Original Article

miR-212-5p suppresses clear cell renal cell carcinoma proliferation and differentiation by targeting heat shock factor 1

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Abstract: Background: Clear cell renal cell carcinoma (ccRCC) is a renal neoplasm with a high morbidity rate. Although many studies on ccRCC are reported and miRNAs are known to play an essential role, the molecular mechanisms underlying tumor occurrence and progression are not completely understood. Methods: Seventeen pairs of tumor and the adjacent normal tissues of ccRCC patients were collected. After cell culture and transfection, the expression of miR-212-5p, HSF1, PI3K, and p-AKT and their relationship in 786-O cells were detected. Results: The relative expression of miR-212-5p significantly decreased in tumor tissues. However, the mRNA level of HSF1 was up-regulated. The relative expression of miR-212-5p in several ccRCC cell lines was decreased, while HSF1 mRNA was determined to be higher in HEK-293 cells using RT-PCR. Combined Target Scan software with dual-luciferase reporter gene assays confirmed that HSF1 was targeted by miR-212-5p. In addition, 786-O cells transfected with a miR-212-5p mimic, showed significantly down-regulated expression of HSF1 at the transcriptional and translational levels. Furthermore, protein levels of PI3K and p-AKT also decreased. Nevertheless, after the miR-212-5p inhibitor was transfected into the 786-O cells, the expression of HSF1/PI3K/p-AKT was significantly up-regulated. Additionally, the proliferation rate notably decreased after the miR-212-5p mimic was transfected into the 786-O cells. However, the result was the opposite following miR-212-5p inhibitor transfection into the 786-O cells. PI3K/AKT signaling was suppressed after HSF1 was inhibited, but elevated when HSF1 was overexpressed. Additionally, the proliferation rate of 786-O cells was statistically lower after PI3K/AKT was inhibited. Conclusion: miR-212-5p may inhibit ccRCC progression by targeting HSF1 through PI3K/AKT signaling.

Keywords: miR-212-5p, HSF1, ccRCC, proliferation, PI3K, AKT

Introduction

It has been reported that renal cell carcinomas (RCC) comprises 90% of kidney tumors. Globally, 116,000 patients die from this malignancy among the 270,000 cases each year [1]. Of the five RCC subtypes, 70-80% are clear cell renal cell carcinoma (ccRCC) [2]. It has been estimated that nearly 30% of RCC eventually deteriorate into invasive disease and metastasize to a variety of other important organs including the lung, brain, bone, and liver [3, 4]. Additionally, there are no available effective treatments for RCC due to metastasis and resistance to chemotherapy. Thus, studying the underlying molecular mechanism and developing potential treatments for the disease is of vital importance.

MicroRNAs (miRNAs) are a class of non-coding single-stranded, endogenous RNA molecules that are 21 to 23 nucleotides in length and regulate gene expression by binding to the 3’-untranslated region (3’-UTR) of an mRNA target to promote mRNA degradation and/or translational repression [5, 6]. It is reported that miRNAs play an essential role in tumor initiation, progression, and metastasis, and are being considered as significant biomarkers in cancer diagnosis and prognosis [7, 8]. Many miRNAs have been investigated for their involvement in RCC. For example, miR-19a correlates with poor prognosis of ccRCC, while miRNA-133b and miRNA-135a induce apoptosis in RCC [9, 10].

Many miRNAs have been reported as biomarkers for ccRCC. Although previous studies suggest that miR-212 is involved in the carcinogenesis and progression of multiple human cancers, the role of miR-212-5p in ccRCC is not yet clear [11, 12]. In this study, we deter-
mired the role of miR-212-5p in human tumor tissue, as well as the signaling pathway involved in ccRCC.

**Materials and methods**

**Tissue sample collection**

From 2015/7/1 to 2016/7/1, ccRCC tissue and the adjacent normal tissue was collected from 17 patients at the Institute of Urology and Nephrology of Guangxi Medical University. Pairs of tumor and the adjacent normal tissues were rapidly frozen in liquid nitrogen and stored at -80°C until RNA isolation. Influencing factors including blood transfusion, chemotherapy, or radiotherapy did not exist prior to surgery. The patients’ written informed consent and the approval of the Ethics Committee of Guangxi Medical University were obtained.

**Cell culture and transfection**

The human ccRCC cell lines, SN12-PM6, A498, and 786-O, as well as the human embryonic kidney cell line, HEK-293, were purchased from the Chinese Academy of Sciences (Shanghai Cell Bank, China). All of the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone) at 37°C in a humidified incubator containing 5% CO₂. The 786-O cells were cultured in 6-well plates and total RNA was extracted from some of the cells after reaching 90-100% confluence, while the remaining cells were used for transfection after reaching 70% confluence. miR-212-5p mimic (50 nM) and its negative control (NC), and 80 nM miR-212-5p inhibitor and its NC (Sangon Biotech, Shanghai, China) were transfected into the cells using lipofectamine 2000 (Invitrogen, USA). After 48 h, the cells were collected to measure luciferase activity with the Dual-Luciferase® Reporter Assay system (Promega). Data are presented as the ratio of experimental (Renilla) luciferase to control (Firefly) luciferase.

**Bioinformatics analysis**

The online TargetScan software program was used to predict the target genes of miR-212-5p by using the human gene sequence, and the predicted genes were intersected for further analysis. The software program website for target gene prediction is the following: TargetScanHuman http://www.targetscan.org/.

**Lentiviral vector over-expressing HSF1**

A full-length genomic fragment of human HSF1 was cloned from the pcDNA3.0 HSF1 packaging plasmid and was amplified by PCR. The sequence of the amplified segments was subcloned into the lentiviral expression vector pCDHEF1-MCS-T2A-copGFP. The recombined lentiviral expression vector pCDH-EF1-HSF1-copGFP was constructed with the help of Vigen Biosciences Science and Technology co., LTD (Shangdong, China). RT-PCR and western blots were used to detect the expression of HSF1 after 786-O cells were transfected by the pCDH-EF1-HSF1-copGFP vector and its NC vector, pCDHEF1-MCS-T2A-copGFP.

**Quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA (1 µg) was reverse transcribed using a reverse transcription kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. Sequences of the primers are as follows: miR-212-5p forward, 5’-GCT TAC
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miR-212-5p was up-regulated, while HSF1 was down-regulated, in ccRCC patient tissues and human ccRCC cell lines. A. In tissues of ccRCC patients, RT-PCR analysis showed that the relative expression of miR-212-5p was significantly decreased (n=17). B. In the three ccRCC cell lines, the relative expression of miR-212-5p was also significantly decreased when compared to HEK-293 cells. C. The relative expression of HSF1 mRNA was significantly elevated in ccRCC patient tissues (n=17). D. The relative expression of HSF1 mRNA was significantly higher in ccRCC cell lines than in HEK-293 cells. *P <0.05, **P <0.01, ***P <0.001.

**Figure 1.** miR-212-5p was up-regulated, while HSF1 was down-regulated, in ccRCC patient tissues and human ccRCC cell lines. A. In tissues of ccRCC patients, RT-PCR analysis showed that the relative expression of miR-212-5p was significantly decreased (n=17). B. In the three ccRCC cell lines, the relative expression of miR-212-5p was also significantly decreased when compared to HEK-293 cells. C. The relative expression of HSF1 mRNA was significantly elevated in ccRCC patient tissues (n=17). D. The relative expression of HSF1 mRNA was significantly higher in ccRCC cell lines than in HEK-293 cells. *P <0.05, **P <0.01, ***P <0.001.

GCT TCG AGC CCA C-3'; miR-212-5p reverse, 5'-GAC ACC ACG GCC CAC TCT GCA-3'; U6 forward, 5'-GCC TGC TCA GCA CTA AGC CGT ACT T-3'; U6 reverse, 5'-CGC ATC TGG CAC TCG CGT GAC AC-3'; HSF1 forward, 5'-ATC ACA CCT GCA TCA CGA AC-3'; HSF1 reverse, 5'-CGT CGT CTC CGA TGC CAG CC-3'; β-actin forward, GCT GTC CTA GTG TGC CTC T-3'; β-actin reverse, TGT CAC GCA CGA TTT CC-3'. Subsequently, 0.8 μL of miR-212-5p or HSF1 primers (Sangon Biotech, Shanghai, China), 10 μL of SYBR green (Takara, Dalian, China), 6.4 μL of DEPC water (Sangon, Shanghai, China), and 2 μL of the cDNA produced were mixed in a total volume of 20 μL for real-time PCR using the LighCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). The following thermal cycling conditions were used: 5 min at 95°C; 40 cycles of 1 sec at 95°C and 20 sec at 65°C. The comparative 2^ΔΔCt cycle threshold method was used for relative
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Figure 2. HSF1 was identified as a target of miR-212-5p using TargetScan software and the dual-luciferase assay system. A. The predicted consequential sequences of miR-212-5p and the 3’ UTR of HSF1 mRNA were found using TargetScan software. B. The sequences on the top are the wild-type, conjoint sequences of miR-212-5p and the 3’ UTR of HSF1 mRNA, while the bottom are the mutated conjoint sequences of miR-212-5p and the 3’ UTR of HSF1 mRNA. C. After the conjoint sequences of the WT 3’ UTR of HSF1 and the miR-212-5p mimic were transfected into HEK-293 cells, the luciferase activity was significantly down-regulated in comparison to the mutated and NC groups and there were no statistical differences between the mutated and control groups. D. The luciferase activity was lower following co-transfection of the miR-212-5p mimic and 3’ UTR of HSF1 into the HEK293 cells. After the miR-212-5p inhibitor and 3’ UTR of HSF1 were co-transfected into the HEK293 cells, the luciferase activity was notably promoted. E. F. HSF1 protein was significantly elevated following miR-212-5p inhibitor transfection into 786-0 cells while there was a declined after miR-212-5p mimic was transfected into the cells. G. Expression of HSF1 was significantly up-regulated or down-regulated after miR-212-5p inhibitor or mimic transfection, into 786-0 cells, respectively. *P < 0.05, **P < 0.01, ***P < 0.001.

quantification of gene expression. Each sample was analyzed at least three times.

Western blot analysis

Total protein was extracted from tissues and cells using RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with 100* proteinase inhibitor and phosphatase inhibitor, followed by separation of the proteins on 10% SDS-PAGE. Afterwards, the gel was transferred to a PVDF membrane (Merck Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk for 2 h at room temperature. The following primary antibodies were all rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA): anti-HSF1 (1:500 dilution), anti-p-AKT (ser473) (1:800 dilution), anti-Pi3K (p85) (1:500 dilution), anti-AKT (1:1000 dilution), and anti-β-actin (1:1000 dilution). The secondary antibody (Beyotime, Shanghai, China) was used at a 1:5000 dilution. Signal was visualized with an ECL kit (Invitrogen, Carlsbad, CA, USA). The gray bands were analyzed with Image J software (NIH, Bethesda, MD, USA) to compare the expression between targeted proteins and internal controls.

786-O cell proliferation assay

In order to detect the proliferation rate of 786-O cells transfected with miR-212-5p mimic, inhibitor, and its NC, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed using the Cell Proliferation Kit I (Sigma-Aldrich, St.Louis, MO, USA) in 96-well plates at a density of 4*10^3 per well, according to the manufacturer’s instructions. After 24, 48, and 72 h of incubation, MTT-formazan production was estimated by VersaMax (Molecular Devices, CA) at 570 nm to evaluate the proliferation rate. The index was determined at 48 and 72 h and normalized to that at 24 h. In addition, the transfected cells were photographed by microscopy (DP72, Olympus, Japan) at 100X magnification.

Statistical analysis

Data are presented as mean ± SEM. All statistical analyses were performed using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA). The differences between the two groups were compared using a Student’s t-test and three or more groups were compared with a one-way analysis of variance (ANOVA), followed by pairwise multiple comparisons to determine any difference between groups. Statistical significance was accepted at P<0.05.

Results

MiR-212-5p was significantly up-regulated, while HSF1 mRNA was down-regulated in tissues of ccRCC patients and ccRCC cell lines

Seventeen pairs of tumor and the adjacent normal tissues of ccRCC patients were collected from July 1 2015 to July 1 2016. The results of qRT-PCR analysis revealed that the relative expression of miR-212-5p significantly decreased in the tumor tissues compared with that in the normal tissues (Figure 1A). In addition, the miR-212-5p expression level also decreased in several of the human ccRCC cell lines including SN12-PM6, A498, and 786-O in comparison to that in the human embryonic kidney 293 cells (HEK-293) (Figure 1B). However, the relative expression of HSF1 mRNA was elevated in the tumor tissues (Figure 1C). Furthermore, HSF1 mRNA was also up-regulated in the three human ccRCC cell lines in com-
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Figure 3. miR-212-5p inhibited ccRCC proliferation and differentiation through the PI3K/AKT signaling pathway. A, B. After miR-212-5p mimic was transfected into 786-O cells, the number growing cells decreased in comparison to the negative control (NC). C, D. After miR-212-5p inhibitor was transfected into the 786-O cells, the number of cells increased in comparison to its NC. E. The MTT assay, revealed a significantly lower proliferation rate in the miR-212-5p mimic transfection group while a higher rate in the miR-212-5p inhibitor transfection group. F, G. In the miR-212-5p mimic-transfected 786-O cells, the expression of PI3K and p-AKT were significantly higher than that in its NC. However, the expression levels were lower after miR-212-5p inhibitor was transfected into the cells. In addition, there was no statistically significant difference in the expression of AKT following transfection with the miR-212-5p mimic or inhibitor. *P < 0.05, ***P < 0.001.

Figure 4. HSF1 plays an important role in the regulation of 786-O cell proliferation through the PI3K/AKT signaling pathway. A. The proliferation rate of 786-O cells was significantly decreased after HSF1 was inhibited while the result was reversed following overexpression of HSF1. B. The expression of miR-212-5p mRNA was statistically higher after HSF1 expression was suppressed while the expression of miR-212-5p was obviously decreased after HSF1 expression was elevated. C, D. The expression of PI3K and p-AKT were significantly decreased after HSF1 was inhibited, while the results were reversed after HSF1 was promoted. **P < 0.01, ***P < 0.001.
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Comparision with HEK-293 cells (Figure 1D), indicating that the expression of miR-212-5p and HSF1 mRNA was opposite in tumor tissues of ccRCC patients and ccRCC cell lines.

Figure 5. PI3K/AKT signaling pathway is intimately associated with the proliferation of 786-O cells. A, B. After the PI3K inhibitor, LY294002, was transfected into 786-O cells, the number of cells was reduced in comparison to its negative control (NC). C. The proliferation rate of 786-O cells was significantly decreased after LY294002 transfection into the cells when comparing to the NC. D, E. After PI3K was statistically suppressed, the p-AKT levels also decreased, but the expression of HSF1 was not statistically different.
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Combined dual-luciferase reporter genes with transfection experiments to confirm that miR-212-5p targets HSF1

In order to further verify the relationship between miR-212-5p and HSF1 in ccRCC, TargetScan software was used to identify HSF1 as one of the target genes of miR-212-5p. In addition, the conserved predicted consequential pairing of the HSF1 3' UTR of miR-212-5p was also confirmed (Figure 2A and 2B). Afterward, HEK-293 cells were transfected with conjoint sequences of HSF1 wild-type (WT) 3' UTR and miR-212-5p mimic, and it was found that the luciferase activity was significantly down-regulated in comparison with the mutated and control groups (Figure 2C). There was no statistical significance between the mutated and control groups (Figure 2C). In addition, the luciferase activity was promoted after the conjoint sequences of miR-212-5p inhibitor and 3' UTR of HSF1 mRNA were transfected into the HEK-293 cells. However, the results were the opposite after the cells were transfected with miR-212-5p mimic (Figure 2D). Furthermore, after 786-O cells were transfected with the miR-212-5p mimic which up-regulated the expression of miR-212-5p, HSF1 protein levels were significantly down-regulated in comparison with NC, while the result was reversed following use of a miR-212-5p inhibitor which lead to down-regulation of miR-212-5p expression (Figure 2E and 2F). In addition, HSF1 mRNA levels were inversely regulated by miR-212-5p (Figure 2G).

MiR-212-5p inhibits 786-O cell proliferation through the PI3K/AKT signaling pathway

In cultured 786-O cells transfected with miR-212-5p mimic, it was found that the number of cells clearly decreased compared with its NC (Figure 3A and 3B). However, after transfection with the miR-212-5p inhibitor, cell proliferation significantly increased in comparison with its NC (Figure 3C and 3D). Furthermore, the proliferation rate was detected with a cell proliferation kit. The results of MTT assay revealed that the proliferation rate was significantly decreased in the miR-212-5p mimic transfection group, but was notably elevated in the miR-212-5p inhibitor group (Figure 3E). In addition, total protein was extracted and the results of western blot analysis revealed that the expression of PI3K and p-AKT was significantly down-regulated in the miR-212-5p mimic group, but was up-regulated in the miR-212-5p inhibitor group. Image J analysis revealed that the expression of PI3K and p-AKT in the miR-212-5p mimic NC group was 1.7-fold more than that in the miR-212-5p mimic group. In addition, the expression of PI3K in the miR-212-5p inhibitor group was 1.2-fold more than in the miR-212-5p inhibitor NC group (Figure 3F and 3G).

Expression of HSF1 regulates 786-O cell proliferation via the PI3K/AKT signaling pathway

In order to confirm that HSF1 indeed plays an essential role in proliferation of ccRCC, the expression of HSF1 was suppressed by HSF1 siRNA. In addition, HSF1 overexpression was also constructed by the pCDH-EF1-HSF1-copGFP vector. The results of RT-PCR and western blot analyses revealed that the proliferation rate of 786-O cells decreased in the HSF1 siRNA group in comparison with its NC group (Figure 4A). However, the proliferation rate was significantly elevated in the pCDH-EF1-HSF1-copGFP group (Figure 4A). At the mRNA level, miR-212-5p was otherwise elevated after the HSF1 expression level decreased, while miR-212-5p was decreased after HSF1 was promoted (Figure 4B). It was illustrated that HSF1 could not regulate miR-212-5p expression. Furthermore, the expression of PI3K and p-AKT were statistically down-regulated in the HSF1 siRNA group but was significantly up-regulated in the pCDH-EF1-HSF1-copGFP group (Figure 4C and 4D). In addition, PI3K and p-AKT expression levels were up-regulated when miR-212-5p was decreased with the use of the miR-212-5p mimic, while the expression was down-regulated with the miR-212-5p inhibitor (Figure 4C and 4D).

PI3K/AKT pathway was involved in proliferation of 786-O cells

In order to further identify that PI3K/AKT signaling pathway was associated with proliferation of 786-O cells, the expression of PI3K was suppressed after the PI3K inhibitor, LY294002, was transfected into the cells. The results of MTT assay revealed that the proliferation rate of 786-O cells was shown to be statistically lower in the LY294002 group than in NC group (Figure 5A-C). The expression of PI3K decreased nearly 40% and p-AKT decreased about 31%, but the HSF1 expression was not
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Our results showed that the expression of miR-212-5p and HSF1 was opposite in ccRCC, which confirmed that HSF1 was one of the target genes of miR-212-5p. After miR-212-5p mimic was transfected into the 786-O cells, the expression of the PI3K pathway protein p-AKT significantly decreased. Thus, the PI3K/AKT signaling pathway has a positive effect on the proliferation of 786-O cells. After 786-O cells were transfected with the miR-212-5p mimic, the expression of HSF1 and the proliferation rate of the cells were both significantly down-regulated, but they both were up-regulated in the miR-212-5p inhibitor group. The results demonstrated that miR-212-5p could regulate ccRCC growth and differentiation by targeting HSF1. In addition, the expression of PI3K/AKT notably decreased in the miR-212-5p mimic group, but not in the miR-212-5p inhibitor group, indicating that PI3K/AKT signaling is involved in the proliferation and differentiation of ccRCC. Furthermore, the proliferation rate and PI3K/AKT signaling in 786-O cells were significantly down-regulated after the expression of HSF1 was inhibited, but they were up-regulated after HSF1 was overexpressed. However, the expression of miR-212-5p was otherwise promoted as the expression of HSF1 was suppressed, but was decreased when HSF1 was constitutively expressed. These results demonstrated that HSF1 is upstream of PI3K/AKT signaling pathway and downstream of miR-212-5p when studying the expression and proliferation of 786-O cells.

In conclusion, in combination with previous studies, data suggests that miR-212-5p suppresses ccRCC proliferation and differentiation by targeting HSF1 through the PI3K/AKT signaling pathway. Furthermore, miR-212-5p could be a therapeutic target in the treatment of ccRCC. However, future studies are required in order to confirm the exact mechanism of action.

Disclosure of conflict of interest

None.

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Statistically different between the LY294-002 group and its NC group (Figure 5D and 5E). The data suggested that the PI3K/AKT signaling pathway was intimately associated with proliferation of ccRCC cells and it could not regulate HSF1 expression in the opposite direction.

Discussion

Though many research studies have been devoted to exploring the exact molecular mechanisms between miRNAs and RCC, the relationship between miR-212-5p and ccRCC has not been determined. ccRCC represents a predominant subtype of RCC and constitutes approximately 75-80% of all cases [13]. In this study, 17 pairs of ccRCC patients’ tumor and the adjacent normal tissues were examined. qRT-PCR analysis revealed that the relative expression of miR-212-5p was significantly down-regulated in tumor tissues. However, the expression of HSF1 was significantly up-regulated in tumor tissues. In addition, miR-212-5p expression was elevated, while HSF1 were closely associated with ccRCC, and their interactions may influence proliferation and differentiation of ccRCC.

The HSF1-mediated stress response has been implicated in protecting organisms from a broad range of pathophysiological conditions, including thermal injury, ischemia/reperfusion, and age-related neurodegeneration [14, 15]. In recent studies, HSF1 has led a dominant role in the development and progression of tumors [16-19]. HSF1 has been regarded as a therapeutic target in the treatment of hepatocellular carcinoma [20]. The increased expression of HSF1 in stromal cells was significantly associated with poor prognosis of esophageal squamous cell carcinoma [21]. The regulation of β-catenin expression by HSF1 may be significant in mammary carcinogenesis [22]. SN12-PM6, 786-O, and A498 cell lines are considered to be ccRCC cell lines in many publications [23]. Therefore, in this manuscript, they were regarded as ccRCC cell lines. In addition, HEK 293 cells, also referred to as 293 cells, or less precisely as HEK cells, are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. In this study, HEK-293 cells were used as a negative control.
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References


