MicroRNA-18a-5p regulates the Warburg effect by targeting hypoxia-inducible factor 1α in the K562/ADM cell line

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Abstract. The Warburg effect is involved in drug resistance and recurrence of cancer, and poses a challenge for the treatment of chronic myelogenous leukemia (CML). Hypoxia-inducible factor 1α (HIF-1α) plays a key role in the Warburg effect. microRNAs (miRs) targeting HIF-1α have potential of regulating such aberrant metabolic process. The present study demonstrated that miR-18a-5p was expressed at a low level in K562/ADM cells via reverse transcription-quantitative PCR (RT-qPCR). The results of the luciferase reporter assay indicated that miR-18a-5p could specifically bind the 3′-untranslated region of HIF-1α. Through RT-qPCR and western blotting, it was revealed that miR-18a-5p downregulated the expression of HIF-1α. By inhibiting HIF-1α, miR-18a-5p suppressed aerobic glycolysis in K562/ADM cells, according to the results produced by glucose uptake, lactate production, pyruvate level and ATP synthesis measurement, along with the results obtained from extracellular acidification rate and oxygen consumption rate assays. These results provided new evidence that miR-18a-5p may suppress the Warburg effect by targeting HIF-1α. Furthermore, via CCK-8 and flow cytometry assays, cells transfected with miR-18a-5p mimics were more sensitive to Adriamycin (AMD) compared with AMD group. Reversing the Warburg effect by miR-30a-5p might provide a potential therapeutic strategy for CML.

Introduction

Leukemia is a common hematological malignant tumor with an incidence rate of 5-8/100,000 per year, endangering human health and life. Chronic myelogenous leukemia (CML) represents one type of leukemia caused by the dysfunction of pluripotent hematopoietic cells in patients resulting in indefinite proliferation of these cells. The development of CML can be divided into chronic, accelerated and acute phases, with a slow progression in the early stage (1). Although treatments such as chemotherapy, interferon therapy and targeted therapy have progressed in recent years, multiple drug resistance (MDR) and relapse still constitute major challenges for curing CML (2). For example, MDR CML is highly resistant to chemotherapeutic drugs (3,4). Some patients can gradually become resistant to imatinib which is a tyrosine kinase inhibitor targeting the Bcr-Abl gene (5). New mechanisms associated with MDR have been found and further investigated in recent years (6), such as autophagy (7) and the Warburg effect (8), providing novel therapeutic targets for CML.

The Warburg effect, which is also called aerobic glycolysis, is an aberrant metabolic process during which glycolysis occurs in cancer cells even in the presence of oxygen. Cancer cells with the Warburg effect exhibit higher glucose uptake and lactate generation (9,10). It has been increasingly recognized that the Warburg effect plays an important role in drug resistance and can be utilized as a potential target for drug development (11,12). The Warburg effect has been reported in different types of cancer, including hepatic carcinoma (13), breast cancer (14), esophageal cancer (15) and leukemia. It has been demonstrated that glucose transporter-1 protein (GLUT) is upregulated and the level of lactate is elevated in pediatric acute lymphoblastic leukemia cells and that these cells are sensitive to glycolytic inhibitors (16). GLUT expression on the surface of Bcr-Abl-positive CML cells can be repressed by imatinib, resulting in decreased glucose uptake (17). Targeting the Warburg effect provides potential therapeutic opportunities for leukemia (18). A clinical trial has been carried out to change the metabolic process in order to enhance drug sensitivity (19). Hypoxia-inducible factor 1α (HIF-1α) plays a key role in the Warburg effect. Glycolytic gene expression can be enhanced by HIF-1α through interaction with hypoxia-responsive factors.
elements of glycolytic gene promoters (20). In addition, overexpression of HIF-1α is closely associated with cancer cell proliferation, apoptosis inhibition, cancer cell infiltration and MDR. Activation of HIF-1α usually indicates poor prognosis of patients with tumors (21). The current study hypothesized that inhibiting HIF-1α in leukemia cells, such as the Adriamycin-resistant CML cell line K562/ADM, may reverse the Warburg effect and improve drug sensitivity.

MicroRNAs (miRNAs/miRs) are short non-coding RNAs with a length of 20-25 nt that participate in regulating various biological effects, such as proliferation, apoptosis and invasion of cancer cells. It has been increasingly demonstrated that miRNAs play regulatory roles in different types of cancer (22,23). miRNAs constitute potential candidates for leukemia diagnosis and therapy (24). miR-18a-5p belongs to the miR-17-92 family (25). aberrant expression of miR-18a-5p has been reported in esophageal squamous cell carcinoma, pancreatic cancer and thyroid cancer, and miR-18a-5p may function as an oncogene or a tumor suppressor (26,27).

Previous studies mainly focused on the impact of miR-18a-5p on proliferation, apoptosis, invasion and migration of cancer cell (28,29). The present study aimed to explore the regulatory role of miR-18a-5p in the Warburg effect in K562/ADM cells, which will provide new insight into the mechanism of miR-18a-5p regulation in leukemia cells and potential targets for CML treatment.

Materials and methods

Materials. RPMI-1640 medium was from Hyclone; Cytiva. FBS, trypsin and penicillin-streptomycin solution were from Thermo Fisher Scientific, Inc. Chronic myelogenous leukemia cell lines K562 and K562/ADM were from American Type Culture Collection. Primary human normal lymphocyte culture was kindly supplied by Dr Shaohua Wang from the Department of Pharmacology at the Medical School of Southeast University and maintained at the Department of Clinical Laboratory in The First Affiliated Hospital of Kunming Medical University. Cell Counting Kit-8 (CCK-8) reagent was from Dojindo Molecular Technologies, Inc. Adriamycin was from Sigma-Aldrich; Merck KGaA. K562/ADM cells were collected from light and tested by a BD flow cytometer (BD Biosciences) and analyzed using FlowJo™ software version 7.6.5 (FlowJo LLC).

Reverse transcription-qPCR (RT-qPCR). Total RNA was purified by RNAiso Plus reagent. For miRNA quantification, the RNA was reverse transcribed using a miR-18a-5p-specific primer (5'-TCTCAGCAGACTTTAGACTTGGAG-3') and a random decamer primer (5'-GATCCCTGAACCTATTCGAG GTGGTATTG-3') were synthesized and cloned into pCMV-3.1 vectors for miR-1α overexpression and knockdown by Sangon Biotech Co., Ltd. miRNAs that could specifically target HIF-1α were screened using TargetsScan 7.2 (http://www.targetscan.org/vert_72/) and Miranda 2.0 (<http://www.miranda.org/>) software. miR-18a-5p mimics (5'-GAUAGAGUGAUCUA CGUGGAU-3') and miR-18a-5p negative control (miR-NC, 5'-UUCCUCCAGGCUACGUCAGAUCG-3') were synthesized by Sangon Biotech Co., Ltd. Lipofectamine® 2000 was from Invitrogen; Thermo Fisher Scientific, Inc. RNAiD® Plus™ was from Takara Biotechnology Co., Ltd. RevertAid™ Reverse Transcriptase was from Thermo Fisher Scientific, Inc. All reverse transcription-quantitative PCR (RT-qPCR) primers were synthesized by Sangon Biotech Co., Ltd. miRNA qPCR Master Mix for miRNA quantification and 2X SGExcel FastSYBR Mixture for miRNA quantification were from Sangon Biotech Co., Ltd. HIF-1α, glucose transporter type 1 (GLUT1), hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDHA) and β-actin antibodies were from Abcam. 

Cell culture and transfection. K562 cells or K562/ADM cells were incubated in RPMI-1640 medium containing 10% fetal calf serum and 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.) in a 37°C, 5% CO2 incubator. 5x10^5 cells/well were seeded into a 6-well plate. When the K562/ADM cell confluency reached >80%, miRNAs, siRNA or overexpression vectors were transfected using Lipofectamin®2000 according to the manufacturer's instructions. miRNAs, siRNA or overexpression plasmid vectors (Sangon Biotech Co., Ltd.) were diluted to a final concentration of 100 nM in FBS-free RPMI-1640 medium and mixed gently. Lipofectamin®2000 (5 µl) was gently mixed, diluted in 250 µl FBS-free RPMI-1640 medium and incubated for 5 min at room temperature. Following gentle mixing, samples were further incubated for 20 min at room temperature. The DNA/lipofectamin®2000 complexes were then added to cells and incubated at 37°C in a CO2 incubator for 4 h.

Cell proliferation analysis. CCK-8 assay was used to analyze cell proliferation. A total of 24 h after miRNA transfection, cells were seeded in a 96-well plate at a density of 5x10^4 cells/well. A total of 1, 2, 3 and 4 days after plating, the CCK-8 reagent was added to the cells at a final concentration of 10% and the cells were incubated in 5% CO2, 37°C incubator for 2 h. The absorbance at 450 nm was measured and recorded at each time point. Finally, the proliferative curve was analyzed to compare the proliferation rate among groups.

Apoptosis analysis. Apoptosis was evaluated using an Annexin V-FITC/PI cell apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd.). K562/ADM cells were collected and stained with PI and Annexin V for 15 min at 25°C away from light and tested by a BD flow cytometer (BD Biosciences) and analyzed using FlowJo™ software version 7.6.5 (FlowJo LLC).
Western blotting. K562/ADM cells were lysed and total proteins were extracted using RIPA buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific, Inc.). Total protein concentration was determined using a BCA protein assay (Thermo Fisher Scientific, Inc.). Total proteins were extracted using RIPA buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific, Inc.). Total mRNA quantification was performed using 2X SGExcel mRNA quantification is as follows: 95˚C for 15 sec; 95˚C for 20 sec; 95˚C for 30 sec; 95˚C for 35 sec with 35 cycles. The relative expression of miR-18a-5p and HIF-1α mRNA was calculated according to the 2^ΔΔCt method (30).

Luciferase reporter assay. The wild type 3’-UTR and mutated 3’-UTR of HIF-1α were cloned into Dual-Glo Luciferase assay plasmids (Promega Corporation). K562/ADM cells cultured in a 6-well plate were transfected with 1 μg plasmids and 50 pmol miR-18a-5p mimics or miR-NC using Lipofectamine®2000. The pRL-CMV Renilla luciferase vector (Promega Corporation) was used to normalize cell numbers and transfection efficiency. After 24 h, the luciferase assay was performed using a luciferase assay kit (Promega Corporation).

ATP analysis. ATP was quantified using an ATP Bioluminescence Assay kit (Roche Diagnostics) according to the manufacturer’s instruction. Briefly, 2x10^5 K562/ADM cells were collected and mixed with 400 μl ATP buffer [100 mM Tris, 4 mM EDTA (pH 7.75)] and the mixture incubated at 100˚C for 2 min. After centrifugation, the supernatant was collected and the protein was quantified by the BCA assay. Luciferase reagent at a volume of 100 μl was added into 100-μl sample. Fluorescence was detected by an ELX808 Absorbance Microplate Reader (BioTek Instruments, Inc.). After subtracting the blank values from the raw data, the ATP concentration was calculated from the standard curve data.

Glucose, lactic acid and pyruvate measurement. For glucose uptake analysis, 2x10^5 K562/ADM cells were seeded into a 96-well plate 48 h after treatment and further incubated at 37°C in a 5% CO₂ incubator for 48 h. 2-NBDG (Invitrogen; Thermo Fisher Scientific, Inc.) was added into the cells at a final concentration of 5 mM. The cells were incubated for another 15 min at 37°C and the absorbance at 488 nm was recorded and normalized to the cell number. The relative glucose uptake for all groups was expressed as the absorbance value normalized to that of the untreated group.

For lactic acid analysis, 1x10^5 K562/ADM cells were seeded into a 12-well plate and incubated at 37°C for 10 h. The media were replaced with FBS-free media. After incubation at 37°C for 1 h, the supernatant was collected for lactic acid measurement using a lactate assay kit (BioVision, Inc.) according to the manufacturer’s protocol. Absorbance at 450 nm was recorded and normalized to the cell number. The relative lactic acid level was expressed as the absorbance value normalized to that of the untreated group.

For pyruvate analysis, 5x10^5 K562/ADM cells were treated with Pyruvate Kinase Assay Buffer (BioVision, Inc.). The extract was collected by centrifugation at 3,000 x g at 4°C for 30 min and measured by Pyruvate Kinase Activity Assay kit (BioVision, Inc.) according to the manufacturer’s protocol. Absorbance at 570 nm was recorded and normalized to the cell number. The relative pyruvate level was expressed as the absorbance value normalized to that of the untreated group.

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) assays. ECAR and OCR were analyzed by Seahorse XF Glycolysis Stress Test kit and Seahorse XF Cell Mito Stress Test kit (Agilent Technologies), respectively, using Seahorse XF®96 Extracellular Flux Analyzer (Seahorse Bioscience; Agilent Technologies). Transfected cells (1x10^5) were inoculated into a microplate and incubated at 37°C for 10 h followed by ECAR and OCR tests. For ECAR analysis, glucose, oligomycin (oxidative phosphorylation inhibitor) and 2-DG (glycolytic inhibitor) were added into the cells according to the manufacturer's protocol. For OCR analysis, oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (reversible inhibitor of oxidative phosphorylation) and the mitochondrial complex I inhibitor rotenone combined with the
miR-18a-5p targets and downregulates HIF-1α in K562/ADM cells. Using bioinformatics software Targetscan and Miranda, miRNAs that could specifically target HIF-1α were firstly screened. It was shown that miR-18a-5p has a binding site in the 3′-UTR in HIF-1α (Fig. 1A). miR-18a-5p expression was further quantified and compared among normal lymphocytes, and K562 and K562/ADM cells by RT-qPCR. miR-18a-5p level was significantly downregulated in K562/ADM cells compared with the K562 cell group and normal lymphocyte group. miR-18a-5p level in K562 cells was significantly lower than that in the normal lymphocyte group, but higher than that in the K562/ADM group (Fig. 1B). Subsequently, miR-18a-5p mimics were transfected into K562/ADM cells and the level of miR-18a-5p increased up to 8-fold compared with that in the miR-NC group (Fig. 1C). In addition, HIF-1α vector transfection could efficiently upregulate the HIF-1α mRNA expression level and siHIF-1α vector transfection could efficiently downregulate the HIF-1α mRNA expression level in K562/ADM cells (Fig. 1D). Overexpression of miR-18a-5p significantly downregulated HIF-1α expression on both mRNA (Fig. 1E) and protein levels (Fig. 1F). Luciferase assay showed that miR-18a-5p significantly attenuated the relative luciferase activity of the wild type 3′-UTR construct but not the mutated 3′-UTR construct (Fig. 1G), indicating that miR-18a-5p suppressed HIF-1α expression by directly targeting the 3′-UTR in HIF-1α.

miR-18a-5p inhibits proliferation and induces apoptosis of K562/ADM cells by targeting HIF-1α. The biological effects, including cell proliferation and apoptosis, that miR-18a-5p exerted on K562/ADM cells by targeting HIF-1α were investigated. As shown in Fig. 2A, transfection with miR-18a-5p mimics or siHIF-1α markedly inhibited cell proliferation compared with the untreated group, miR-NC group and HIF-1α overexpressing group. Furthermore, overexpression of HIF-1α ameliorated the inhibitory effect of miR-18a-5p on cell proliferation. Similarly, both miR-18a-5p mimics and siHIF-1α efficiently induced cell apoptosis, and restoration of HIF-1α attenuated the apoptosis induced by miR-18a-5p (Fig. 2B). Accordingly, the expression level of apoptosis-associated protein C-caspase-3 was markedly upregulated by miR-18a-5p and restored by HIF-1α overexpression (Fig. 2C). The results indicated that HIF-1α was a target of miR-18a-5p to inhibit proliferation and induce apoptosis of K562/ADM cells.

miR-18a-5p suppresses the warburg effect by targeting HIF-1α. The Warburg effect is a hallmark of cancer cells exhibiting an increased glucose uptake, lactate fermentation, increased
glycolysis rate and decreased respiration, even in the presence of oxygen. Using this aberrant metabolic process, cancer cells can acquire enough energy for proliferation, migration and survival (33). It has been widely recognized that the transcription...
factor HIF-1α plays an essential role in the Warburg effect concerning the glucose uptake, lactate production, pyruvate metabolism and respiration, providing a valuable target for cancer treatment and insight into the mechanism conducive to novel drug discovery (34). The present study investigated the Warburg effect on a cellular level and a strategy of improving the sensitivity of leukemia cells to Adriamycin by targeting HIF-1α.

miRNAs take part in a series of biological processes of cancer cells, including proliferation, differentiation and apoptosis (35). According to the complementary base pairing principle, miRNAs bind and degrade target genes at the post-transcriptional level (36). An array of miRNAs have been identified to be involved in regulating aerobic glycolysis. For example, miRNAs can inhibit the Warburg effect through targeting the 3'UTR of LDHA or HK2 (37). Zhu et al (38) reported that expression of miRNA-31-5p was positively associated with the Warburg effect in two lung cancer cell lines by specifically targeting HIF-1α inhibitor mRNA, which indicated the important regulatory role of HIF-1α in the Warburg effect. In the present study, miR-18a-5p was identified as a direct inhibitor of HIF-1α. Hence, it was hypothesized that miR-18a-5p may play a
Figure 3. Warburg effect analysis. (A) miR-18a-5p level. (B) HIF-1α protein level. (C) Glucose uptake. (D) Lactate production. (E) Pyruvate level. (F) ATP synthesis. (G) ECAR. (H) OCR. (I) Expression of Warburg effect-associated proteins in K562/ADM cells. *P<0.05 vs. miR-NC; †P<0.05 vs miR-18a-5p + HIF-1α. n=3. miR, microRNA; HIF-1α, hypoxia-inducible factor 1α; NC, negative control; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; GLUT, glucose transporter-1 protein; HK2, hexokinase 2; PKM2, pyruvate kinase M2; LDHA, lactate dehydrogenase A; si, small interfering RNA.
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Figure 4. Effect of miR-18a-5p combined with ADM on K562/ADM cells. (A) Proliferation of K562/ADM cells treated with miR-NC, miR-18a-5p, ADM and miR-18a-5p + ADM analyzed by Cell Counting Kit-8. *P<0.05 vs. miR-NC. n=3. (B) Apoptosis of K562/ADM cells treated with miR-NC, miR-18a-5p, ADM and miR-18a-5p + ADM analyzed by flow cytometry. *P<0.05 vs. miR-NC. n=3. (C) Caspase-3 protein level analyzed by western blot analysis. ADM, Adriamycin; miR, microRNA; NC, negative control; c-caspase-3, cleaved caspase-3.

Functional role in the Warburg effect. Aberrant expression of miR-18a-5p has been observed in cancer cell lines and tumor tissues, and its regulatory mechanism may be varied in different types of cancer. In HCT116 colon cancer cells, miR-18a-5p can upregulate autophagy and ataxia telangiectasia mutated gene, with the potential for colon cancer inhibition (39). However, miR-18a-5p is upregulated in non-small cell lung cancer (NSCLC) tissues and NSCLC cell lines and functions as an oncogene by directly targeting interferon regulatory factor 2 (28). Downregulating
miR-18a-5p using long non-coding RNA FENDRR also inhibits prostate cancer progression (29).

The present study demonstrated that the level of miR-18a-5p in the K562/ADM cell line was significantly lower than that in the normal lymphocytes, indicating that miR-18a-5p may act as a suppressor in CML. Bioinformatics analysis revealed that HIF-1α may be a potential target of miR-18a-5p. The miR-18a-5p expression level in K562/ADM cells was significantly downregulated compared with that in the K562 cells and normal lymphocytes. However, the miR-18a-5p expression level in K562 cells was relatively high compared with that in K562/ADM cells. The function of miR-18a-5p in K562 cells, which is a limitation of the present study, will be explored in our future work. The present study indicated that the mRNA and protein levels of HIF-1α can be decreased by miR-18a-5p overexpression. Transfection of miR-18a-5p mimics inhibited cell proliferation and induced apoptosis in K562/ADM cells, and restoration of HIF-1α expression reversed the biological effects of miR-18a-5p. The above results indicated that miR-18a-5p can inhibit K562/ADM cells by targeting HIF-1α. The Warburg effect associated with miR-18a-5p was further explored in the present study; miR-18a-5p could inhibit the ATP level, glucose uptake, lactate production and pyruvate level in K562/ADM cells. ECAR and OCR assays were performed to analyze the overall glycolytic flux and mitochondrial oxidative respiration changes (40,41). The results revealed that ECAR was decreased and OCR was enhanced by miR-18a-5p mimics. Furthermore, expression of downstream molecules of HIF-1α, including GLUT1, HK2, PKM2 and LDHA, which are closely associated with the Warburg effect, was affected by miR-18a-5p transfection. However, overexpression of HIF-1α could counteract the effects on the Warburg effect exerted by miR-18a-5p mimics. The results indicated that miR-18a-5p was able to inhibit the Warburg effect by targeting HIF-1α. The Warburg effect has been shown to be involved in drug resistance by increasing drug efflux, DNA damage repair, drug inactivation, and epigenetic alterations or mutations in drug targets (42). Therapeutic strategies, such as RNA interference targeting the Warburg effect combined with chemotherapeutic drugs, are expected to combat chemoresistance of tumors. The present study aimed to sensitize K562/ADM cells, which are commonly utilized as the MDR CML cell model, to Adriamycin. In conclusion, the present study demonstrated that the level of miR-18a-5p is relatively high compared with that in K562 cells. The exogenous miR-18a-5p expression could inhibit the ATP level, glucose uptake, and lactate production in K562/ADM cells. Moreover, the Warburg effect associated with miR-18a-5p was downregulated by miR-18a-5p overexpression. The results suggested that miR-18a-5p mimics can inhibit the Warburg effect by downregulating HIF-1α in K562/ADM cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KW and YZ conceived and designed the study. SJC, CG and YZ analyzed and interpreted the data, and drafted the manuscript. YXL, BN, JRY, QZ, SJC and YHL performed the experiments. All authors participated in the writing and revision of the manuscript. YZ and BN supervised the project. All authors read and approved the final manuscript. KW and CG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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