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-1α) is widely considered to be one of the key regulators in cancer cells. Here, we investigated a microRNA regulating expression of HIF-1α 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-1α as a direct target of miR-138. The effects of miR-138 or si-HIF-1α on ccRCC 786-O cells were also estimated by apoptosis analysis and cell migration assay. **Results:** The data showed HIF-1α to be one target of miR-138. Furthermore, inhibition of the expression of HIF-1α 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-1α and regulate the apoptosis and migration of ccRCC cells.

**Keywords:** miR-138 - suppressive influence - HIF-1α - ccRCC - apoptosis - migration
Materials and Methods

Cell Lines

The 786-O cell line was propagated in Dulbecco’s Modified 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 lipofetamine 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 oligo (dT) (Promega) or microRNA specific primers (invitrogen). Samples were incubated at 65°C for 10 min with 5 µl of 5x 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 2x SYBR green PCR mix (Fermentas), 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, biologend), 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 1x106 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).
MiR-138 was chosen for further analysis. To verify whether HIF-1α is a direct target of miR-138, a dual-luciferase reporter system was first employed. The 3′-UTR of HIF-1α 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 downregulate the luciferase activity of the reporter (Figure 1A). In order to further prove its reliability, mutants of the HIF-1α 3′-UTR was constructed by deleting the miR-138 target site (Figure 1B) and cotransfected with miR-138 or NC. The result showed that the luciferase activity of the mutant HIF-1α 3′-UTR was not regulated by miR-138. This suggested that the 3′UTR of HIF-1α was an exact regulation site of miR-138.

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

To examine the potential role of miR-138 in tumorigenesis, we first evaluated the effect of miR-138 on 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. The result showed that silencing of HIF-1α with si-HIF increased the apoptosis of 786-O cells and the cells show the same phenotype after transfected with miR-138 (Figure 3A). This results indicated that the alterations of 786-O cells were due to miR-138 through HIF-1α pathway.
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 suggest 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 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 suppressor 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 (Zhao 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 apoptosis and decrease migration of 786-O cells. In addition, the role of miR-138 in the apoptosis 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 apoptosis 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

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