MicroRNA-30a-5p suppresses tumor cell proliferation of human renal cancer via the MTDH/PTEN/AKT pathway

JIANMIN LI1, CHANGYING LI1, HONGJIE LI2, TING ZHANG1, XIAODONG HAO1,3, JIWU CHANG1 and YONG XU1,3

1Cancer Immunity Research Laboratory, Tianjin Institute of Urology, The Second Hospital of Tianjin Medical University, Tianjin Medical University, Tianjin 300211; 2School of Basic Medical Sciences, North China University of Science and Technology, Tangshan, Hebei 063000; 3Department of Urology, The Second Hospital of Tianjin Medical University, Tianjin Medical University, Tianjin 300211, P.R. China

Received November 23, 2016; Accepted October 2, 2017

DOI: 10.3892/ijmm.2017.3269

Abstract. The present study aimed to explore the effects of microRNA (miRNA)-30a-5p on tumor proliferation and to seek a potential therapeutic target for the treatment of human renal cancer. The results demonstrated that the expression levels of miRNA-30a-5p were reduced in tumor samples from patients with renal cancer compared with in normal tissue samples. Overall survival and disease-free survival were increased in patients with renal cancer and high miRNA-30a-5p expression compared with in those with low miRNA-30a-5p. Furthermore, overexpression of miRNA-30a-5p suppressed cell proliferation, induced apoptosis, and promoted caspase-3/9 activities and B-cell lymphoma 2-associated X protein (Bax) protein expression in Caki-2 cells. In addition, the results confirmed that overexpression of miRNA-30a-5p inhibited metadherin (MTDH), upregulated phosphatase and tensin homolog (PTEN) and suppressed phosphorylated (p)-protein kinase B (AKT) protein expression levels in Caki-2 cells. Knockdown of MTDH expression also upregulated PTEN and suppressed p-AKT protein expression in Caki-2 cells. In conclusion, the present study is the first, to the best of our knowledge, to provide evidence suggesting that miRNA-30a-5p suppresses tumor human renal cancer cell proliferation via the MTDH/PTEN/AKT pathway.

Introduction

Renal cell carcinoma (RCC) is the most common space-occupying lesion in adult kidneys and accounts for ~3% of all tumors. In addition, the mortality rate of RCC is ≤40% (1). RCC is derived from renal proximal tubule cells, and clear cell carcinoma is the most common subtype, which accounts for 80-85% of metastatic RCC (2). With regards to treatment, RCC is not sensitive to radiotherapy or chemotherapy. Although surgical tumor resection is the optimal treatment strategy at present, it is associated with 20-40% postoperative relapse. Since RCC is resistant to radiotherapy and chemotherapy, effective postoperative adjuvant treatment for RCC is lacking (3). In addition, the lack of biochemical markers for the early diagnosis of RCC, as well as follow-up data, hinders the timely diagnosis of RCC. Therefore, there is an urgent need to understand the development of RCC at molecular and genetic levels, and to identify biochemical markers that can postoperatively predict the early metastasis of RCC (4).

MicroRNA (miRNA) expression profile screening is one of the most advanced methods used to research tumor-associated molecules (5). miRNAs are endogenous, non-coding, single-stranded RNAs. The mechanism of action of miRNAs is one kind of the epigenetic regulation and control mechanism, and miRNAs serve important roles in the regulation of gene expression (6). It has been estimated that one miRNA can regulate the expression of ~100 target genes, and miRNAs of >10,000 genes are directly regulated by miRNAs (7). miRNAs are highly conserved among various species and regulate numerous biological functions (6). In addition, miRNAs are able to simultaneously regulate the expression of oncogenes and tumor suppressor genes through regulating critical cellular activities, including metabolism, differentiation, development and apoptosis. It has previously been reported that miRNAs serve a crucial regulatory role in carcinogenesis of prostate cancer, lung cancer, breast cancer and colon cancer, and miRNAs are also considered to be involved in the pathogenesis of RCC (8,9). Numerous RCC-specific miRNAs have been identified through the screening of miRNA expression profiles, the majority of which exert their functions by disrupting the balance between oncogene and tumor suppressor
gene expression (8). A recent study indicated that the regulatory mechanisms of miRNAs in RCC are complex, and are associated with numerous biological mechanisms, including mutations, epigenetic alterations, chromosomal abnormalities and deletions (10).

miRNA-30a-5p has been reported to inhibit metadherin (MTDH) expression, which serves an important role in tumor development. The MTDH gene is located in chromosome 8q22, and exhibits high expression in numerous tumor types, including breast cancer, prostate cancer, neuroblastoma and astrocytoma, where it is associated with poor prognosis (11). MTDH mediates several forms of chemotherapy resistance, and it has previously been demonstrated that the activation of MTDH expression can increase the death of tumor cells mediated by drugs, including cisplatin and doxorubicin (7). In addition, MTDH can enhance the invasion of tumor cells and increase the expression levels of relevant adhesion molecules via the nuclear factor-kB pathway (12). MTDH is the downstream gene of Ha-ras, which induces upregulation of MTDH via the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/glycogen synthase kinase 3β/c-Myc pathway (13).

Among the numerous signal transduction pathways that mediate tumor cell apoptosis, the PI3K/phosphatase and tensin homolog (PTEN)/AKT signal transduction pathway is essential for growth regulation, and has an important role in the regulation of apoptosis. Activation of the PI3K/AKT signal transduction pathway can inhibit apoptosis induced by numerous stimuli and promote cell cycle progression, thus promoting cell survival and proliferation. Furthermore, this pathway participates in angiogenesis, serves an important role in tumor formation, and participates in tumor invasion and metastasis (14). As well as being able to induce the survival and differentiation of tumor cells, angiogenesis, and malignant development, the PI3K/AKT signal transduction pathway is associated with therapeutic resistance (15). At present, tumor treatments that target this pathway have gained extensive attention and abnormal activation of the PI3K/PTEN/AKT signaling pathway has been detected in several human tumor types, including lung cancer, pancreatic cancer, leukemia, liver cancer, multiple myeloma, prostate cancer and RCC (16). The present study aimed to investigate the effects of miRNA-30a-5p on tumor proliferation and to seek a potential therapeutic target for the treatment of human renal cancer.

Materials and methods

Tissue samples. A total of 229 patients with RCC from the Second Hospital of Tianjin Medical University (Tianjin, China) were included in the present study from March 2004 to October 2005; detailed clinicopathological characteristics of the patients are summarized in Table I. Renal parenchyma (RP) samples (5 cm) and RCC samples were collected from the patients. The RCC samples were divided into the following categories: TNM stage I + II, TNM stage III + IV and metastatic. The present study was approved by the Ethics Committee of The Second Hospital of Tianjin Medical University, Tianjin Medical University (Tianjin, China) and informed consent was obtained from all participants.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cancer samples and RP using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA (500 ng) was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer’s protocol. The miRNA-30a-5p, si-MTDH and negative plasmids were obtained from Tianjin Sainie Biologineering Technology Co., Ltd. (Tianjin, China). The sequence of miRNA-30a-5p was: miR-30a-5p, UGUAAACAU CCUCGACUGGAA. qPCR was performed on an Applied Biosystems PCR7900 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR-Green PCR Master Mix reagent kit (Takara Bio, Inc., Otsu, Japan). U6 was used as an internal control. The qPCR thermocycling conditions were as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec, 57°C for 60 sec and 72°C for 30 sec. Relative miRNA-30a-5p expression levels were calculated using the 2ΔΔCt method (17).

Table I. Clinical characteristics of the patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RCC (n=219)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>122</td>
</tr>
<tr>
<td>Female</td>
<td>97</td>
</tr>
<tr>
<td>Age at surgery [mean (range)]</td>
<td>64 (33-79)</td>
</tr>
<tr>
<td>TNM stage, n (%)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>76 (34.70)</td>
</tr>
<tr>
<td>II</td>
<td>27 (12.33)</td>
</tr>
<tr>
<td>III</td>
<td>99 (45.20)</td>
</tr>
<tr>
<td>IV</td>
<td>8 (3.65)</td>
</tr>
<tr>
<td>Not available</td>
<td>9 (4.11)</td>
</tr>
<tr>
<td>Metastasis, n (%)</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>191 (87.21)</td>
</tr>
<tr>
<td>M1</td>
<td>3 (1.37)</td>
</tr>
<tr>
<td>NA</td>
<td>25 (11.42)</td>
</tr>
</tbody>
</table>

Cell culture and transfection. Caki-2 cells, purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (all Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. The miRNA-30a-5p and negative plasmids were obtained from Tianjin Sainie Biologineering Technology Co., Ltd. Caki-2 cells (1x10⁶) were seeded in a 6-well plate and were transfected with 100 ng miRNA-30a-5p and 100 ng negative plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Transfected cells were maintained at 37°C in a 5% CO2 atmosphere for 4 h, and then old DMEM was removed and new DMEM was added into the cells at 37°C in a 5% CO2 atmosphere.

MTT assay. Post-transfection for 24 h, Caki-2 cells were adjusted to 1x10⁴ cells/ml, inoculated in a 96-well plate and cultured for an additional 48 h. Subsequently, 10 µl MTT was added to each well for 4 h, after which dimethyl sulfoxide
was added to each well for 20 min. The absorbance of each well was detected at 490 nm using an Orion II microplate luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The overexpression of miR-30a-5p significantly suppressed cell proliferation and induced apoptosis of Caki-2 cells at 48 h.

Apoptosis assay. Post-transfection for 48 h, Caki-2 cells were adjusted to 1×10⁶ cells/ml, inoculated in a 6-well plate and proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Nanjing, China). Protein concentrations were determined using Micro BCA Protein Assay kit (EMD Millipore). After incubation with 5% non-fat milk in TBST for 1 h at 37°C, the membranes were incubated with the following specific primary antibodies: B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; 1:2,000; sc-7985-R; Santa Cruz, CA, USA), phosphorylated (p)-AKT (1:1,000; 7074; Cell Signaling Technology, Inc.), MTDH (1:1,000; sc-517220; Santa Cruz Biotechnology), PTEN (1:1,000; sc-6817-R; Santa Cruz Biotechnology), caspase-3 (C1116) and caspase-9 (C1158) activities kit (Beyotime Institute of Biotechnology, Haimen, China) for 2 h at 37°C. The absorbance of each well was detected at 405 nm using an Orion II microplate luminometer (Berthold Technologies GmbH & Co. KG).

Western blot analysis. Post-transfection for 48 h, Caki-2 cells were adjusted to 1×10⁶ cells/ml, inoculated in a 6-well plate and proteins were extracted using RIPA buffer. Protein concentrations were determined using Micro BCA Protein Assay kit (EMD Millipore). Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore). After incubation with 5% non-fat milk in TBST at 4°C overnight, the membranes were incubated with the following specific primary antibodies: Bax (1:2,000; 14796; Cell Signaling Technology, Inc., Beverly, MA, USA), Bcl-2 (1:1,000; 3498; Cell Signaling Technology, Inc.), MTDH (1:1,000; sc-517220; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PTEN (1:1,000; sc-6817-R; Santa Cruz Biotechnology), phosphorylated (p)-AKT (1:1,000; sc-7985-R; Santa Cruz Biotechnology) and GAPDH (1:2,000; 5174; Cell Signaling Technology, Inc.) at 4°C overnight. Subsequently, membranes were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000; 7074; Cell Signaling Technology, Inc.) for 1 h at 37°C. An enhanced chemiluminescence western blotting detection system (EMD Millipore) was used to analyze protein expression levels. An enhanced chemiluminescence western blotting detection system (EMD Millipore) was used to analyze protein expression levels and results were semi-quantified using Image-ProPlus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

miRNA-30a-5p expression. The present study detected the expression levels of miRNA-30a-5p using RT-qPCR. As presented in Fig. 1A, miRNA-30a-5p expression was lower in RCC tissue samples compared with in RP tissue samples. In addition, miRNA-30a-5p expression was much lower in samples from patients with TNM stage III + IV RCC compared with in the RP tissue samples (Fig. 1B).

Overall survival (OS) and disease-free survival (DFS) are associated with miRNA-30a-5p expression. The present study also determined whether OS and DFS were associated with miRNA-30a-5p expression in patients with RCC. OS and DFS were increased in patients with RCC and high miRNA-30a-5p expression compared with in those with low miRNA-30a-5p expression. These results indicated that miRNA-30a-5p expression may affect RCC (Fig. 2).

Overexpression of miRNA-30a-5p suppresses cell proliferation and induces apoptosis of Caki-2 cells. To determine the effects of elevated miRNA-30a-5p expression on cell proliferation and apoptosis of Caki-2 cells, MTT assay and flow cytometry were conducted. As presented in Fig. 3, overexpression of miRNA-30a-5p significantly suppressed cell proliferation and induced apoptosis of Caki-2 cells.

Effects of miRNA-30a-5p overexpression on Bax and MTDH protein expression levels in Caki-2 cells. To determine whether miRNA-30a-5p expression had an effect on MTDH and Bax protein expression in Caki-2 cells, western blot analysis was used to analyze the protein expression levels of Bax and MTDH. The results indicated that overexpression of miRNA-30a-5p increased the protein expression levels of Bax and inhibited MTDH protein expression in Caki-2 cells (Fig. 5).
Overexpression of miRNA-30a-5p inhibits MTDH protein expression in Caki-2 cells. The present study also analyzed whether miRNA-30a-5p affected MTDH protein expression in Caki-2 cells by immunocytofluorescence. Overexpression of miRNA-30a-5p markedly inhibited MTDH protein expression in Caki-2 cells, as determined using immunocytofluorescence (Fig. 6).

Overexpression of miRNA-30a-5p affects PTEN and p-AKT protein expression in Caki-2 cells. In order to elucidate whether miRNA-30a-5p affects p-AKT and PTEN protein expression in Caki-2 cells, western blotting was conducted. As shown in Fig. 7, overexpression of miRNA-30a-5p significantly induced...
the protein expression levels of PTEN and suppressed p-AKT protein expression in Caki-2 cells.

Small interfering RNA (si)-MTDH increases the effects of miRNA-30a-5p on MTDH protein expression in Caki-2 cells. The present study aimed to determine how MTDH regulates the effects of miRNA-30a-5p in RCC. As presented in Fig. 8, knockdown of MTDH, alongside miRNA-30a-5p overexpression, significantly inhibited MTDH protein expression in Caki-2 cells compared with in the control group.

Figure 5. Effects of miRNA-30a-5p overexpression on Bax and MTDH protein expression levels in Caki-2 cells. (A) Bax and MTDH protein expression were determined by western blotting. Overexpression of miRNA-30a-5p (B) increased Bax and (C) inhibited MTDH protein expression levels in Caki-2 cells, as determined by statistical analysis. Control, control group; Negative, negative control group; miRNA-30a-5p, miRNA-30a-5p overexpression group. **P<0.05 compared with the Control group. Bax, B-cell lymphoma 2-associated X protein; miRNA-30a-5p, microRNA-30a-5p; MTDH, metadherin.

Figure 6. Overexpression of miRNA-30a-5p inhibits MTDH protein expression in Caki-2 cells, as determined using immunocytofluorescence (magnification, x5). Control, control group; Negative, negative control group; miRNA-30a-5p, miRNA-30a-5p overexpression group. miRNA/miR-30a-5p, microRNA-30a-5p; MTDH, metadherin.
in Fig. 9, knockdown of MTDH, alongside miRNA-30a-5p overexpression, inhibited cell proliferation and increased apoptosis of Caki-2 cells compared with in the control group.

**si-MTDH increases the effects of miRNA-30a-5p on caspase-3/9 activities of Caki-2 cells.** The present study investigated the mechanism underlying the effects of miRNA-30a-5p on Caki-2 cells; caspase-3/9 activities were analyzed using ELISA kits. Caspase-3/9 activities were significantly reduced in response to miRNA-30a-5p overexpression and knockdown of MTDH in Caki-2 cells compared with in the control group (Fig. 10).
si-MTDH increases the effects of miRNA-30a-5p on Bax, PTEN and p-AKT protein expression in Caki-2 cells. The present study aimed to determine the effects of si-MTDH on miRNA-30a-5p-regulated expression of Bax, PTEN and p-AKT in Caki-2 cells. The protein expression levels of Bax and PTEN were significantly promoted, whereas p-AKT protein expression was significantly suppressed in Caki-2 cells in response to miRNA-30a-5p overexpression and MTDH knockdown compared with in the control group (Fig. 11).

Discussion

RCC is the most common type of kidney cancer, which accounts for ~3% of all body tumors. In addition, the morbidity of RCC has increased in the past 20 years (18). Surgery remains the main treatment strategy for RCC; however, ~30% of patients develop recurrence within 3 years postsurgery. The 5-year survival rate of RCC is <10% and ~25% of patients have metastasis at the time of diagnosis (19). Therefore, the identification of reliable biological markers is of importance for the early diagnosis of RCC, the judgment of patient prognosis and the instruction of individualized treatment (20). miRNAs exert their important functions by influencing tumor proliferation, migration and invasion (8). The results of the present study demonstrated that miRNA-30a-5p expression was lower in tumor samples from patients with RCC compared with in RP tissue samples. In addition, miRNA-30a-5p expression was much lower in tumor samples from patients with TNM stage III + IV RCC compared with in RP tissue samples.

RCC is associated with numerous genes and its pathogenesis is complex; therefore, the molecular and biological foundation for its etiology and development remains unclear (21). However, with in-depth research regarding the molecular mechanisms underlying tumor cell growth, proliferation and apoptosis, it has been reported that an imbalance in the internal environment is an important factor for tumor development; the main cause of disturbances to the internal environment is imbalances in numerous intracellular signal pathways (22). It has previously been demonstrated that abnormalities in the signal transduction pathways that control cell proliferation and differentiation results in cellular growth disorders; in particular, increased signaling of pathways that promote cell proliferation, or reduced signaling of pathways that inhibit cell proliferation and promote cell apoptosis, at the cellular level during tumor formation may lead to tumor formation or reduced tumor cell apoptosis (23). In the present study, OS and DFS were increased in patients with RCC and high miRNA-30a-5p expression compared with in those with low miRNA-30a-5p expression.

The present study demonstrated that overexpression of miRNA-30a-5p inhibited MTDH, upregulated PTEN, and suppressed p-AKT protein expression levels in Caki-2 cells. Our results were similar to results from previous studies (24-26), which showed that miRNA-30a-5p regulated MTDH/PTEN/AKT signal pathway to induce renal cancer cell

![Figure 10](image1.png)

![Figure 11](image2.png)

Figure 10. Knockdown of MTDH increases the effects of miRNA-30a-5p on caspase-3/9 activities in Caki-2 cells. Control, control group; Negative, negative control group; miRNA-30a-5p, microRNA-30a-5p overexpression group; si-MTDH + 30a-5p, si-MTDH + miRNA-30a-5p overexpression group. *P<0.05 compared with the Control group; **P<0.05 compared with the miRNA-30a-5p group. miRNA-30a-5p, microRNA-30a-5p; MTDH, metadherin; si-MTDH, small interfering RNA-MTDH.

Figure 11. Knockdown of MTDH increases the effects of miRNA-30a-5p on Bax, PTEN and p-AKT protein expression in Caki-2 cells. si-MTDH increased the effects of miRNA-30a-5p on Bax, PTEN and p-AKT protein expression in Caki-2 cells, as determined using (A) western blot analysis and (B-D) statistical analysis. **P<0.05 compared with the Control group; ***P<0.05 compared with the miRNA-30a-5p group. Control, control group; Negative, negative control group; miRNA-30a-5p, microRNA-30a-5p overexpression group; si-MTDH + 30a-5p, si-MTDH + miRNA-30a-5p overexpression group. Bax, B-cell lymphoma 2-associated X protein; miRNA-30a-5p, microRNA-30a-5p; MTDH, metadherin; p-AKT, phosphorylated-protein kinase B; PTEN, phosphatase and tensin homolog; si-MTDH, small interfering RNA-MTDH.
apoptosis. The present study demonstrated that overexpression of miRNA-30a-5p inhibited MTDH, upregulated PTEN, and suppressed p-AKT protein expression levels in Caki-2 cells. The present study demonstrated that overexpression of miRNA-30a-5p inhibited MTDH, upregulated PTEN, and suppressed p-AKT protein expression levels in Caki-2 cells, which showed that miRNA-30a-5p regulates MTDH/PTEN/AKT pathway to suppress cell growth in human renal cancer.

A recent study demonstrated that the main function of AKT is the direct inhibition of cell apoptosis (27). The Bcl-2 family is also closely associated with cell apoptosis, numerous members of which, including Bcl-2-associated death promoter (Bad), exert proapoptotic effects. AKT can phosphorylate the Ser136/Ser112 residue of the Bad protein, thus resulting in its disaggregation from Bcl-2 or Bcl-extra large, Bad then binds and suppressed p-AKT protein expression levels in Caki-2 cells.

Akt can directly catalyze and phosphorylate Ser196 of caspase-9, resulting in its inactivation, thus reducing its proapoptotic effect. In addition, when the AKT kinase domain is activated, cells can resist penicillin-induced apoptosis, suggesting that only AKT with the complete kinase activity domain can promote the anti-apoptotic effect (29). The present study demonstrated that si-MTDH increased the effects of miRNA-30a-5p on the inhibition of cell proliferation and the promotion of apoptosis, caspase-9/3 activities and Bax protein expression in Caki-2 cells. The present study demonstrated that si-MTDH increased the effects of miRNA-30a-5p on the inhibition of cell proliferation and the promotion of apoptosis, caspase-9/3 activities and Bax protein expression in Caki-2 cells. MTDH is an important role in the anti-effect of miRNA-30a-5p on human renal cancer.

A previous study indicated that MTDH can activate numerous signaling pathways, regulate physiological and pathological cellular processes, and mediate the proliferation, invasion, metastasis, angiogenesis and chemotherapeutic resistance of tumor cells (30). MTDH is a downstream target gene of AKT, as well as an upstream activator of the PI3K/AKT pathway; PI3K/AKT and c-Myc can induce MTDH expression, which can further activate PI3K/AKT and upregulate c-Myc expression, resulting in the expression of N-Myc in a neuroblastoma cell line, further enhancing oncogenic effects, and forming a vicious cycle during tumor formation (31). In the present study suppression of MTDH expression upregulated PTEN and suppressed p-AKT protein expression levels in Caki-2 cells. In the present study suppression of MTDH expression upregulated PTEN and suppressed p-AKT protein expression levels in Caki-2 cells by miRNA-30a-5p. MTDH/PTEN/AKT pathway participated in the anti-effect of miRNA-30a-5p on human renal cancer.

In conclusion, the present study demonstrated that miRNA-30a-5p may suppress human RCC cell proliferation via the MTDH/PTEN/AKT pathway. Further studies aim to determine whether miRNAs can be practically applied in the treatment of RCC.

References


