MicroRNA-18b inhibits the growth of malignant melanoma via inhibition of HIF-1α-mediated glycolysis

YAO CHEN1, ZIQING ZHANG1, CHENGQUN LUO2, ZIZI CHEN2 and JIANDA ZHOU2

1Department of Plastic and Cosmetic Surgery, Longgang Orthopetics Hospital of Shenzhen, Shenzhen, Guangdong 518000; 2Department of Plastic Surgery, The Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, P.R. China

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Abstract. MicroRNAs (miRs) have been demonstrated to play critical roles in the development and progression of malignant melanoma (MM). However, the exact role and underlying mechanism of miR-18b in MM growth remains unclear. In the present study, real-time PCR data indicated that miR-18b was significantly downregulated in MM tissues compared to their matched adjacent non-tumor tissues. Low miR-18b expression was significantly associated with the tumor thickness and stage, although no significant association was observed between the miR-18b expression and the age, gender, or lymph node metastasis. Besides, miR-18b was also significantly downregulated in MM B16 and A375 cells compared to normal skin HACAT cells. Ectopic expression of miR-18b decreased the proliferation of A375 and B16 cells, while induced a remarkable cell cycle arrest at G1 stage. Besides, miR-18b overexpression also inhibited the glycolysis in A375 and B16 cells. HIF-1α, a key regulator in glycolysis, was then identified as a target gene of miR-18b, and its expression was negatively downregulated in MM B16 and A375 cells. Overexpression of HIF-1α rescued the suppressive effect of miR-18b on MM cell proliferation and glycolysis. In vivo study further showed that overexpression of miR-18b inhibited the MM growth as well as the tumor-related death, accompanied with HIF-1α downregulation. Taken together, the present study suggests that miR-18b inhibits the growth of MM cells in vitro and in vivo through directly targeting HIF-1α.

Introduction

Malignant melanoma (MM), a malignant tumor of melanocytes, is characterized by a rapid progression and metastasis (1). It ranks as the fifth most common cancer for male and the seventh most common malignancy in female (2,3). In recent years, a large number of studies have focused on the pathogenesis of MM, and deregulation of oncogenes and tumor suppressors have been reported to play key roles in the development and progression of MM (4,5). However, the detailed molecular mechanism underlying MM still remains largely unclear.

Cancer cells preferentially utilize glycolytic metabolism to produce ATP, even in the presence of oxygen, a phenomenon termed aerobic glycolysis (6,7). Inhibition of glycolysis has been suggested to improve the outcomes for cancer therapy (8,9). Hypoxia-inducible factor (HIF)-1α, a heterodimer composed of an α and a β subunit, plays a critical role in cellular and systemic homeostatic response to hypoxia by activating transcription of many genes involved in energy metabolism, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia (10,11). Our previous study reported that HIF-1α played an oncogenic role in MM (12). However, the regulatory mechanism of HIF-1α in MM remains largely unknown.

MicroRNAs (miRs) are a class of small non-coding RNAs. They can inhibit the gene expression via directly binding to the 3’ untranslated region (UTR) of their target mRNA, leading to translational repression (13,14). Moreover, miRs have been demonstrated to mediate various biological processes, such as cell proliferation, cell cycle progression, glycolysis, as well as tumorigenesis (15,16). miR-18b plays an oncogenic or tumor suppressive role in different cancer types (17). For instance, miR-18b is upregulated in HCC, and higher miR-18b level predicates poor prognosis (18). Moreover, overexpression of miR-18b increased HCC cell proliferation, while inhibited cell adhesion ability, indicating that miR-18b acts as an oncogene in HCC (18). On the contrary, miR-18b was recently reported to inhibit MM (19). It was downregulated in MM by virtue of hypermethylation, and overexpression of miR-18b decreased MM cell viability, induced cell apoptosis, and inhibited tumor growth in vivo (19). However, the exact role of miR-18b in the regulation of energy metabolism in MM has not been reported.

Therefore, the present study aimed to investigate the expression of miR-18b in MM tissues and its association with clinicopathological features. Moreover, we also studied the exact role and underlying mechanism of miR-18b in regulating MM growth and energy metabolism.
Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of Central South University, Changsha, China.

Tissue samples. A total of 68 pairs of MM tissues and adjacent normal tissues were collected. The clinicopathological information is summarized in Table I. These MM patients received neither radiation therapy nor chemotherapy before surgical resection. Written informed consents had been obtained. Tissues were snap-frozen in liquid nitrogen, then stored at -80°C before use.

Cell culture. Human embryonic kidney HEK293 cells, MM B16 and A375 cell lines, and normal skin HACAT cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS; Life Technologies) in a 37°C humidified atmosphere of 5% CO₂.

Real-time RT-PCR assay. Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer’s instruction. For miR detection analysis, real-time PCR was performed using PrimeScript® miRNA RT-PCR Kit (Takara, Dalian, China) added with 10% fetal bovine serum (FBS; Life Technologies) in a 37°C humidified atmosphere of 5% CO₂. The proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Life Technologies). The PVDF membrane was incubated with phosphate-buffered saline containing 5% milk overnight at 4°C. Then, the PVDF membrane was incubated with rabbit polyclonal anti-human HIF-1α antibody (1:100, ab2185; Abcam, Cambridge, MA, USA) and rabbit monoclonal anti-human GAPDH antibody (1:200, ab181602; Abcam) at room temperature for 3 h. After washed by PBS for 3 times, the membrane was incubated with goat anti-rabbit secondary antibody (1:10,000, ab7090; Abcam) at room temperature for 1 h. After three washes by PBS, an ECL kit (Pierce Biotechnology, Rockford, IL, USA) was used to perform chemiluminescence detection. The relative protein expression was analyzed by Image-Pro® Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), represented as the density ratio vs. GAPDH.

MiR mimic, inhibitor and plasmid. Scramble miR mimic (miR-NC), miR-18b mimic, blank pCDNA3.1 vector, pCDNA3.1-HIF-1α plasmid, and blank pLVTH vector, pLVTHmiR-18b lentiviral plasmid were purchased from Amspring Co., (Changsha, China).

Cell transfection. Cells (1x10⁵ cells/well) were seeded in 24-well plates, and cell transfection was performed using 100 nM of diluted Lipofectamine 2000 (Life Technologies), according to the manufacturer's instruction. Cells were then cultured in a cell incubator containing 5% CO₂ at 37°C, and harvested at 48 h post-transfection for further analysis.

Cell proliferation analysis. Cells (5,000 cells/well) were cultured in a 96-well plate, each well had 100 µl fresh serum-free medium with 0.5 g/l MTT. Following incubation
at 37°C for 0, 24, 48 and 72 h, the medium was removed by aspiration and 50 µl dimethyl sulfoxide was added to each well. Following incubation at 37°C for 10 min, the A570 of each sample was measured using a plate reader.

Cell cycle distribution analysis. Cells (2x10^5) were washed twice with DPBS and resuspended in 70% ethanol. After fixed overnight at -20°C, cells were pelleted, washed twice in PBS with 3% BSA and pelleted. Cells were then resuspended and incubated for 30 min at room temperature in propidium iodide staining buffer containing 3% BSA, 40 µg/ml propidium iodide, and 0.2 mg/ml RNase in PBS. DNA content analyses were carried out using the flow cytometry (FACSCalibur; Beckman Coulter).

Glucose uptake analysis. After cultured for 24 or 48 h, the medium supernatant was collected and diluted 1:4,000 in DPBS. The amount of glucose in the supernatant was then detected using the Glucose Uptake Colorimetric assay kit (Sigma-Aldrich) in accordance with the manufacturer's protocol. The absorbance was detected at 412 nm with ELx800 type ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA).

Lactate secretion analysis. Metabolites were quantified from medium supernatant using a Lactate Assay kit (Sigma-Aldrich) after cultured for 24 or 48 h. Assays were performed according to the manufacturer's instructions. The concentrations were normalized to protein contents determined by a BCA protein assay (Pierce Biotechnology) using bovine serum albumin (Sigma-Aldrich) as a standard protein.

Bioinformatics analysis. Targetscan software (www.targetscan.org/) was used to analyze the putative target genes of miR-18b.

 Luciferase reporter assay. Mutations of miR-18b binding sites in the HIF-1α 3'-untranslated region (UTR) were introduced using Easy Mutagenesis System kit (Promega, Madison, WI, USA), according to the manufacturer's instruction. The wild-type (WT) or mutant type (MT) of HIF-1α 3'UTR was cloned downstream of the firefly luciferase coding region of pmirGLO™ Luciferase vector (Promega), generating the WT or MT HIF-1α 3'UTR reporter plasmid, respectively.

HEK293 cells were co-transfected with WT-HIF-1α-3'UTR or MUT-HIF-1α-3'UTR reporter plasmid, and miR-18b mimic or miR-NC, respectively. After cultured at 37°C for 48 h, cells were lysed using the lysis buffer (Promega), and Dual-luciferase reporter assay system (Promega) was used to detect the luciferase activity, according to the manufacturer's instruction.

Tumor growth in vivo. In the control group, B16 cells were stably transfected with the blank pLVTH vector. In the experiment group, B16 cells were stably transfected with the pLVTH-miR-18b lentiviral plasmid. Male BALB/C-nu/nu nude mice (n=6) were injected subcutaneously in the dorsal flank with 1x10^7 cells of each group. The mice still alive were sacrificed on day 50 after tumor implantation. Tumor volume was calculated by using the formula V (mm^3) = 0.5 x a x b^2 (a maximum length to diameter, b maximum transverse diameter). Tumor weight was also recorded.

Statistical analysis. Data are expressed as mean ± SD. GraphPad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Data were analyzed by using a Student's t-test for two-group comparison and one-way ANOVA for multiple-group comparison. The contingency data were analyzed by using the Chi-squared test. P-value <0.05 was considered statistically significant.

Results

miR-18b is downregulated in MM. In the present study, we first performed qPCR to examine the miR-18b level in a total of 68 pairs of MM tissues and their matched adjacent non-tumor tissues. As demonstrated in Fig. 1A, the expression of miR-18b was significantly lower in MM tissues, when compared with that in adjacent non-tumor tissues. These MM patients were then divided into two groups, low miR-18b expression group and low miR-18b expression group, according to the mean value of the miR-18b expression as the cut-off point. The association between the miR-18b expression and the clinicopathological features of MM were then analyzed. We found no significant association between the miR-18b expression and the age, gender, or lymph node metastasis (Table I). However, low miR-18b level was significantly associated with the increased tumor thickness as well as advanced tumor stage (Table I), suggesting that the downregulation of miR-18b may
play a role in MM progression. In addition, we then examined the miR-18b level in MM B16 and A375 cell lines. Human normal skin HACAT cells were used as control. As shown in Fig. 1B, the expression of miR-18b was also markedly reduced.
in B16 and A375 cells compared to HACAT cells. Therefore, miR-18b was significantly downregulated in MM.

miR-18b overexpression decreases cell proliferation, induces cell cycle arrest and inhibits glycolysis in MM cells. We further used B16 and A375 cells to study the role of miR-18b in MM. B16 and A375 cells were transfected with miR-NC or miR-18b mimic, respectively. Real-time qPCR data showed that the miR-18b level was significantly increased after transfection with miR-18b mimic, but not with miR-NC, compared to the control group (Fig. 2A and B). We further performed MTT assay to examine cell proliferation, and found that overexpression of miR-18b significantly decreased the proliferation of B16 and A375 cells compared to the control group (Fig. 2C and D).

As cell cycle progression is crucial for cell proliferation (21), we further examined the cell cycle distribution in each group. As indicated in Fig. 3A and B, ectopic expression of miR-18b...
induced a significant cell arrest at G1 stage, contributing to the suppressive effect of miR-18b on MM cell proliferation. In addition, energy metabolism is also important for cancer cell proliferation. Thus, we further examined the glycolysis level of MM cells with or without miR-18b upregulation. As shown in Fig. 3C-F, the glucose uptake and lactate secretion were significantly decreased in MM cells transfected with miR-18b mimic, indicating that the glycolysis was suppressed after miR-18b overexpression.

**HIF-1α is a direct target gene of miR-18b in MM cells.** As HIF-1α is a key regulator in glycolysis (22), we further examined the mRNA and protein expression of HIF-1α in MM cells with or without miR-18b overexpression. No significant difference was observed in the mRNA level of HIF-1α in each group (Fig. 4A and B). However, miR-18b upregulation caused a remarkable reduction in the protein level of HIF-1α in B16 and A375 cells, when compared to the control group (Fig. 4C and D), suggesting that miR-18b negatively regu-
lates the HIF-1α expression at a post-transcriptional level. Therefore, HIF-1α may be involved in the miR-18b-mediated MM cell glycolysis. To further reveal the relationship between miR-18b and HIF-1α, bioinformatics analysis was performed, and TargetScan software data showed a perfect base pairing between the 3' UTR of HIF-1α mRNA and the seed sequence of miR-18b (Fig. 4E), suggesting that HIF-1α is a target gene of miR-18b. To further confirm the targeting relationship between miR-18b and HIF-1α, the WT or MT of HIF-1α 3' UTR (Fig. 4F) was cloned into luciferase reporter vector, generating the WT or MT HIF-1α 3' UTR reporter plasmid, respectively (Fig. 4G). Luciferase reporter assay data showed that co-transfection with WT HIF-1α 3' UTR reporter plasmid and miR-18b mimic caused a significant decrease in the luciferase activity, which was rescued by transfection with MT-HIF-1α 3' UTR reporter plasmid (Fig. 4H). Accordingly, HIF-1α is a direct target gene of miR-18b, and the protein expression of HIF-1α is negatively regulated by miR-18b in MM cells.

Overexpression of HIF-1α rescues the inhibitory effect of miR-18b on MM cell proliferation and glycolysis. We further studied whether HIF-1α was involved in the miR-18b-mediated MM cell proliferation and glycolysis. pCDNA3.1-HIF-1α plasmid was transfected into the miR-18b-overexpressing B16 and A375 cells. After transfection, the protein level of HIF-1α was significantly increased (Fig. 5A and B). MTT

Figure 6. (A) B16 cells were stably transfected with blank pLVTH vector or pLVTH-miR-18b lentiviral plasmid, respectively. Real-time qPCR was conducted to examine the miR-18b level. Nude mice were subcutaneously implanted with these cells. (B) All surviving mice were sacrificed on day 50 after implantation, and the xenograft was obtained. (C) The survival curve was made. (D and E) The tumor volume and weight were examined. (F) Western blot analysis was conducted to examine the protein levels of HIF-1α in the tumor xenograft. *P<0.01 vs. Blank.
assay was then conducted to examine the cell proliferation. Our data showed that the proliferation was higher in B16 and A375 cells co-transfected with miR-18b mimic and HIF-1α plasmid, when compared to miR-18b-overexpressing B16 and A375 cells (Fig. 5C and D). The glycolysis level was then analyzed. As indicated in Fig. 5E-H, the glucose uptake and lactate secretion were also higher in MM cells co-transfected with miR-18b mimic and HIF-1α plasmid, when compared to cells transfected with only miR-18b mimic. Therefore, our data demonstrate that overexpression of HIF-1α rescued the inhibitory effect of miR-18b on MM cell proliferation and glycolysis.

miR-18b upregulation reduces MM growth and glycolysis in vivo. To further reveal the role of miR-18b in MM in vivo, pLVTH-miR-18b lentiviral plasmid was stably transfected into B16 cells. After transfection, the miR-18b level was significantly increased compared to the control B16 cells transfected with blank pLVTH vector (Fig. 6A). Then, nude mice were subcutaneously implanted with these cells. The mice were sacrificed on day 50 after implantation and the xenografts were obtained (Fig. 6B). The survival curve data indicated that overexpression of miR-18b decreased the death rate caused by tumor growth (Fig. 6C). The tumor volume and weight were then determined. As indicated in Fig. 6D and E, the tumor volume and weight were significantly decreased in the miR-18b overexpression group compared to the control group. We also examined the protein expression of HIF-1α in the tumor xenografts. Our data showed that the protein level of HIF-1α was also lower in the miR-18b overexpression group (Fig. 6F). Therefore, our findings suggest that miR-18b has a suppressive effect on the MM growth in vivo, probably through inhibition of HIF-1α-mediated glycolysis.

Discussion

miR-18b has recently been demonstrated to play a suppressive role in MM (19). However, the underlying mechanism remains largely unknown. In the present study, we found that miR-18b was significantly downregulated in MM tissues and cell lines. Moreover, low miR-18b expression was significantly associated with the tumor thickness and stage. Overexpression of miR-18b decreased cell proliferation, induced cell cycle arrest, and inhibited glycolysis in A375 and B16 cells. HIF-1α, a key regulator in glycolysis, was further identified as a target gene of miR-18b in MM cells. Overexpression of HIF-1α rescued the suppressive effect of miR-18b on MM cell proliferation and glycolysis. Moreover, in vitro study showed that overexpression of miR-18b inhibited the MM growth as well as the tumor-related death, accompanied with HIF-1α downregulation. These data demonstrate that miR-18b inhibits the growth and glycolysis of MM cells in vitro and in vivo through directly targeting HIF-1α.

In recent years, many miRs have been implicated in the development and progression of MM (23,24). For instance, miR-203 was significantly downregulated in MM, and upregulation of miR-203 suppressed A375 cell migration via inhibition of versican (25). Overexpression of miR-193b repressed MM cell proliferation (26). In the present study, we found that miR-18b was also downregulated in MM tissues and cell lines. Moreover, low expression of miR-18b was significantly associated with the increased tumor thickness and advanced tumor stage. These data suggest that miR-18b was involved in the malignant progression of MM. The sample size of tumor tissues was not very large, and expanding it will further help confirm the relationship between the miR-18b expression and clinicopathological features of MM patients. In addition, the association between the miR-18b expression and the survival time of patients with MM should also be investigated in future. To further reveal the role of miR-18b in MM, B16 and A375 cells were transfected with miR-18b mimic to upregulate its expression level. Overexpression of miR-18b significantly decreased the proliferation of B16 and A375 cells, suggesting that miR-18b may have inhibitory effect on MM growth. Another study reported that overexpression of miR-18b caused suppression of MM cell colony formation (19). Furthermore, we examined the cell cycle distribution in B16 and A375 cells with or without miR-18b overexpression, and found that overexpression of miR-18b induced a remarkable cell cycle arrest at G1 stage. Our data suggest that the miR-18b-induced cell cycle arrest contributes to the decreased cell proliferation.

Increased glycolysis in tumor cells compared to normal tissues is observed in most cancers, which is in accordance with the Warburg effect that the aerobic glycolysis is a major source of energy in cancer cells (27). As no previous study has focused on the effect of miR-18b on the energy metabolism in MM, we then examined the glycolysis level in B16 and A375 cells with or without overexpression of miR-18b. Interestingly, our data showed that overexpression of miR-18b led to a significant decrease in the glucose uptake and lactate secretion, indicating that miR-18b upregulation suppressed the glycolysis in MM cells. In fact, several other miRs were also found to be involved in the regulation of glycolysis in human cancers. For instance, overexpression of miR-144 enhanced glucose uptake and lactate production in ovarian cancer cells, leading to a rapid growth of ovarian cancer cells (28). Xu et al (29) reported that inhibition of miR-340 increased Glut1 expression, leading to an increase in lactate secretion and glucose uptake rate. Moreover, let-7a was found to decrease key anabolic enzymes and increase both oxidative phosphorylation and glycolysis in metastatic MM cells (30). However, evidence is rare in the role of miRs in regulating glycolysis in MM. Thus, the present study expands the understanding of the function of miRs in MM glycolysis.

Then we focused on the molecular mechanism underlying miR-18b inhibited glycolysis in MM. HIF-1α has been demonstrated to play a key role in the development and progression of different cancer types through regulating the cellular and systemic homeostatic responses to hypoxia (31). Moreover, HIF-1α was found to be significantly upregulated in MM, and the increased HIF-1α expression predicated poor prognosis in MM (32,33). In the present study, HIF-1α was found to be significantly upregulated in MM, and the increased HIF-1α expression predicated poor prognosis in MM (32,33). In the present study, HIF-1α was found to be significantly upregulated in MM, and the increased HIF-1α expression predicated poor prognosis in MM (32,33).
inhibited MM growth in vivo, accompanied with HIF-1α downregulation, further suggesting that miR-18b may become a promising candidate for the treatment of MM.

To the best of our knowledge, the present study for the first time demonstrates that miR-18b can inhibit the growth and glycosylation of MM cells, partly at least, via direct inhibition of the HIF-1α expression. Therefore, we suggest that the miR-18b/HIF-1α may serve as a potential therapeutic target for MM.

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