# Hypoxia-induced hsa-miR-101 promotes glycolysis by targeting TIGAR mRNA in clear cell renal cell carcinoma

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**Abstract.** Increasing evidence suggests that microRNAs (miRNAs) are essential in carcinogenesis, therefore, the present study investigated the role of hsa-miR-101 in renal tumorigenesis and cancer development. On identification of its expression pattern, it may serve as a diagnostic biomarker for clear cell renal cell carcinoma (ccRCC). In the present study, 10 pairs of ccRCC and noncancerous tissue samples were obtained to examine whether the expression of hsa-miR-101 is linked to cancer. The data obtained were validated using reverse transcription-quantitative polymerase chain reaction analysis. The levels of hsa-miR-101 were examined following exposure to hypoxia in ACHN and HK-2 cells. As a predicted target, the mRNA and protein levels of TP53-induced glycolysis and apoptosis regulator (TIGAR) were then assessed. A pcDNA-GFP-miR-101 plasmid was stably transfected into ACHN and HK-2 cells, following which the effects of hsa-miR-101 on the expression of TIGAR and inhibition of glycolysis were investigated. The present study also examined the association between the level of hsa-miR-101 and kidney tumors. It was identified that the expression level was significantly higher in the ccRCC tissues, compared with that in the corresponding noncancerous tissues. The expression values for the upregulated miRNA ranged between 4.6- and 67.9-fold. On demonstrating the functional link between hypoxia and the expression of miRNAs changes in the expression of hsa-miR-101 were examined following hypoxia exposure in kidney tumor and non-tumor cell lines. It was shown that hypoxia exposure significantly induced hsa-miR-101. The hypoxia-induced upregulation of hsa-miR-101 repressed the activity of TIGAR by targeting TIGAR mRNA and promoting glycolysis. The results showed that the upregulation of hsa-miR-101 in ccRCC was induced by hypoxia. Its expression deceased the protein expression of TIGAR and promoted glycolysis. This regulatory pathway may represent a novel mechanism of carcinogenesis and requires further investigation.

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*Key words:* clear cell renal cell carcinoma, hsa-miR-101, TP53-induced glycolysis and apoptosis regulator, glycolysis, hypoxia

## Introduction

Renal cell carcinoma (RCC) is a common urologic malignancy, which accounts for 2-3% of adult malignancies, and clear cell RCC (ccRCC) is the most frequent of all types of RCC, which accounts for 85-95% of cases (1). The mortality rate of ccRCC is >90,000/year worldwide, and particularly high in China. Histological subtype detection shows ccRCC represents ~75-80% of all cases of RCC (1). Over the past decade, the incidence of ccRCC has increased significantly with an mortality rate of ~40% (2). In previous years, studies investigating the biological mechanisms, which cause ccRCC carcinogenesis focussed on genomic mutations, expression of protein-coding genes and epigenetic changes (3). However, there is increasing evidence suggesting that the microRNAs (miRNAs) are closely associated with cancer, and that the changes in their expression profiles may be considered as biomarkers for early detection, accounting for early diagnosis. Therefore, an improved understanding of miRNAs associated with the progression of ccRCC may lead to more accurate prognosis and therapeutic strategies (4).

miRNAs are a class of small noncoding RNAs, which have been shown to regulate gene expression by binding through partial sequence homology to the 3' untranslated region (3' UTR), causing target mRNA degradation or translational inhibition (5). The majority of miRNAs are transcribed as long monocistronic, bicistronic or polycistronic primary transcription units (pri-miRNAs) by RNA polymerase II and, following a series of cellular processing events, mature miRNAs are synthesized (6). The mature miRNAs are incorporated into the RNA-induced silencing complex and drives the selection of mRNAs of interest containing antisense sequences matching those of the miRNAs (7). As a result of their gene expression-associated activity, miRNAs have been identified as key regulators of several biological processes, including development, differentiation, apoptosis, proliferation and carcinogenesis (8-10). miRNAs are found aberrantly expressed or mutated in cancer, suggesting their role as a novel class of factors involved in promoting or inhibiting carcinogenesis (11,12). In

several types of cancer, miRNAs show significant differences, including prostate, lung, breast, colon and kidney malignancies (13), and evidence supports the promising use of miRNAs for diagnostic, prognostic and predictive purposes (14).

Hypoxia is an essential feature of the neoplastic microenvironment. The oxygen concentration in tumor tissue is substantially lower, compared with that in the surrounding non-tumor tissue. For example, in solid tumors of the breast, the $O_2$ pressure is ~10 mmHg, whereas normal breast tissue has an $O_2$ pressure of >60 mmHg (15). The regulation of hypoxia in cells occurs through affecting the expression of certain genes, which are sensitive to $O_2$ concentration. The molecular mechanisms responsible for the hypoxic survival of cancer cells remains to be fully elucidated, and additional information on this process may lead to novel strategies for pharmacological intervention.

In order to determine the possible role of hsa-miR-101 in hypoxic gene regulation and to examine this hypoxically-regulated miRNA, which may promote glycolysis by inhibiting TP53-induced glycolysis and apoptosis regulator (TIGAR), the present study examined changes in miRNA expression levels in response to hypoxia. The results obtained characterized in detail the hypoxic regulation of hsa-miR-101 and the mechanism underlying its regulation of TIGAR and promotion of glycolysis. The data also revealed the association between the level of hsa-miR-101 and the pathogenesis and prognosis of ccRCC.

## Materials and methods

*Patients and tissue samples*. In total, 15 pairs of ccRCC tumor tissue and normal tissue samples were collected between July 2012 and May 2013 at the department of Urological Surgery, First People's Hospital of Beijing (Beijing, China). All patients who donated tissue were diagnosed with ccRCC via post-operative pathology. The current study was approved by the Ethics Committee of Xutong Hospital (Xutong, China).

Of the 15 tissue samples from the patients with ccRCC, seven were from men and eight were from women, with a mean age of 58 years (range, 36-71 years). Of these patients, nine were in clinical stage I and six were in stage II.

*Cell culture and induction of hypoxia*. In the present study, the normal HK-2 epithelial cell line (frozen in the laboratory) and the ACHN ccRCC cell line were used, obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown in Eagle's minimum essential medium (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies; Thermo Fisher Scientific, Inc.) in a 5% $CO_2$ atmosphere at 37˚C according to standard procedures. Hypoxia exposure was established by changing the medium, which had been gassed to equilibrium with 0.1% $O_2$, decreasing $CO_2$ flow rates to 10 $cm^3$/sec in specifically designed chambers obtained from Oxold (Adelaide, Australia).

*Transfection*. To generate transiently-expressing constructs, the genomic region surrounding the pri-miRNA sequence of hsa-miR-101 was amplified using primers the following primers: Forward 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCAGTTAT-3' and reverse 5'-ACACTCCAGCTGGGTACAGTACTGTGATAA-3'. The thermocycling protocol was as follows: 95˚C for 5 min, then 30 cycles of amplification, each cycle consisted of 94˚C for 30 sec, 55˚C for 45 sec, and 72˚C for 1 min. This generated a polymerase chain reaction (PCR) product of ~500 bp, which was directionally cloned into pLNCX (Clontech Laboratories, Mountain View, CA, USA) using *Not*I and *Xho*I (Takara Bio, Inc., Shiga, Japan). The ligated construct was transformed into the DH5α *E. coli* strain (ATCC) chemically by preparing endotoxic-free plasmid using Maxipreps (Qiagen, Hildern, Germany), packed with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) and mixed with Phoenix packaging cells (Orbigen, Inc., San Diego, CA, USA).. Sequencing was performed by Qingkezixi (Chengdu, China) to confirm the insertion of the target sequence into the plasmid following the cytomegalovirus promoter. The supernatant was obtained following centrifugation at 800 x g for 10 min at 4˚C was collected and used to infect the target cells, which were incubated in a 5% $CO_2$ atmosphere at 37˚C, and these cells were harvested 48 h later, for further analysis.

*RNA extraction and cDNA synthesis*. Total RNA was extracted from the tissues by homogenizing 100 mg of the frozen tissue with a homogenizer (Thermo Fisher Scientific, Inc.), followed by isolation using the mir-VANA miRNA Isolation kit (Ambion, Austin, TX, USA). For reverse transcription (RT), 1 $\mu$g of the isolated miRNAs were incubated with RT mixture from Reverse-Transcriptional Fast kit (RiboBio Co., Ltd., Guangzhou, China) to simultaneously convert all small RNAs into detectable cDNAs for PCR analysis.

*TaqMan RT-qPCR and quantitative (q)PCR*. The method was optimized for miRNA, and all reagents, primers and probes were obtained from Applied Biosystems; Thermo Fisher Scientific, Inc. Human 28S rRNA was used to normalize all RNA samples. The RT reactions and qPCR analyses were performed according to the manufacturer's protocols. The previously prepared cDNA samples were run in duplicate in an Applied Biosystems 9700 thermocycler. The Taqman probe for hsa-miR-101 was 5'-CGGCGGTACAGTACTGTGATAA-3'; miR-93 5'-CGGCGGTGGAGTGTGACAATGG-3'. The thermocycling conditions were: 95˚ for 5 min, followed by 35 cycles at 95˚C for 10 sec, 55˚C for 10 sec and 72˚C for 1 min. The gene expression levels were quantified using the ABI Prism 7900HT sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Analysis was performed in duplicate, including controls containing no template. The relative expression was calculated using the comparative quantification cycle method (16).

Total RNA was extracted from the cells using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.), and a 1 $\mu$g from each cell line were used for RT (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequent qPCR was performed in the Bio-Rad CFX qPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. The protocol used was as follows: 98˚C 5 min, followed by 40 cycles of 98˚C for 10 sec and 60˚C for 1 min. The prediction of potential target for microRNAs was performed by using TargetScan (www.targetscan.org). The primers used

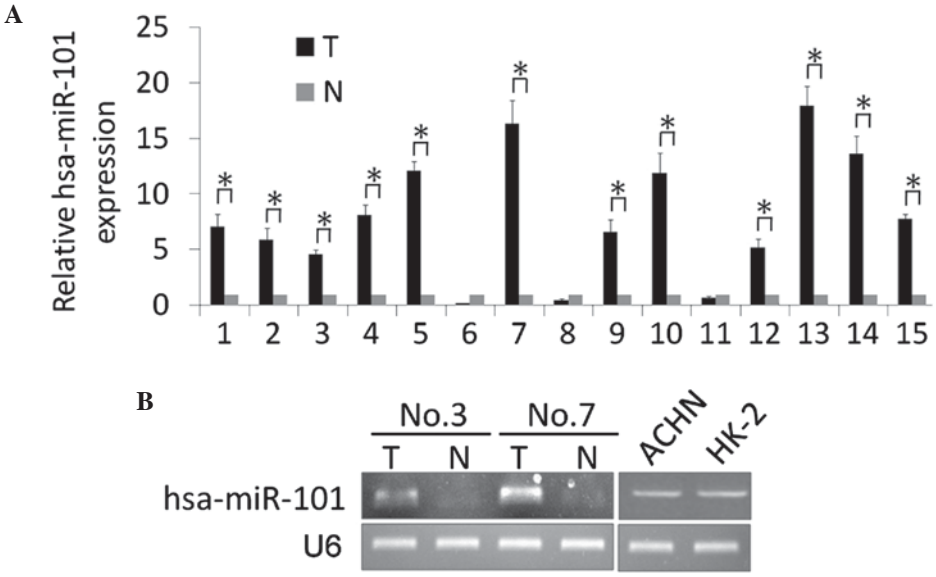


Figure 1. Detection of expression levels of hsa-miR-101 in ccRCC and adjacent tissue samples. (A) RT-qPCR analysis of the expression of hsa-miR-101 in 15 pairs of ccRCC and corresponding noncancerous tissue samples. (B) Conventional stem-loop semi-quantitative PCR analysis of the expression of hsa-miR-101 in ccRCC and corresponding noncancerous tissue samples, and ACHN and HK-2 kidney cell lines. U6 RNA was used as an internal control. All experiments were performed in triplicate. *P<0.01. ccRCC, clear cell renal cell carcinoma; T, tumor tissue; N, noncancerous tissue; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

for qPCR were as follows: TIGAR, forward 5'-GTGAGGACT ACGCAGCATCA-3' and reverse 5'-GCATCAGAACCGTGA TATATTCT-3'; β-actin, forward 5'-GCGCGTGCCTTCATC AC-3' and reverse 5'-TCTGCGCCATAAGGTGGTAG-3'.

*Semi-quantitative western blot analysis*. To prepare the total protein samples, the cells were trypsinized and homogenized on ice in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl and 100 mM KCl. The crude lysate was centrifuged at 20,000 x g for 45 min at 4˚C and ~200 $\mu$l of clear supernatant was collected. A bicinchoninic acid assay was performed to identify the total protein concentration of the collected supernatant. A final concentration of 2 $\mu$g/$\mu$l total protein was adjusted with the addition of $ddH_2O$ and 1X laemmli buffer and boiled at 98˚C for 10 min. To fractionate the proteins, 4-12% gradient SDS-PAGE gels were used. Immunoblotting was performed with specific antibodies, including mouse anti-TIGAR antibody (cat. no. ab64622; 1:1,000; Abcam, Cambridge, UK), anti-β-actin antibody (cat. no. ab8226; 1:1,000; Abcam, Cambridge, UK) at 4˚C overnight. The PVDF membranes were then incubated with goat anti-mouse horseradish peroxidase-labeled secondary antibody (cat. no ab97040; 1:5,000; Abcam, Cambridge, UK) incubated at room temperature.

*Cell proliferation assay*. Cell proliferation was detected using 3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The cells were seeded into 24-well plates ($1.2x10^4$ cells/well) and allowed to attach overnight in a 5% $CO_2$ atmosphere at 37˚C. After 24, 48, 72 and 96 h, cell viability was assessed using an MTT assay. The absorbance at 490 nM (A490) of each well was read on a spectrophotometer. Three independent experiments were performed in quadruplicate.

*Metabolite determination*. To measure the levels of fructose (Fru)-2,6-$P_2$, the treated cells were homogenized in homogenizing buffer (100 mM NaOH and 0.1% Triton X-100), following which the crude lysate was heated to 80˚C for 10 min and centrifuged at 20,000 x g for 10 min at 4˚C. The supernatant was transferred to a fresh 1.5 ml tube. Fru-2,6-$P_2$ was identified in the supernatants by its ability to activate pyrophosphate-dependent phosphofructokinase-1 from potato tubers, as described previously (17). Standard enzymatic methods for measuring lactate spectrophotometrically in the neutralized perchloric extracts were performed according to the protocol described previously (18).

*Statistical analysis*. All statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The differences between the tumor and adjacent noncancerous tissues were assessed using the paired samples *t*-test. The differences between the tissues of patients with ccRCC were assessed using an independent samples *t*-test or non-parametric test. The clinical correlation analysis was performed using the variance test or Kruskal-Wallis test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*hsa-miR-101 is significantly downregulated in human ccRCC tissues*. In the present study, a stem-loop RT-qPCR assay was performed to identify the expression level of hss-miR-101 in 15 pairs of matched ccRCC and noncancerous kidney tissue samples. As shown in Fig. 1A, significantly upregulated expression levels of hsa-miR-101 were detected in the ccRCC tissues, compared with the noncancerous tissue. The expression levels of hsa-miR-101 in 12 ccRCC tissue samples were upregulated between 4.6- and 17.9-fold, whereas the levels in three ccRCC tissue samples were downregulated between 0.2- and 0.7-fold. Two pairs of the matched ccRCC and noncancerous tissue samples, and the normal HK-2 epithelial cell line and ACHN

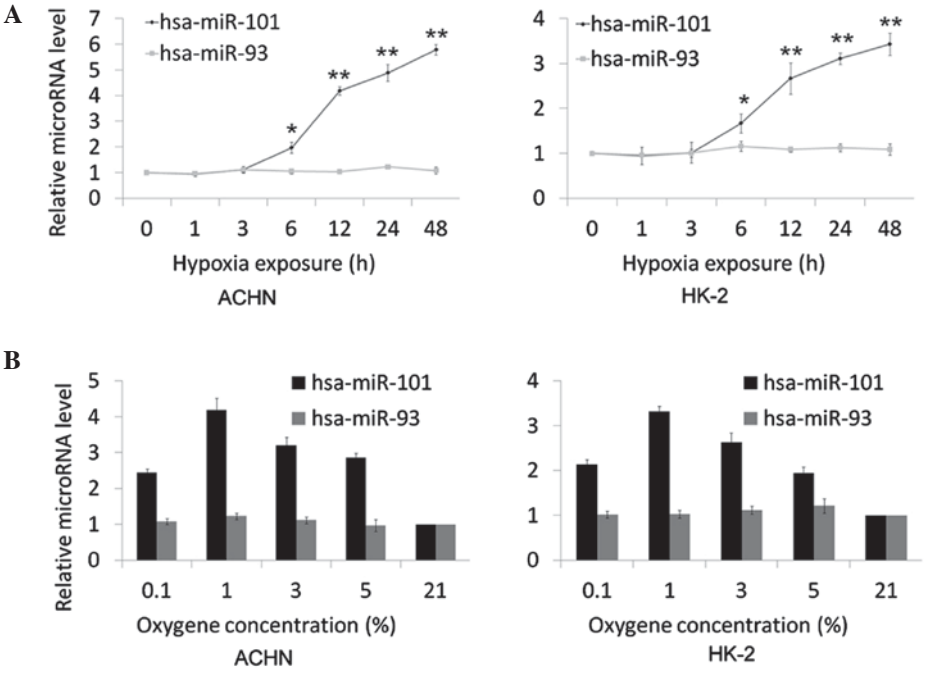


Figure 2. Time course and oxygen sensitivity of hsa-miR-101 following the induction of hypoxia. (A) AHCN and HK-2 cells were exposed to 1% oxygen for 0, 1, 3, 6, 12, 24 and 48 h. All time points were performed in triplicate. (B) AHCN and HK-2 cells were exposed for 16 h to oxygen concentrations of 0.1, 1, 3, 5 and 21%. All concentrations were performed in triplicate. hsa-miR-93 was considered a negative control, which is not affected by hypoxia. The expression levels of hsa-miR-101 and hsa-miR-93 were measured using reverse transcription-quantitative polymerase chain reaction analysis. The data are expressed as the mean fold difference in microRNA levels between the treated and untreated samples. $^{*}$P<0.05 and $^{**}$P<0.01. miR, microRNA.

ccRCC cell line were used for further conventional RT-qPCR analyses, and the results were analyzed by gel electrophoresis. The results showed that the expression of hsa-miR-101 was upregulated in the ccRCC tissues (Fig. 1B). Of note, no significant differences were found in the expression of hsa-miR-101 in the ACHN cells, compared with the HK-2 cells, indicating the induction of hsa-miR-101 by other factors.

*Confirmation of the hypoxic upregulation of hsa-miR-101 in ccRCC cells using RT-qPCR analysis*. The present study hypothesized that hsa-miR-101 is involved in the hypoxic response, and this was investigated by examining the level of hsa-miR-101 following hypoxic exposure. The time course of the induction of hsa-miR-101 by hypoxia was examined. The ACHN and HK-2 cells were cultured in hypoxia (1% $O_2$) for 1, 3, 6, 12, 24 and 48 h. The expression of hsa-miR-101 was analyzed using TaqMan RT-qPCR analysis. Following incubation for 6 h, the upregulation of hsa-miR-101 by hypoxia was detected and showed a progressive increase in expression (Fig. 2A). Following hypoxia exposure for 12 h, the upregulation of hsa-miR-101 was significant (ACHN, 4.19±0.17; HK-2, 2.67±0.35; P<0.001) and was maximal at the 48 h time point (ACHN, 5.79±0.21; HK-2, 3.43±0.25; P<0.001). As a negative control, the expression of hsa-miR-93, which is not affected by hypoxic incubation (19), was completely unaffected (data not shown). To investigate the oxygen-dependent regulation of hsa-miR-101, a range of oxygen concentrations (0.1, 1, 3, 5 and 21% oxygen) were used for cell maintenance. The induction of hsa-miR-101 was most marked at 1% oxygen, with more modest regulation at 0.1, 3 and 5% oxygen, following hypoxic exposure for 24 h (Fig. 2B). No detectable change in the expression of hsa-miR-93 was observed following hypoxia exposure.

*Overexpression of hsa-miR-101 knocks down the expression of TIGAR, and affects kidney cell metabolism and growth rate*. According to the stem-loop character of hsa-miR-101 and cross-species comparison, computational algorithms have been developed. TargetScan was used to generate a potential miRNA with a high probability of binding to the 3'UTR of TIGAR. To further confirm the predicted result, a hsa-miR-101-expressing plasmid was constructed and transfected into ACHN and HK-2 cells. Total RNA was extracted from the stably transfected cells, and RT-qPCR analysis was performed. The data showed that the overexpression of hsa-miR-101 reduced the expression of TIGAR at the mRNA and protein levels (Fig. 3A and B).

TIGAR is important as a regulator of oxidative stress, therefore, the present study next examined the biological consequences of the specific knockdown of TIGAR by hsa-miR-101 in ACHN and HK-2 cells. Metabolic parameters associated with the function of TIGAR were determined, and the levels of Fru-2, 6-$P_2$ and lactate were significantly increased in the hsa-miR-101-overexpressing cells following transfection (Fig. 3C). The present study also evaluated the effects of the overexpression of hsa-miR-101 on the growth rate of the transfected ACHN and HK-2 cells. As shown in Fig. 3D, the ACHN/hsa-miR-101 and HK-2/hsa-miR-101 cell lines had significantly increased cell viability, compared with the mock-transfected ACHN and HK-2 cells (P<0.05).

## Discussion

Increasing reports have shown the close association between disturbances in the expression levels of miRNAs and the carcinogenic process (20-22). These miRNAs, the mutation or

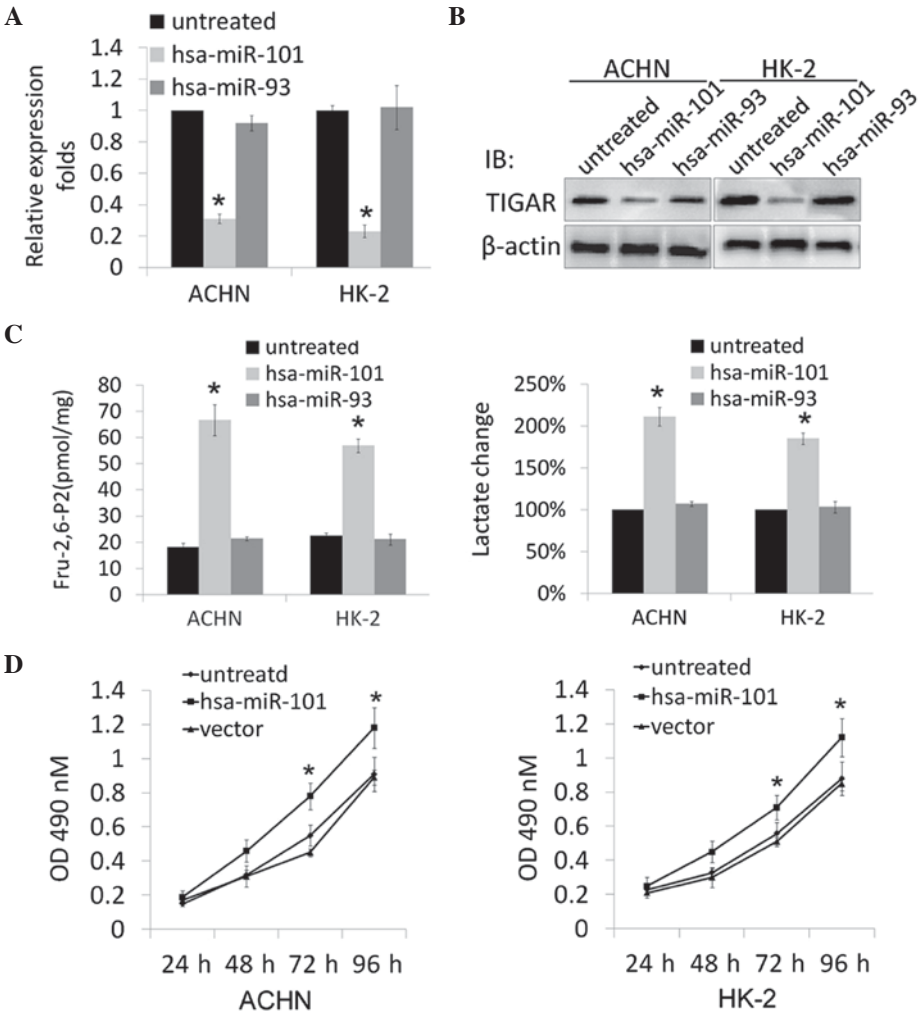


Figure 3. Overexpression of hsa-miR-101 affects the metabolism and growth rate of clear cell renal cell carcinoma and normal kidney cell lines. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analyses of the expression of TIGAR in mock-, hsa-miR-101- or hsa-miR-93-stably transfected ACHN and HK-2 cells. (C) Intracellular Fru-2, 6-P2 and lactate were detected. (D) Proliferation rates of cells were assayed by performing a 3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. All experiments were performed in triplicate. *P<0.01 vs. untreated cells. microRNA; TIGAR, TP53-induced glycolysis and apoptosis regulator; OD, optical density.

misexpression of which correlate with various types of human cancer, are referred to as oncomiRs (11). They can be involved in tumor suppression or formation.

The expression pattern of miRNAs may be altered during the progressive development of ccRCC. Gowrishankar *et al* (23) examined the differences in expression levels of >700 human miRNAs in a series of 94 ccRCC samples, and found that the accumulation of miR-21-5p and miR-142-3p were reduced. The overexpression of these miRNAs leads to proliferation and decreased cell death, suggesting their role as oncogenes. Previously, Cheng *et al* (24) showed that miR-34a and miR-224 were upregulated and had an anti-apoptotic effect in ccRCC. This suggests that these miRNAs may be involved in tumor suppression.

The effects of TIGAR on cell proliferation and glycolysis are considered to be cell- and context-dependent. Following moderate levels of stress, TIGAR is induced for repairing DNA damage and inhibiting glycolysis. Peña-Rico *et al* (25) showed that the silencing of TIGAR in glioblastoma cell lines causes higher levels of Fru-2,6-$P_2$ in cells, consequently increasing glycolysis and leading to the accumulation of reactive oxygen species, which promotes cell death. In tumor tissues, TIGAR has been found to be markedly downregulated (26), which is predominantly caused by p53 dysfunction. However, the mechanism underlying the downregulation of TIGAR in tumors with functioning p53 remains to be elucidated, however, it is hypothesized to be involved with miRNAs.

The aim of the present study was to evaluate whether there is an association between hypoxia exposure and altered expression patterns of hsa-miR-101 in ccRCC. The results showed that hsa-miR-101 was frequently downregulated in human ccRCC tissues, compared with corresponding noncancerous kidney tissues, which is consistent with the results of Wotschofsky *et al* (27). In addition, hsa-miR-101 has been reported to be upregulated in HepG2 cells (28) and periodontal ligament cells (29), indicating its close association with different types of tumors. However, the mechanism causing the upregulation of hsa-miR-101 and the effect of the higher level of this miRNA remains to be elucidated.

Due to the close association between the induction of miRNA with hypoxia, the present study investigated whether

hsa-miR-101 is induced by hypoxia. The resulting data showed that hsa-miR-101 was induced by hypoxia *in vitro*. Consistent with previous results, hsa-miR-93 showed no responsiveness to hypoxia *in vitro*. The effects of alterations in the levels of hsa-miR-101 remain to be fully elucidated. Resultant modifications in target gene expression are possible, however, the relative effects on transcription, mRNA post-transcriptional regulation and translation remain to be elucidated. The computational prediction of mRNA targets for hsa-miR-101 binding and action generates a number of potential targeted mRNA sequences (www.MicroRNAs.org/microRNAs/home.do and www.targetscan.org). As TIGAR mRNA showed the highest binding affinity, it was selected for further experiments. The data obtained showed that the overexpression of hsa-miR-101 decreased the mRNA and protein levels of TIGAR, causing the stimulation of glycolysis and proliferation. The correlation between the levels of hsa-miR-101 and ccRCC was founs to be marked. This may be due to the direct effect of hsa-miR-101 on tumor biology through its induction by tumor hypoxia. Of note, as a feature of a solid tumors, hypoxia induces hsa-miR-101 to target TIGAR, causing changes in metabolism. This indicates one mechanism involved in the regulation responses of miRNAs to microenvironmental factors.

The results of the present study showed that a significantly high proportion of hsa-miR-101 is overexpressed in human tumor tissues, and the alteration of this miRNA is caused by hypoxia *in vitro* and *in vivo*. By regulating its target gene, TIGAR, the overexpression of hsa-miR-101 stimulates glycolysis and increases proliferation. In conclusion, the present study may provide a novel therapeutic target site for ccRCC through targeting hsa-miR-101.

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