miR-137 inhibits renal cell carcinoma growth in vitro and in vivo

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Abstract. MicroRNA (miR)-137 has been reported to be underexpressed and involved in various cell processes and to have antitumor effects in a range of tumors, but so far not in renal cell carcinoma (RCC). The aim of the present study was to investigate the clinical significance and role of miR-137 in RCC. The expression levels of miR-137 were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in 50 cases of paired RCC tissues and adjacent normal tissues, and in RCC cell lines. The role of miR-137 in the growth and survival of RCC cells was assessed with several in vitro approaches and in nude mice models. The results of the RT-qPCR showed that the miR-137 expression was down-regulated in the RCC tissues and cell lines. The in vitro assay showed that ectopic expression of miR-137 robustly impaired RCC cell proliferation, migratory and invasive properties, and increased the induction of cell apoptosis properties. The in vivo assay demonstrated that enforced miR-137 suppressed tumor growth in xenograft nude mice models. In addition, miR-137 was indicated to inhibit the activation of phosphoinositide 3 kinase/protein kinase B signal pathway, which contributes to the inhibition of RCC growth. These findings indicate that miR-137 functions as tumor suppressor in RCC, suggesting that miR-137 may be a potential therapeutic target for RCC.

Introduction

Renal cell carcinoma (RCC) accounts for 3% of all adult malignancies and is the third most common urological cancer worldwide, with the highest mortality rate of RCC at >40% (1). Approximately 65,150 novel cases and 13,680 mortalities were estimated for 2013 in the USA (1). Clear cell RCC (ccRCC) is the largest subtype of RCC and accounts for 70% of RCCs (2). Despite the increasingly early detection of RCC and more frequent use of surgery, the mortality rate has not changed significantly since 1990 and ~30% patients with localized RCC develop post-operative metastatic recurrence (3). The current adjuvant therapy for metastatic RCC remains extremely limited, as RCC is relatively unaffected by chemotherapy or radiotherapy (4). Therefore, a better understanding of the mechanisms involved in the pathogenesis of RCC and more effective therapeutic approaches are urgently required.

MicroRNAs (miRNAs) are a small class of non-coding RNAs of 19-25 nucleotides in length. miRNAs are known to be important in the pathogenesis of cancer, from initiation to metastasis, primarily through interaction with the 3' untranslated region (3'UTR) of various target genes, which results in target gene translational silence or cleavage (5). miRNAs are known to be involved in multiple tumorigenic steps in human cancers, including in cell growth, apoptosis, migration and invasion (6-9). Of these miRNAs, miR-137 is an notable member that is located on chromosome 1p22 and has been indicated to be underexpressed in non-small lung (10), gastric (11), colorectal (12) and breast cancer (13). The restoration of miR-137 expression has been shown to inhibit cancer cell growth, migration and invasion, and to induce cancer cell apoptosis (10-13). These previous studies suggest that miR-137 could act as a diagnostic and prognostic marker in human cancers. However, the clinical significance and role of miR-137 in RCC remain unknown at present.

Therefore, the aim of the present study was to analyze the expression pattern of miR-137 in clinical RCC samples and examine the effects of miR-137 on the proliferation, migration, invasion, apoptosis and cell cycle of RCC cell lines, and to evaluate the effects of miR-137 on tumor growth in 786-O xenograft nude mice models.

Materials and methods

RCC clinical specimens. Subsequent to obtaining informed consent from all patients, surgical specimens (paired normal and cancerous tissue) were collected from 50 patients with RCC in the Department of Urology, China-Japan Union Hospital of Jilin University (Changchun, China) between August 2012 and August 2014. Samples were flash frozen in liquid nitrogen and stored at ~80°C until required. The present study was approved by the ethics committee of Jilin University. Clinical and pathological information from patient records was also gathered and is listed in Table I.

Cell culture. The human RCC A498 and 786-O cell lines and normal renal HK-2 cell line were purchased from the Type
RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of tissues and cells was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. The concentration and quality of RNA was determined using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) with 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in 5% CO₂.

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Table I. Association between the relative level of miR-137 in the tumor tissues of patients with RCC and the clinicopathological features of RCC.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Patients, n</th>
<th>miR-137 expression level</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;60</td>
<td>22</td>
<td>0.54±0.06</td>
<td>0.797</td>
</tr>
<tr>
<td>≥60</td>
<td>28</td>
<td>0.56±0.07</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>26</td>
<td>0.55±0.08</td>
<td>0.914</td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
<td>0.56±0.07</td>
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<tr>
<td>TNM stage</td>
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<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>I-II</td>
<td>34</td>
<td>0.68±0.12</td>
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</tr>
<tr>
<td>III-IV</td>
<td>16</td>
<td>0.35±0.05</td>
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</tr>
<tr>
<td>Tumor size</td>
<td></td>
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<td>0.006</td>
</tr>
<tr>
<td>&lt;5 cm</td>
<td>30</td>
<td>0.62±0.11</td>
<td></td>
</tr>
<tr>
<td>≥5 cm</td>
<td>20</td>
<td>0.47±0.07</td>
<td></td>
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<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>35</td>
<td>0.70±0.14</td>
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<tr>
<td>Yes</td>
<td>15</td>
<td>0.23±0.04</td>
<td></td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± standard deviation. Measured as tumor diameter. RCC, renal cell carcinoma; TNM, tumor-node-metastasis.

Cell transfection. miR-137 mimic and corresponding negative control miRNA (miR-NC) were purchased from Shanghai GenePharma (Shanghai, China), and were transiently transfected into 786-O cells in 6-well plates at a concentration of 100 nM using Oligofectamine™ Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

Cell viability. Cell viability was assessed at 24, 48 and 72 h after transfection with miR-137 or miR-NC using the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) method (Sigma-Aldrich). In brief, transfected cells (5x10⁴) were seeded in 96-well plates in triplicate, with MTT working solution (Sigma-Aldrich) and cultured for 4 h at 37°C. The medium was then removed and 200 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals. Cell viability was assessed at an absorbance of 570 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cells transfected with PBS were used as a control. All experiments were repeated three times.

Cell cycle and cell apoptosis assay. Cell cycle and cell apoptosis assays were performed by flow cytometry. First, cells were transfected with either miR-137 or miR-NC and cultured at 37°C in 5% CO₂ for 48 h. For cell cycle analysis, the cells were harvested by trypsinisation, and the fixed cells were incubated with DNA binding dye propidium iodide (PI; 20 µg/ml; Sigma-Aldrich) and RNase (1.0 mg/ml; Sigma-Aldrich) for 30 min at 37°C in the dark, and then analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For the cell apoptosis assay, apoptosis was determined by PE Annexin V/Annexin V Apoptosis Detection Kit I (BD Pharmlingen, San Diego, CA, USA) according to the manufacturer's protocol, and analyzed by fluorescence activated cell sorting in flow cytometry (FACSCalibur 4 Colour; BD Biosciences). Flow cytometry data were analyzed using FlowJo software (version 7.65; FlowJo, LLC, Ashland, OR, USA). Experiments were performed in triplicate.

Cell migration and invasion assay. A 24-well Transwell plate with 8-µm pore polycarbonate membrane inserts (BD Biosciences) was used to analyze the migration and invasive potential of cells transfected with miR-137 mimic or miR-NC. For the invasion assay, the membrane was coated with the Matrigel (200 µg/ml; BD Biosciences). Transfected cells (2.5x10⁴) suspended in serum free DMEM were added to the upper chamber. In the bottom chamber, medium containing 10% FBS was added as a chemoattractant. Subsequent to 24 h for the migration assay or 48 h for the invasion assay, migrated or invaded cells were fixed with 70% ethanol (Sigma-Aldrich) for 30 min and stained with 0.2% crystal violet (Sigma-Aldrich) for 10 min. Migrating or invading cells were counted by taking photomicrographs in randomly five fields under a light microscope (ECLIPSE TS100; Nikon Corporation, Tokyo, Japan).
Western blot analysis. Protein was extracted from tissues and cells using radioimmunoprecipitation assay lysis buffer containing proteinase inhibitor (Sigma-Aldrich) supplemented with a protease inhibitor mixture stock solution (Roche Molecular Biochemicals, Mannheim, Germany) and phenylmethanesulfonyl fluoride (Sigma-Aldrich). Concentrations of total cellular protein were determined using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein (20 μg) was separated by a 10% sodium dodecylsulfate-polyacrylamide gel, and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories GmbH, Munich, Germany). The membrane was incubated with 5% nonfat skim milk to block nonspecific binding. The membranes were incubated overnight at 4°C with the following antibodies: Mouse anti-human monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH; dilution, 1:5,000; catalog no. sc-365062; Cell Signaling Technology, Inc., Danvers, MA, USA); mouse anti-human monoclonal phosphoinositide 3-kinase (PI3K; dilution, 1:2,000; catalog no. sc-23962; Cell Signaling Technology, Inc.); rabbit anti-mouse monoclonal phosphorylated-PI3K (p-PI3K) (Tyr458; dilution, 1:500; catalog no. 4228; Cell Signaling Technology, Inc.); mouse anti-human monoclonal protein kinase B (AKT; dilution, 1:2,000; catalog no. sc-5298; Cell Signaling Technology, Inc.); and mouse anti-human monoclonal phosphorylated-AKT (p-AKT; Ser473; dilution, 1:1,500; catalog no. sc-293125; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (dilution, 1:5,000; catalog no. sc-2004; Santa Cruz Biotechnology, Inc.) or goat anti-rabbit IgG (dilution, 1:5,000; catalog no. sc-2005; Santa Cruz Biotechnology, Inc) for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; catalog no. 32209; Thermo Fisher Scientific, Inc.). Blots were stripped and reprobed with anti-GAPDH to control for loading variations.

Tumor growth in vivo. A total of 30 male BALB/c mice, 5-6 weeks old, were purchased from the Experiments Animal Center of Changchun Biological Institute (Changchun, China), maintained under specific pathogen-free conditions and provided with food and water ad libitum. The present study was approved by the Animal Ethics Committee of Jilin University (Changchun, China).

For animal xenograft model assays, 2x10⁴ cells stably expressing miR-137 or miR-NC were injected into the flanks of mice (n=10 per group). The control group was injected with 2x10⁶ 786-O cells into the flanks of mice (n=10). The tumors were measured using vernier calipers every week, and tumor volume was calculated according to the formula: Volume (mm³) = width² x length / 2. At 30 days subsequent to injection, the mice were sacrificed and the tumors were dissected and weighed. Total RNAs of tumor tissues were extracted to measure the miR-137 level using the aforementioned RT-qPCR method.

Statistical analysis. Data from at least three independent experiments were expressed as mean ± standard deviation. Statistical analysis between two samples was performed using two-sided Student's t-test, and more than two groups was performed using one-way analysis of variance followed a Tukey's post-hoc test. All data were analyzed using the GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-137 is frequently reduced in RCC tissues and cell lines. To determine whether miR-137 expression is associated with RCC pathogenesis, the expression levels of miR-137 were determined using RT-qPCR analysis in 50 pairs of RCC and adjacent normal tissues. Notably, there was a significant decrease in miR-137 expression in ~78% (39/50) of tumor tissues compared with normal tissues (Fig. 1A). In addition, the levels of miR-137 expression in the human RCC A498 and 786-O cell lines and normal renal HK-2 cell line were examined by qRT-PCR (Fig. 1B). The expression level of miR-137 in the RCC cell lines was found to be decreased compared with the expression in the normal renal HK-2 cell line (Fig. 1B). The 786-O cell line, which possessed the lower level of miR-137 expression of the two RCC cell lines (Fig. 1B), was selected for further studies.

The association between miR-137 expression and the clinicopathological parameters of the patients, including age, gender, TNM stage, tumor diameter and metastasis was assessed (Table I). The level of miR-137 expression in tissues was found to be negatively associated with a larger tumor diameter, advanced TNM stage and metastasis (P<0.01), which are all indicators of poor prognosis. There was no correction between miR-137 expression and age and sex. These data suggested that miR-137 might play a key role in RCC development.

Overexpression of miR-137 attenuates RCC cell proliferation and motility and induces cell apoptosis. To explore the biological significance of miR-137, we stably overexpressed miR-137 in 786-O cell by transfection with miR-137 mimic or miR-NC. The efficacy of transfection was tested by qRT-PCR (Fig. 2A). It was found that miR-137 expression in miR-137 group was elevated in 786-O cells compared to miR-NC group (Fig. 2B). As proliferation directly linked to cell cycle distribution, we investigate the effect of miR-137 on RCC cell cycle progression, and found that overexpression of miR-137 caused cell-cycle arrest at G0-G1 phase and decreased the S-phase population (Fig. 2C). Furthermore, to reveal the biological role of miR-137 on migration and invasion, transwell assay were performed. It was found that overexpression of miR-137 markedly impaired RCC cell migration (Fig. 2D) and invasiveness (Fig. 2E) compared with miR-NC (P<0.01). In addition, PE Annexin-V staining was used to reveal miR-137 on apoptosis in RCC cells, and found that overexpression of miR-137 markedly induced cell apoptosis compared with the miR-NC group (Fig. 2F). These findings suggest that miR-137 acts as a tumor suppressor in RCC by inhibiting cell proliferation and motility and inducing cell apoptosis.
study showed that miR-137 functions as a tumor suppressor in gastric cancer cell through targeting Cyclooxygenase-2 (Cox-2) through inhibiting the activation of PI3K/AKT signaling pathway (11). Thus, in this study, we wonder whether miR-137 affects activation PI3K/AKT signaling pathway. Measurements of the phosphorylation/activation pattern of PI3K and AKT was performed by western blot. Western blot assay showed that overexpression of miR-137 markedly inhibited the phosphorylation of PI3K and p-AKT expression compared to untreated group and miR-NC group, without altering the total protein levels of PI3K or AKT in 786-O cells (Fig. 3), suggesting that miR-137 suppressed RCC growth in vitro and in vivo, at least in part, via inhibiting activation of PI3K/AKT signaling pathway.

**Overexpression of miR-137 suppresses tumor growth in vivo.**

As we showed that miR-137 acts as a tumor suppressor in RCC
by inhibiting cell proliferation and motility and inducing cell apoptosis, we further investigated whether miR-137 would have similar antitumor effect in vivo. 786-O cells stable expression miR-NC or miR-137 were subcutaneously inoculated in nude mice (n=10 for each group). The volumes of tumors established in mice were measured with caliper every weeks. It was found that overexpression of miR-137 significantly inhibited tumor volumes than controls group and miR-NC group (Fig. 4A). The tumors were extracted 28 days after implantation, tumor weight were measured. The result showed that tumors tissue weight was significantly lower in miR-137 mimic group relative to control group and miR-NC group (Fig. 4B and C). In addition, miR-137 expression level in xenograft tumors was determined by qRT-PCR. The results of qRT-PCR showed that miR-137 expression was higher in the xenograft tumors of miR-137 mimic group than that of the xenograft tumors of miR-NC group and control group (P<0.05; Fig. 4D). These results might imply that overexpression of miR-137 could inhibit RCC growth in vitro and suppressed tumor growth in vivo.

**Discussion**

Recently, miRNAs have been reported to play key roles in the initiation and maintenance of human various cancers (7). It has drawn more attention in the recent years because of it role in the gene transcriptional and posttranscriptional regulation. Growing evidence showed that miRNAs could act as novel biomarkers or therapy agent for various cancers, inculding RCC (17). For instance, the expression of was miR-129-3p significantly decreased in RCC, as a molecular diagnostic marker to distinguish RCC from benign tumors and normal tissues and a potential prognostic marker for RCC (18). Chen et al showed that miR-141 serves as a potential biomarker for discriminating RCC from normal tissues and a crucial suppressor of RCC cell proliferation and metastasis by modulating the EphA2/p-FAK/p-AKT/MMPs signaling cascade (19). Lu et al demonstrated that the expression of miR-145 was downregulated in RCC compared to their normal adjacent tissues, and that restoring miR-145 expression in RCC cell lines dramatically suppressed cell proliferation, migration and invasion, and induced cell apoptosis and G2-phase arrest (20). In this study, we found that the expression of miR-137 was downregulated in RCC compared to their normal adjacent tissues, and that overexpression of miR-137 in RCC cells dramatically suppressed cell proliferation, migration and invasion, and induced cell apoptosis in vitro and suppressed tumor growth in vivo. These studies and together with our study provide a solid foundation for RCC utilization of miRNAs in diagnostic and anticancer therapy in future.

Downregulation of miR-137 has been frequently observed in several cancer cells including non-small lung cancer (10), gastric cancer (11), and oral cancer cells (21) colorectal cancer (12) and breast cancer (13), glioblastoma cells (22). However, recently a study showed that miR-137 expresion was upregulated in squamous cells carcinoma of the tongue (23), which suggests that miR-137 may play different roles depending on different tumor microenvironments. In this study, our results demonstrate that miR-137 expression was frequently decreased in clinical specimens of RCC and RCC cell lines, and its expression level was significantly associated

**Figure 4.** Overexpression of miR-137 suppressed tumor growth in a 786-O xenograft model. (A) Growth curves for tumor volumes in xenografts of nude mice. (B) Images of the tumor specimens of xenograft models at day 28. (C) Tumor weights of various groups at day 28. (D) Reverse transcription-quantitative polymerase chain reaction analyzed miR-137 expression in tumor tissue. *P<0.05 and **P<0.01 vs. control group. miR, microRNA; miR-NC, negative control microRNA.
with poor prognostic clinicopathological parameters, such as larger tumor diameter, advanced TNM stage, and metastasis. We also showed that restored expression of miR-137 in RCC cell line 786-O resulted in the inhibition of cell proliferation, migration and invasion, and induction cell apoptosis in vitro. In addition, in vivo assay demonstrated that enforced miR-137 suppressed tumor growth and decreased tumor volume and weight in xenograft nude mice models. These results suggest that miR-137 functions as a tumor suppressor in RCC.

PI3K/AKT signaling is dysregulated in numerous cancers, including RCC (24), and the activation of this pathway has been suggested to correlate with aggressive behavior and a poor prognosis in RCC tumors (25). Inhibition of the PI3K/AKT signaling pathway using small molecule compounds acts as an attractive potential therapeutic approach for RCC (15,16). A recent study showed that the overexpression of miR-137 in gastric cancer may inhibit the activation of PI3K/AKT signaling pathway (11). Consistent with this result, the results of the present study demonstrated that the overexpression of miR-137 significantly inhibited the phosphorylation of PI3K and p-AKT expression without altering the total protein levels of PI3K or AKT in 786-O cells; which indicates that miR-137 suppressed the growth of RCC in vitro and in vivo, at least in part, by inhibiting the activation of the PI3K/AKT signaling pathway.

In summary, the results of the present study demonstrate that miR-137 was frequently reduced in clinical specimens of RCC and in RCC cell lines. In addition, the expression level of miR-137 was significantly associated with poor prognostic clinicopathological parameters, including larger tumor diameter, advanced TNM stage and metastasis. miR-137 showed a significant suppressive effect on RCC proliferation, migration and invasion in vitro and on the growth of tumors in xenograft nude mice models, suggesting that miR-137 functions as a tumor suppressor in RCC. In addition, miR-137 also inhibited the activation of the PI3K/AKT signaling pathway, which may contribute to the inhibition of RCC cell growth. These findings indicate that miR-137 may present a useful biomarker for poor prognosis and a therapeutic target for patients with RCC.

References