RESEARCH COMMUNICATION

MiR-138 Suppresses Expression of Hypoxia-inducible factor 1α (HIF-1α) in Clear Cell Renal Cell Carcinoma 786-O Cells

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Abstract

Objectives: Hypoxia-inducible factor-1alpha (HIF-1a) is widely considered to be one of the key regulators in cancer cells. Here, we investigated a microRNA regulating expression of HIF-1a and explored its functions in clear cell renal cell carcinoma (ccRCC) cells. Methods and materials:Western blot and reporter assays were used to assess HIF-1a as a direct target of miR-138. The effects of miR-138 or si-HIF-1a on ccRCC 786-O cells were also estimated by apoptosis analysis and cell migration assay. Results: The data showed HIF-1a to be one target of miR-138. Futhermore, inhibition of the expression of HIF-1a with specific siRNA or miR-138 could increase apoptosis and reduce the migration of 786-O cells. Conclusions: miR-138 could inhibit the expression of HIF-1a and regulate the apoptosis and migration of ccRCC cells.

Keywords: miR-138 - suppressive influence - HIF-1a - ccRCC - apoptosis - migration

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Introduction

Kidney cancer, like most solid malignancies, is nearly always fatal when a patient develops advanced disease. Renal cell carcinoma(RCC) is the most common type of kidney cancer in adults, responsible for approximately 80% of cases (Mulders et al., 2008) and it has been reported that the advanced RCC is very poor with a 5-year survival rate of 5%–10% (Hadoux et al., 2010). RCC is represented by 80% by clear cell RCC (ccRCC), originating from the renal proximal tubule (Dormoy et al., 2009).I nitial treatment is most commonly a radical or partial nephrectomy and remains the mainstay of curative treatment (Rini et al., 2008). The most effective therapy for RCC localized to the kidney is surgery and a metastatic tumor is practically incurable. There is a low response to biological modifiers and the treatment is generally only palliative (Kovacs, 1996). It has been reported that 20% to 30% of the patients who undergo curative surgery will develop metastatic disease during follow-up (Zisman at al., 2002). It is resistant to radiation therapy and chemotherapy, although some cases respond to immunotherapy. Targeted cancer therapies such as sunitinib, temsirolimus, bevacizumab, interferon-alpha, and possibly sorafenib have improved the outlook for RCC (progression-free survival), although they have not yet demonstrated improved survival.

Hypoxia-inducible factor-1alpha (HIF-1a) is widely considered to be one of the key regulators of tumor angiogenesis, including ccRCC (Maxwell, et al., 1997; Semenza, 2003; Kaelin and Ratcliffe, 2008; Dormoy et al., 2009). In addition, the reduced HIF-1a levels was found to followed by suppressed tumor angiogenesis, growth, and metastasis (Cha et al., 2010). In renal cancer, HIF-1a is involved in the VHL-HIF signaling pathway and inhibits mitochondrial biogenesis and cellular respiration.

MicroRNA microRNAs (miRNAs) are a class of small non-coding RNAs in animals and plants. They can bind to the 3‘ untranslated regions (UTRs) of target mRNAs and regulate genes expression at posttranslational and translational levels (He and Hannon, 2004). Lots of evidence show that miRNAs play the important role in multiple cellular and biological process (He and Hannon, 2004). Deregulation of miRNAs is involved in many kinds of human diseases, including cancer (Garzon et al., 2006).

Here, we found that miR-138 could negatively regulate HIF-1a in ccRCC 786-O cell. Furthermaor, the proliferation and mobility of ccRCC 786-O cell were decreased after treated by miR-138 or si-HIF-1a. Our results indicate HIF-1a was one of targets of miR-138 and miR-138 could increase the apoptosis and reduce the migration of 786-O cells via down-regulating the expression level of HIF-1a. Our findings will help to further understand the functions of miRNAs in cancer and suggest that miR-138 may be employed as therapeutic for ccRCC.

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Materials and Methods

Cell Lines

The 786-O cell line was propagated in Dulbecco's Modifed Eagle Medium (DMEM) (Gibco). The medium was supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37°C in 5% CO2.

Transfection

The mimic microRNAs, interfering RNA complex (si-HIF) and negative control RNA duplex (denoted NC) were synthesized by Genepharma (Shanghai,China). Cells were transfected using Lipofetamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 786-O cells were plated to 6-well plate to 60% confluence. Mixture containing the miRNA, siRNA or NC and lipofectamine 2000 were prepared according to the recommended protocol and added directly to cells at a final oligonucleotide concentration of 60 nM. Cells were harvested 72 h after transfection. Total RNAs and proteins were extracted for qRT-PCR or western blot analysis.

Quantitative reverse transcription PCR

Total RNAs was extracted from cells using Trizol (Invitrogen) according to the manufacturer's protocol. For cDNA synthesis ,1 μg of RNA was mixed with 500 ng of olig (dT) (Promega) or microRNA specific primers (invitrogen). Samples were incubated at 65°C for 10 min with 5 μl of 5×first-strand buffer, 2 μl of 5 mM dNTP, 20 U of RNasin (Takara), 1 μl of M-MLV reverse transcriptase (Promega) and distilled water to a total volume of 25 μl. The qPCR reaction mixture contained 12.5 μl of 2×SYBR green PCR mix (Fermetas), 0.3 μM of gene-specific forward and reverse primers, and 1 μl of cDNA template, made up to a final volume of 25 μl with distilled water. Cycling parameters were set as follows: initial activation step at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s. Melting curve analysis was performed at from 58°C to 95°C with stepwise fluorescence acquisition at every 1°C s-1. The levels of gene expression were calculated by relative quantification using GAPDH or U6 snRNA as the endogenous reference genes. All samples were amplified in triplicate and the data analysis was carried out using the MxPro qPCR system software (Stratagene). Western blotting analysis

The 786-O cells were harvested and washed twice with PBS. The cell pellets were lysed in RIPA Lysis Buffer (50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% Sodium deoxycholate) for 30 min on ice. Lysates were centrifuged (12,000 g, 20 min, 4°C). Protein at the same amount were separated by 12% SDS polyacrylamide gel electrophoresis and transferred electrophoretically to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Membranes were blocked for 1 h at 37°C with 5% non-fat dry milk, then were probed with mouse anti-HIF-1a (at 1:500 dilution, biolegend), GAPDH (at 1:400 dilution, santa cruz) in 5% non-fat dry milk for 1 h at 37°C. After washing in PBS with 0.5% Tween 20 (PBST), the membrane was incubated in a 1:5,000 solution of HRP-conjugated goat anti-mouse secondary antibody at room temperature for 1 h. After further washing with PBST, the membrane was assayed by the enhanced chemiluminescence (ECL) Western blotting detection system.

Vector Construction and Luciferase Reporter Assay

To create a luciferase reporter construct, 3'UTR fragment of HIF-1a containing putative binding sites for miR-138 was inserted downstream of firefly luciferase in pGL3. Mutant 3'UTR, which carried the mutated sequence in the complementary site for miR-138, was generated using the fusion PCR method inserted downstream of firefly luciferase in pGL3. The 786-O cells grown in a 48-well plate were cotransfected miR-138 with luciferase reporter comprising wildtype or mutant 3' UTR of target gene. The luciferase assay was performed
as reported 786-O cells were cotransfected with miRNAs and 3'UTR or mutant 3'UTR luciferase reporter, using pRL-TK as control vector. At 48 h after transfection, Luciferase activity was measured using the Dual-Luciferase Assay kit (Promega) with a beta-counter luminometer. Relative luciferase activity was calculated as ratio of the raw firefly luciferase activity and the renilla luciferase activity.

Apoptosis analysis

Cells were transfected and cultured in DMEM containing 10% FBS. Prior to harvesting, the cells were washed twice with PBS, trypsinized, and pelleted. Then cells were resuspended at a concentration of 1×106 cells/ml in Binding Buffer (0.01 M HEPES/NaOH, pH 7.4, 14 mM NaCl, 0.25 mM CaCl2). Cells (500 µl) were added into FACS tubes and mixed with 25 ng/ml fluorescein isothiocyanate–labeled annexin V and 10 mg/ml propidium iodide (PI) to incubation for 15 min at room temperature in the dark. Then the cells were analyzed immediately by flow cytometry.

Cell migration assay

Cells were transfected and cultured for 24 h in DMEM containing 0.1% FBS. And then 1 X 105 cells were harvested and added to upper chamber (8 µm pore size polycarbonate membrane, Corning) of 24-well plate in serum free medium (300 µl). After incubated for 24 h at 37°C in 5% CO2, invasive cells on lower surface of the membrane were stained with 0.1% violet staining solution for 30 min, and counted by photographing the membrane through the microscope (× 100 magnifications).

Statistical analysis

All quantitative data were analyzed using Student t-tests. All tests performed were two-sided. $P<0.05$ was considered to be statistically significant.

Results

BmiR-138 directly inhibits HIF-1a through it 3'UTR
In order to find the miRNAs which regulate the expression of HIF-1a, predicted regulating miRNAs of HIF-1a were retrieved using publicly available databases (TargetScan).

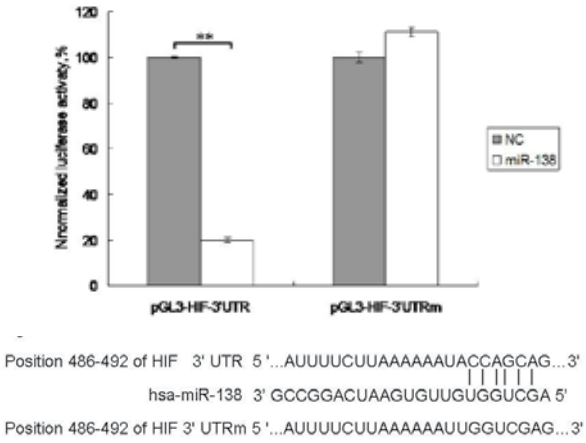

Figure 1. miR-138 could significantly suppress the luciferase activities of HIF-1a 3'UTR-luciferase report gene. A: A luciferase expression vector with the 3'-UTR of HIF-1a was transfected into 786-O cells with miR-138. The luciferase activity of the mutant HIF-1a 3'UTR report genes were not regulated by miR-138. **:P<0.01 B) Predicted duplex formation between miR-138 and HIF-1a 3' UTR. HIF-1a 3'UTR mutant is showed in lower panel

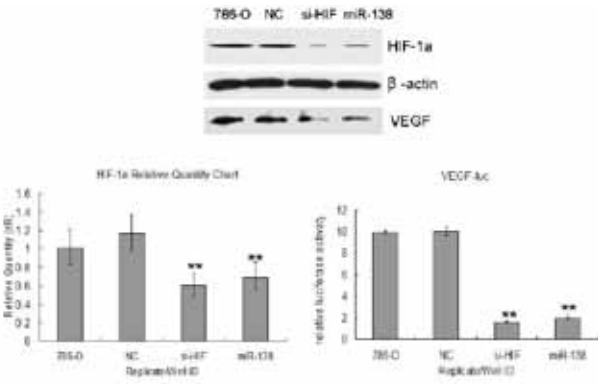

Figure 2. miR-138 Decreases the Level of HIF-1a. (upper panel) Detection the level of HIF-1a and VEGF protein by Western blot. miR-138, si-HIF or NC was transfected into 786-O cells and total proteins were detected by Western blot with β-actin as an internal control (lower panel,left) Relative expression levels of HIF-1a mRNA in786-O cells after transient transfection with si-HIF or miR-138 for 72 h. β-actin served as internal controls. (lower panel,right) The VEGF-luc reporter plasmid was constructed and cotransfected to 786-O cells with miR-138 or si-HIF-1a. The VEGF-luc activity was strongly inhibited after the cells transfected with miR-138 or si-HIF-1a** P<0.01

MiR-138 was chosen for further analysis. To verify whether HIF-1a is direct targets of miR-138, a dual-luciferase reporter system was first employed. The 3'UTR of HIF-1a was inserted downstream of the luciferase gene and transfected into 786-O cells together with miRNAs or negative-control(NC) and pRL-TK to normalize transfection. The result showed that miR-138 could down-regulate the luciferase activity of the reporter (Figure 1A). In order to further proved its reliability, mutants of HIF-1a 3'UTR was constructed by deleting the miR-138 targets site (Figure 1B) and cotransfected into 786-O cells together with miR-138. The luciferase expression of mutant UTR of HIF-1a was no longer subject to be regulated by miR-138 (Figure 1A). This suggested that this site in the 3`UTR of HIF-1a was exact regulation site of miR-138.

miR-138 inhibits the expression of HIF-1a protein

The effect of miR-138 on endogenous expressions of HIF-1a was subsequently examined in 786-O cells. To verify whether HIF-1a protein expression was indeed regulated by miR-138. miR-138, or si-HIF (siRNA targeting to HIF-1a) was transfected into 786-O cell. The western blot result showed that the level of expression of HIF-1a protein of 786-O cell was significant reduced after treated with miR-138 or si-HIF (Figure 2). This results further confirmed that the HIF-1a protein was one of targets of miR-138 and miR-138 could directly modulate the expression level of HIF-1a. To further verify the conclusion, the VEGF-luc reporter plasmid was constructed and cotransfected to 786-O cells with miR-138 or si-HIF-1a. We found that the VEGF-luc activity was strongly inhibited after the cells transfected with miR-138 or si-HIF-1a (Figure 2). In the same time, the level of VEGF was detected by western blot. The result showed that it was decreased significantly after the cells were transfected with miR-138 or si-HIF-1a (Figure 2). The results indicated that the alterations of 786-O cells were due to miR-138 through HIF-1a pathway.

miR-138 could induce cell apoptosis and suppresses migration of 786-O cells by decreasing the level of HIF-1a To examine the potential role of miR-138 in tumorigenesis, we first evaluated the effect of miR-138 on the apoptosis and migration of ccRCC cells. The 786-O cells was transfected with NC, miR-138 or si-HIF, and cells were harvested at 72 h after transfection. Then the apoptosis of cells was detected by flow cytometry. We found that silencing of HIF-1a with si-HIF increased the apoptosis of 786-O cells and the cells show the same phenotype after transfected by miR-138(Figure 3A). Then the 786-

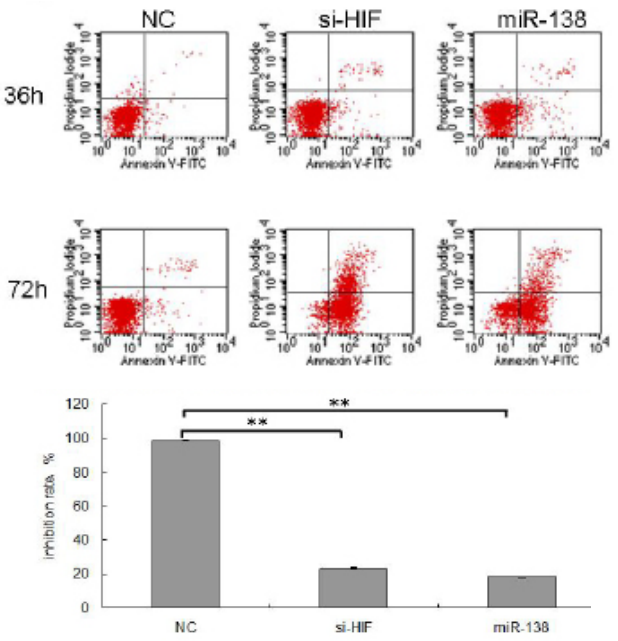

Figure 3. miR-138 Reduces Apoptosis and Suppresses Migration of 786-O Cell by means of HIF-1a. A) miR-138 and si-HIF could increase the apopotosis of 786-O cells B) Number of cells invaded at 24 h time point. Values represent the mean ± SD of three independent experiments (**P <0.01).

O cell migration was measured by wound healing assay and transwell chemotaxis assay. In wound healing assay, the cells were observed at 0 h, 24 h and 48 h respectively after transfection with miR-138 or si-HIF. The result showed that the cellular motility was obviously inhibited after treated with miR-138 or si-HIF compared with NC groups. We further analyzed the effect of miR-138 on cell migration in the further transwell assay and found that the number of cells migrating across the membranes in miR-138 or si-HIF groups decreased dramatically compared with the controls (Figure 3B). The above findings were further confirmed that miR-138 significantly inhibits tumorigenicity by decreasing the level of HIF-1a.

Discussion

MiRNAs are post-transcriptional regulators that bind to the 3‘ untranslated regions (UTRs) of target mRNAs, usually resulting in translational repression and gene silencing (Calin et al., 2004). MicroRNA expression correlates with various cancers. A recent study showed that approximately 50% of annotated human miRNAs were associated with cancer (DeVere et al., 2009). Recent investigations have revealed that miRNAs have unique expression profiles in different cancer types at different stages and play an important role in the initiation and progression of many diseases (Li et al., 2009).These sugguest that miRNAs might have a crucial function in cancer occurrence and development. These features make miRNAs ideal candidates for use as both biomarkers and therapeutic. Many miRNAs are reported to be involved in the process of tumorigenesis and tumor metastasis. However, the mechanism by which miRNAs regulate cancer cells still unclear (Negrini et al., 2009). MiR-138, a highly conserved miRNA, was found involved in tongue, stomach, colon, pancreas and thyroid ca cancer (Mitomo et al., 2008). Down-regulation of microRNA-138 has been frequently observed in several cancers (Wong et al., 2008; Liu et al., 2009; Jiang,et al., 2010). And it acts as a tumor suppresser to suppress invasion and promote apoptosis of cancer cells (Semenza,1999; Wong et al., 2008). In this case, we have identified that HIF-1a is one of target of miR-138. In 786-O cells, miR-138 can negatively regulate HIF-1a expression. HIF-1a is involved in the expression of genes involved in cell growth and differentiation (Wiesener et al., 2001). Upregulation of both HIF-1α and HIF-2α has been identified in ccRCCs (Zhang et al., 2006; Bartel, 2009). The metastasis and invasive growth is the transition of tumour cells from an epithelial to a mesenchymal morphology (Dasgupta et al., 2009). Our data show that miR-138 can induce the apototosis and decrease migration of 786-O cells. In addition, the role of miR-138 in the apototosis and migration of the cancer cells was due to its involvement in HIF-1a pathway.

In a word, our findings support that miR-138 can inhibit the expression of HIF-1a and regulate the apopotosis and migration of ccRCC. Our findings will help to further understand the functions of miRNAs in cancer cells. And miR-138 may be employed as therapeutic for ccRCC. The results might provide insight for the development of novel tumor markers or new therapeutic strategies.

Acknowledgements

The authors declare that there is no conflict of interest with this work.

References

Bartel DP (2009). MicroRNAs: target recognition and regulatory functions. *Cell*, **136**, 215-33.

Calin GA, Sevignani C, Dumitru CD, et al (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* , **101**, 2999-3004.

Cha ST, Chen PS, Johansson G, et al(2010). Suppresses hypoxia-inducible factor-1alpha expression and tumor angiogenesis. *Cancer Res* , **70**, 2675-85.

Dasgupta P, Rizwani W, Pillai S, et al(2009). Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines. *Int J Cancer*, **124**, 36-45.

DeVere White RW, Vinall RL, Tepper CG, et al(2009). MicroRNAs and their Potential for Translation in Prostate Cancer. *Urol Oncol*, **27**, 307-11.

Dormoy V, Danilin S, Lindner V, et al(2009). The sonic hedgehog signaling pathway is reactivated in human renal cell carcinoma and plays orchestral role in tumor growth. *Mol Cancer*, **8**,123.

Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM(2006). MicroRNA expression and function in cancer. *Trends Mol Med*, **12**, 580-7.

Hadoux J, Vignot S, De La Motte Rouge T(2010). Renal Cell Carcinoma: Focus on Safety and Efficacy of Temsirolimus. *Clin Med Insights Oncol*, **4**,143-54.

He L, Hannon GJ(2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*, **5**, 522-31.

Jiang L, Liu X, Kolokythas A, et al (2010). Downregulation of the Rho GTPase signaling pathway is involved in the microRNA-138-mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. *Int J Cancer*, **127**, 505-12.

Kaelin WG Jr, Ratcliffe PJ(2008). Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. *Mol Cell*, **30**, 393–402.

Kovacs G(1996). Molecular genetics of human renal cell tumors. *Nephrol Dial Transplant*, **11**, 62–65.

Li S, Fu H, Wang Y, et al(2009).MicroRNA-101 Regulates Expression of the v-fos FBJ Murine Osteosarcoma Viral Oncogene Homolog (FOS) Oncogene in Human Hepatocellular Carcinoma. *Hepatology*, **49**, 1194-202.

Liu X, Jiang L, Wang A, et al(2009). MicroRNA-138 suppresses invasion and promotes apoptosis in head and neck squamous cell carcinoma cell lines. *Cancer Lett*, **286**, 217-22.

Maxwell PH, Dachs GU, Gleadle JM, et al(1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA*, **94**, 8104-9.

Mitomo S, Maesawa C, Ogasawara S, et al (2008).Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. *Cancer Sci*, **99**, 280-6.

Mulders PF, Brouwers AH, Hulsbergen-van der Kaa CA, et al(2008). Guideline ‘Renal cell carcinoma’. *Ned Tijdschr Geneeskd*,**152**,376–80.

Negrini M, Nicoloso MS, Calin GA(2009). MicroRNAs and cancer—new paradigms in molecular oncology. *Curr Opin Cell Biol*, **21**, 470-9.

Rini BI, Rathmell WK, Godley P(2008). Renal cell carcinoma.

Curr Opin Oncol, **20**, 300–6.
Semenza GL(1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol*,**15**,551–578.
Semenza GL(2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3**, 721–732.
Wiesener MS, Munchenhagen PM, Berger I, et al(2001). Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1alpha in clear cell renal carcinomas. *Cancer Res*, **61**, 5215–5222.
Wong TS, Liu XB, Wong BY, et al(2008). Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue. *Clin Cancer Res*,**14**, 2588–2592.
Zhang N, Gong K, Yang XY, et al (2006). Expression of hypoxia-inducible factor-1-alpha, hypoxia-inducible factor-2alpha and vascular endothelial growth factor in sporadic clear cell renal cell renal cell carcinoma and their significance in the pathogenesis thereof. *Zhonghua Yi Xue Za Zhi*, **86**,1526–1529.
Zisman A, Pantuck AJ, Wieder J, et al(2002). Risk group assessment and clinical outcome algorithm to predict the natural history of patients with surgically resected renal cell carcinoma. *J Clin Oncol*, **20**, 4559–66.