Original Article

The putative tumour suppressor microRNA-145, repressed by c-jun, modulates clear cell renal cell carcinoma aggressiveness by repressing ROCK1

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Abstract: Aberrant expression of miR-145 has frequently been reported in cancer studies; however, its role in renal cell carcinoma (RCC) has not been examined in detail. Here, we showed that miR-145 was downregulated in RCC cells and tissues and associated with a poor prognostic phenotype in RCC patients. Restoration of miR-145 suppressed proliferation and invasion and induced apoptosis in clear cell RCC (ccRCC) cells. By luciferase and western blot assays we identified ROCK1 as a direct target gene for miR-145. Furthermore, a reverse correlation between miR-145 and ROCK1 expression was observed in ccRCC tissues. In addition, we revealed that miR-145 downregulation in ccRCC cells was due to EZH2-mediated histone methylation. Altogether, these results identify a crucial tumor suppressive role of miR-145 in the progression of ccRCC and suggest that miR-145 might be an anticancer therapeutic target for ccRCC patients.

Keywords: Clear cell renal cell carcinoma, miR-145, ROCK1, c-jun, A498

Introduction

Renal cell carcinoma (RCC), accounting for 90% to 95% of all renal tumors in adults [1], comprises a heterogeneous group of epithelial neoplasms with diverse biologic potential and variable clinical outcomes [2]. From the three main subtypes of RCC, clear cell, chromophobe and papillary RCC, clear cell renal cell carcinoma (ccRCC) is the most common type and associated with a worse prognosis [3]. Radical or partial nephrectomy remains the most important treatment for localized RCC, but alone it has limited benefit in patients with an aggressive form of the disease [4]. In addition, in most cases RCC is resistant to chemotherapy and immunotherapy [5]. Recently, there has been a rapid development in molecular targeted therapy for the treatment of advanced RCC. However, most of the treatments are not curative and side effects associated with targeted therapy cannot be ignored [6-8]. Thus, new correlative markers and therapeutic agents for RCC diagnostics and treatment are urgently needed.

MicroRNAs (miRNAs), single-stranded 19-25 nucleotide long non-coding RNAs, function as negative posttranscriptional regulators of their target genes [9-11]. Recent studies suggest that miRNAs are aberrantly expressed during the development and/or progression of a variety of human cancers [12, 13]. A recent miRNA expression signature of ccRCC revealed that miR-145 is downregulated in cancer tissues, suggesting that it might be a candidate tumor suppressor in ccRCC [14]. The tumor suppressive function of miR-145 has been reported in several types of cancer, such as gastric cancer [15], neuroblastoma [16], and ovarian cancer [17]. However, the biological functions of miR-145 in the control of ccRCC have not been well characterized. Here, we sought to investigate the potential role of miR-145 in the development and progression of ccRCC.

Materials and methods

Cell lines and patient samples

A498 cell line was purchased from the Institute of Biochemistry and Cell Biology of the Chinese
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Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified 37°C incubator with 5% CO₂. ccRCC and adjacent normal tissue samples from patients were obtained with informed consent under institutional review board-approved protocols. The samples were collected at the Affiliated Hospital of Jining Medical University, Jining city, Shandong province, China. The study was approved by the Institutional Review Boards of Affiliated Hospital of Jining Medical University and written informed consent was obtained from each patient.

Quantitative realtime PCR (qRT-PCR)

Total RNA was extracted with TRizol reagent (Invitrogen, Carlsbad, California, USA). For reverse transcription and qRT-PCR analysis, One Step PrimeScript® miRNA cDNA Synthesis Kit and SYBR Premix Ex Taq (TaKaRa, Dalian, China) were used. The primers for miR-145 were 5’-TCAAGAGCAATAACGAAAAATGT-3’ (forward) and 5’-GCTGTCAACGATACGCTACGT-3’ (reverse). U6 snRNA was used as normalization control. The primers for ROCK1 were 5’-AGAGGGACATATTAGTCCCT-3’ (forward) and 5’-AGACGATAGTTGGGTCCCGGC-3’ (reverse). Primers for GAPDH were 5’-AACGTGTCAGTGGTGGACCTG-3’ (forward) and 5’-AGTGGGTGTCGCTGTTGAAGT-3’ (reverse). The relative expression of each gene was calculated and normalized using the 2^ΔΔCt method relative to U6 snRNA or GAPDH. PCR amplification was repeated three times for each gene.

Lentivirus production and infection

The pLV-hsa-miR-145 plasmid together with the control pLV-miRNA-vector, were obtained from Biosettia Inc. (Biosettia, San Diego, USA). Lentivirus packaging and transfection was performed according to standard protocols. We named the packaged lentiviruses LV-miR-145 and LV-ctrl.

Plasmid construction and luciferase reporter assays

For the luciferase reporter assay, the 3’-UTR segment of ROCK1 containing the miR-145-binding sites was amplified and inserted into the pGL3-control vector (Promega, Madison, WI, USA). Site-directed mutagenesis of the miR-145 seed sequence in the ROCK1 3’-UTR (Mut) was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Subsequently, LV-miR-145 cells were transfected with WT or Mut reporter plasmids, followed by measuring the luciferase activity with the Dual-Luciferase Reporter Assay System (Promega) 48 hours later. Full-length ROCK1 cDNA entirely lacking the 3’-UTR was purchased from GeneCopoeia (Rockville, MD, USA) and subcloned into the eukaryotic expression vector pcDNA.3 (Invitrogen).

MTT, colony formation and cell invasion assays

MTT assay was used to analyze cell proliferation. Briefly, 4×10^3 cells were seeded into 96-well culture plates. Subsequently, the cells were incubated with MTT. Then the culture supernatant was removed and DMSO was added to solubilize the crystals. The absorbance value (OD) of each well was measured at 490 nm. Experiments were performed three times.

For colony formation, twenty-four hours after infection, 300 infected cells were placed in a fresh six-well plate and maintained in DMEM medium containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 min. Experiments were performed three times.

To perform the invasion assay, 10^5 cells were added to a Matrigel invasion chamber (BD Biosciences), which was present in the insert of a 24-well culture plate. Cells were plated in medium without serum, and in the lower chamber medium containing 10% FBS served as chemoattractant. After the cells were incubated for 8 h at 37°C in a 5% CO₂ atmosphere, the insert was washed with PBS, and cells on the top surface of the insert were removed with a cotton swab. Invasive cells located on the lower side of the chamber were stained with Crystal Violet, air-dried and photographed. Experiments were performed three times.

Apoptosis assay

Apoptosis was measured by using an Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit (Keygen, China) according to the manufacturer’s instructions. Data acquisition
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and analyses were done on a Becton Dickinson FACSCalibur (Franklin Lakes, NJ, USA) using Cell Quest software (Becton Dickinson, USA). Each experiment was performed in triplicate.

Western blot analysis

Cells were lysed in RIPA Buffer in the presence of a proteinase inhibitor cocktail. Then the total protein extracts were separated and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk and incubated with primary antibodies against ROCK1 and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. Then membranes were incubated for 1 h at room temperature with an appropriate secondary antibody, followed by detection with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, USA).

Chromatin immunoprecipitation (CHIP) assay

We performed the CHIP assay according to the manufacturer’s instructions by a ChIP assay kit (Millipore). Briefly, the cells were fixed with 1% formaldehyde to covalently crosslink proteins to DNA followed by harvesting chromatin from the cells. Subsequently, the cross linked DNA (sheared to 200-1,000 base pairs in length) that linked with sonication were processed to an immunoselection process. Then the PCR assay was performed to measure enrichment of DNA fragments in the putative c-JUN-binding sites in the miR-145 promoter.

In vivo tumorigenesis

All animal procedures were performed in accordance with institutional guidelines. 4×10⁶ A498 cells were injected into the flanks of Nude mice. Mice were sacrificed for examination 21 days after tumor inoculation. The tumor volume was calculated by using the formula Volume (mm³) = L×W²/2 (length L, mm; width W, mm). All treatment of animals was in strict accordance with the guidelines of the Animal Center of Jining.

Figure 1. Lower levels of miR-145 are frequently detected in ccRCC tissues and are associated with a poor prognostic phenotype. A. The expression of miR-145 was significantly downregulated in ccRCCs when compared with adjacent non-neoplastic renal tissues. B. ccRCC patients with miR-145 expression levels below the median had shorter survival times than those whose levels were higher than the median.

Figure 2. ROCK1 is a direct target of miR-145 in A498 cells. A. Sequence alignment of miR-145 and the ROCK1 3′-untranslated region (UTR), which contains one predicted miR-145-binding site. The seed-recognizing sites in the ROCK1 3′-UTR are indicated in red, while the mutant ROCK1 3′-UTRs are indicated in blue. B. Luciferase assay in A498 cells co-transfected with miR-145 and luciferase reporter containing ROCK1 wild type (WT) or mutant (Mut) 3′-UTR. C. miR-145 transfection affects ROCK1 mRNA levels. D. miR-145 transfection affects ROCK1 protein levels.
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Medical College., and all animal experimental procedures were approved by the Experimental Animal Ethical Committee of Jining Medical College.

Statistical analysis

All values are presented as the mean ± S.E.M. One-way ANOVA or two-tailed Student’s t-test was used for comparisons between groups. Kaplan-Meier method was used to perform the survival analysis. SPSS software package (SPSS Standard version 16.0, SPSS Inc) was used for statistical analysis and P<0.05 was considered statistically significant.

Results

Lower levels of miR-145 in ccRCC tissue are associated with a poor prognosis

The expression levels of miR-145 were first examined by real-time PCR in 26 pairs of ccRCC and adjacent non-neoplastic renal tissues. The results showed that miR-145 was significantly down-regulated in ccRCC tissues when compared with adjacent non-neoplastic renal tissues (Figure 1A, P<0.05). Furthermore, the relationship between miR-145 expression and overall survival was determined through Kaplan-Meier survival curve analysis with a log-rank comparison of 98 ccRCC patients. We observed that ccRCC patients with miR-145 expression levels lower than the median were associated with poorer survival (Figure 1B).

MiR-145 targets ROCK1 in ccRCC cells and tissue

It is generally accepted that miRNAs exert their function by regulating the expression of their downstream target gene(s). MiRanda and TargetScan algorithms were used to search for target genes of miR-145. Analysis revealed that the 3'-UTR of ROCK1 mRNA contains a comple-
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Figure 2A. Subsequently, we subcloned ROCK1 wild-type (WT) or mutant 3'-UTR (MUT) (Figure 2A) into a luciferase reporter vector. Then A498 cells were co-transfected with miR-145 or scrambled control and ROCK1 wild-type (WT) or mutant 3'-UTR (MUT). It was found that miR-145 significantly inhibited the luciferase activity of the ROCK1 WUT 3'-UTR but not of the mutant in A498 cells (Figure 2B, P<0.05). Furthermore, overexpression of miR-145 significantly downregulated ROCK1 mRNA and protein levels in A498 cells (Figure 2C and 2D).

Exogenous overexpression of miR-145 suppresses proliferation and invasion and induces apoptosis in ccRCC cells

We asked whether restoration of miR-145 may suppress proliferation and invasion in ccRCC cells. As shown in Figure 3A and 3B, miR-145 overexpression significantly inhibited ccRCC cells proliferation and colony formation ability. However, over-expression of ROCK1-overexpressing vector (pCDNA.3-ROCK1) can counteract this effect.

The matrigel invasion assay demonstrated that exogenous overexpression of miR-145 markedly reduced invasiveness of ccRCC cells, while ROCK1 restoration could partially block these effects (Figure 3C).

We further examined whether restoration of miR-145 may affect apoptosis in ccRCC cells.

Figure 3D. miR-145 overexpression induced apoptosis in A498 cells, while exogenetic overexpression of ROCK1 blocked the effect of miR-145 on apoptosis.

MiR-145 levels are inversely correlated with mRNA expression of ROCK1 in ccRCC tissues

We further examined miR-145 and the mRNA expression of ROCK1 in 39 cases of ccRCC tissues. We found a significant inverse correlation between the levels of miR-145 and ROCK1 mRNA expression in ccRCC tissues (Figure 4, P<0.05).

C-jun repressed miR-145 expression in ccRCC cells

We used UCSC and PROMO bioinformatics software to analyze a 0.8-kb region upstream of the transcription start site of miR-145. We identified four c-jun-binding motifs inside the putative promoter region upstream of the miR-145 transcriptional start site (TSS). These transcription factor-binding sites (TFBSs) were named A, B, C and D, respectively (Figure 5A). Si-RNAs were used to knock down c-jun expression and we found that miR-145 expression was significantly increased in these cells when c-jun was down-regulated (Figure 5B). c-jun down-regulation also increased miR-145 promoter luciferase activity (Figure 5C). Finally, the Chromatin immunoprecipitation (ChIP) assay confirmed that c-jun protein was recruited to all the four binding sites in the putative miR-145 promoter (Figure 5D).

MiR-145 inhibits tumor growth in vivo

To examine the role of miR-145 in ccRCC tumor development, we used a SCID xenograft mouse model in which mice were transplanted with miR-145 overexpressing or scramble control A498 cells. After 3 weeks, miR-145 overexpressing tumors were significantly smaller than those of mice transplanted with the scrambled control (Figure 6A). Furthermore, overexpression of miR-145 significantly reduced xenograft tumor volume (Figure 5B) and tumor weight (Figure 5C). Taken together, these results showed that miR-145 can inhibit tumor growth in vivo.
Discussion

In this study, we focused on miR-145 and investigated its tumor suppressive function and how miR-145-mediated cancer pathways contribute to ccRCC progression. We observed that miR-145 expression is decreased in human ccRCC tissues when compared with matching adjacent normal tissue. Furthermore, miR-145 suppressed tumor growth both in vitro and in vivo.

MiRNAs not only contribute to diverse biological processes but are also implicated in the progression and metastasis of human cancers [13, 18]. Insights into the roles of miRNAs in cancer have made miRNAs targets for novel therapeutic approaches and several miRNA-targeted therapeutics have reached clinical development [19]. Xu et al. reported that miR-145 suppresses gastric cancer cell invasion and metastasis through directly targeting the specificity protein 1 (SP1) and Bcl-w (BCL2L2) mRNA transcripts [20]. Recently, researchers found that miR-145 acts as a tumor suppressor in prostate cancer [21]. Consistent with the above findings, we found that restoration of miR-145 could suppress proliferation and invasion and induce apoptosis in ccRCC cells. By dual-luciferase and Western Blot assay ROCK1 was confirmed as a direct target of miR-145.

ROCK1 is a serine/threonine kinase that belongs to the Rho family of GTPase proteins that facilitate the reorganization of the actin cytoskeleton, a pivotal event during cell motion and invasion [22]. ROCK1 is over-expressed in various cancers, including ccRCC [23, 24]. ROCK1 mediates multiple pathways regulating cell proliferation,
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invasion, and apoptosis [25, 26]. Our findings showed that miR-145 exert its tumor suppressor function partially through ROCK1 in ccRCC cells. Importantly, we further found that miR-145 levels are inversely correlated with mRNA expression of ROCK1 in ccRCC tissues.

The transcription factor c-jun is a key regulator of cell growth [27] and metastasis [28] in cancer. We revealed four putative binding sites of c-jun in the region upstream of miR-145 locus. The subsequent experiment demonstrated that c-jun could negative regulate miR-145 expression by directly binding at its promoter. Taken together, our findings revealed that c-jun was responsible for miR-145 un-regulation in ccRCC.

Taken together, this study identified miR-145 as growth-suppressive miRNA in human ccRCC both in vitro and in vivo. As miR-145 is down-regulated in ccRCC, reintroduction of this mature miRNA into the tumor tissue could provide a therapeutic strategy. Although miRNA-based therapeutics are still in their infancy, our findings on miR-145 are encouraging and suggest that this miRNA could be a potential target for the treatment of ccRCC in the future.

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Disclosure of conflict of interest

None.

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