibited the viability of RCC cells. (D and E) Wound healing and Transwell assays in FoxM1-siRNA or FoxM1-NC transfected RCC cells. *P<0.05, **P<0.01. RCC, renal cell carcinoma; FoxM1, forkhead box protein M1; NC, negative control; siRNA, small interfering RNA; RT-qPCR, quantitative reverse transcription-polymerase chain reaction.

Figure 5. Restoration of FoxM1 expression reverses the miR-320a-mediated suppression of the pro-tumour effect of FoxM1. (A) The expression of FoxM1 in cells transfected with NC, miR-320a mimics, FoxM1 and miR-320a+FoxM1. (B-D) FoxM1 overexpression attenuated the suppressive function of miR-320a-mediated cell growth rate, invasion and migration. *P<0.05, **P<0.01. FoxM1, forkhead box protein M1; miR/miRNA, microRNA; NC, negative control.
miR-320a in BALB/c nude mice. ACHN cells transfected with miR-320a mimics or NC were subcutaneously injected into the flank regions of nude mice. The growth of the miR-320a-treated xenograft was significantly slower compared with that of the NC xenograft (at the 35-day time-point, Fig. 6A). The size and weight of the miR-320a group were smaller than those of the NC xenograft (Fig. 6B and C). The present study also determined the FoxM1 levels in tumour tissues and revealed that the expression of FoxM1 was decreased in the miR-320a xenograft (Fig. 6D and E). Based on the aforementioned data, the results demonstrated that miR-320a may impede tumorigenicity in vivo.

Discussion

RCC is one of the major causes of cancer-related mortalities worldwide and despite current treatments, the 5-year survival rate of patients is still poor. Previous studies have identified some prognostic markers to assess and predict patient survival outcomes. miR-320a has demonstrated anti-tumour effects in various types of human cancers (23-27). It was previously ascertained that miR-320a could suppress the cell malignant phenotype by interacting with Ras-related protein Rab-(RAB)11A, RAB14 and metadherin in breast cancer (28-30), and the miR-320a/STAT3 signalling pathway in lung adenocarcinoma (15). These results indicated that miR-320a may be a new tumour prediction molecule.

The mechanism underlying the miR-320a-associated regulation of FoxM1 expression and function in RCC progression was verified in the present study. Firstly, the expression of miR-320a in RCC tissues and cells was determined. miR-320a levels were decreased in RCC tissues and were negatively correlated with tumour TNM stage, tumour grade and poor survival. miR-320a suppressed cell growth and invasion in RCC cells. These results demonstrated that altering the expression of FoxM1 in RCC, which is mediated partly by miR-320a, could modulate RCC progression by suppressing the proliferation and invasion of RCC cells.

FoxM1 is an important biomarker of development and cell cycle, which exerts a pro-survival role in many types of human cancer cells (16,31). The FoxM1 gene belongs to the forkhead box superfamily of transcription factors, which are known to be regulators of cell proliferation, differentiation, apoptosis and invasion (32). High expression of FoxM1 has been revealed to be positively correlated with the RCC grade, advanced stage, and poor survival of RCC patients (33,34). A recent study revealed that FoxM1 regulated glucose metabolism in epithelial ovarian cells (35). In renal cancer cells, FoxM1 could modulate the cell cycle by targeting polo-like kinase 1 (36) and promoted colorectal cancer cell invasion and migration by regulating heat shock protein family A member 5 transactivation (37).

MMP-2 exerts a vital role in mesenchymal phenotypes in RCC (38). It has been reported that RCC exhibited a mesenchymal subtype and the levels of MMP-2 may be directly correlated with mesenchymal transition (39). FoxM1 induced the epithelial-mesenchymal transition (EMT) process by regulating the extracellular signal-regulated kinase signalling pathway in non-small cell lung carcinoma (40). Overexpression of FoxM1 promoted the metastasis of hepatocellular carcinoma by targeting snail family transcriptional repressor 1 and was involved in the EMT process (41). Overexpression of FoxM1 was also revealed to modify the cancer stem cell phenotype, which is in part regulated by miR-200b (42). These results indicated that miR-320a may play a vital role in tumour cell migration and EMT.

In conclusion, the present study detected and ascertained that FoxM1 was downregulated in RCC and was a direct downstream target of miR-320a in human RCC cells. The results revealed that miR-320a was downregulated in RCC, and high miR-320a expression decreased cell proliferation and invasion by targeting FoxM1. These findings indicated that
miR-320a could be a novel therapeutic strategy for the early diagnosis and therapy of RCC.

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Availability of data and materials
The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions
SZ, YaW, YL, YoW, JS, ML, WL and LM took part equally in the conception and design of the study, acquisition and interpretation of data, drafting the article and final approval of the version to be published.

Ethics approval and consent to participate
Written informed consent for research purposes was obtained from each patient in the present study. All procedures were subjected to the Declaration of Helsinki. And all applicable international, national and/or institutional guidelines for the care and use of animals were followed. The study was approved by the Institutional Review Board of the Second Affiliated Hospital of Jilin University (Changchun, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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