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MiR-182 promotes glucose metabolism by upregulating hypoxia-inducible factor 1α in NSCLC cells

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ABSTRACT

Objective: This study aims to demonstrate the role of miR-182 in the glucose metabolism of NSCLC cells and the potential mechanism.
Methods: MTT Cytotoxicity Assay was used to measure the function of differentially expressed miR-182 on two NSCLC cell lines proliferation. Metabolite analysis was introduced to monitor the glucose consumption, lactate release and glycolytic intermediate metabolites. The mRNA level of critical genes involved in glycolysis was detected by qRT-PCR. The 3′UTRs of predicted gene with a miR-182 binding site and their seed-sequence-mutated version were cloned downstream to the ORF of a Renilla luciferase reporter gene and the ability of miR-182 to downregulate luciferase expression was assessed.
Results: MiR-182 had significantly improved proliferation of NSCLC cell lines. Metabolite analysis of the cells with strengthened miR-182 revealed significantly increased glucose consumption and lactate release, as well as glycolytic intermediate metabolites, or conversely. Among a panel of genes controlling glucose metabolism, miR-182 exhibited significantly influence on ENO1, GLUT1, HIF-1α, HK1, HK2, LDHA and PDK1, especially HIF-1α. For the predicted target gene HIF1AN, the wild-type but not mutated 3′UTR, responded to miR-182 by directing ~45% reduction of reporter gene expression.
Conclusion: MiR-182 promotes glucose metabolism by upregulating HIF-1α in NSCLC cells.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 80% cases of lung related carcinoma, the most common solid tumor and the leading cause of cancer-related mortality worldwide [1]. One basic character of tumor is the disorders in energy metabolism regulations which facilitate tumor cells to fast growth and proliferation [2]. Warburg effect [3] holds that most cancer cells predominantly produce their energy through a high rate of glycolysis followed by lactic acid fermentation even in the presence of abundant oxygen. Aerobic glycolysis is less efficient than oxidative phosphorylation in terms of adenosine triphosphate production, but leads to the increased generation of additional metabolites that may particularly benefit proliferating cells [3]. To compensate for the consequent reduction in ATP production, cancer cells often adopt mechanisms to increase glucose consumption and utilization. One mechanism involves the regulation of glucose transporters, for example GLUT1, a basal element of glucose consumption of cells [4], can be upregulated by abnormally activated signaling of cancer cells. Additionally, hypoxia can stimulate glucose consumption and metabolism through hypoxia-inducible factor 1 (HIF-1) by inducing glycolytic genes, such as pyruvate dehydrogenase kinase 1 (PDK1) [5], lactate dehydrogenase A (LDHA) [6], hexokinase 2 (HK2) [7], as well as GLUT1 [8]. The activity of HIF1 can be inhibited by the asparagine hydroxylase factor inhibiting HIF (HIF1AN, also known as FIH), which hydroxylates asparagines 803 in the carboxy-terminal transactivation domain of human HIF-1α [9].

MicroRNA (miRNA) is a set of small non-coding RNA molecules containing about 22 nucleotides that functions in RNA silencing and post-transcriptional regulation of gene expression [10,11]. MicroRNAs can function as tumor suppressors or oncogenes and thus are deemed to play a crucial role in the initiation and progression of human cancer [12]. MiR-182 is a member of miR-183 family, dysregulation of which was uncovered in several cancers

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[13–18]. It has been proved that miR-182 is up-regulated in NSCLC tumor tissues and high expression of tumor and serum miR-182 was associated with overall poor survival in patients with lung cancer [19]. Here we focus on the role of miR-182 in the glucose metabolism of NSCLC cells and explore the potential mechanism.

2. Materials and methods

2.1. Cell culture

A549 and 95C cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin. The cells were originally obtained from the American Type Culture Collection.

Cultures were maintained at 37 °C in a humidified incubator, incubated for 12 h under normoxic (20% O2, 5% CO2) or hypoxic (1% O2, 5% CO2, and 94% N2), and were used in subsequent experiments. 100 nM Digoxin was used as an inhibitor of HIF-1α as previously described [20].

2.2. RNA oligoribonucleotides and transfection

MiR-182 mimic (MSY0000259), inhibitors (MIN0000259), and AllStars negative control siRNA (SI03650318) were synthesized by Qiagen (Germany). The transfection was conducted using lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

2.3. Plasmid construction and luciferase reporter assay

The putative miR-182 binding site in the 3′ UTR of target gene HIF1AN (wt or mt, as shown in Fig. 4G) were cloned into psi-CHECK (Promega) vector downstream of firefly luciferase 3′ UTR as a primary luciferase signal with rellina luciferase as the normalization signal. The psi-CHECK vector itself provided renilla luciferase signal as normalization to compensate the differences between transfection and harvested efficiencies. Transfection was performed using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after transfection. Luciferase assays were performed by using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Luciferase activity was measured on a luminometer (Berthold Technologies).

2.4. MTT cytotoxicity assay

Cells were plated in 96 well plates 12 h before the treatment. The cells were then transfected with RNA Oligoribonucleotides or treated with digoxin for indicated hours. At the end of the treatment, 10% v/v of 5-mg/ml solution of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) agent was added for 2 h. The medium was then removed and the cells were dissolved in DMSO (Sigma-Aldrich). Relative cytotoxicity was determined by measuring the absorbance at 570 nm using a luminometer (Berthold Technologies).

2.5. Metabolite analysis

Glucose uptake (MAK083), lactate release (MAK064)and intermediate products of glucolysis (Glucose-6P: MAK014, Fructose-6P: MAK020, 2 P-Glycerate: MAK180, P-enolpyruvate: MAK102, Pyruvate: MAK071)were measured by assay kits from Sigma-Aldrich, and Fructose-1,6 B P was measured by assay kit from Solarbio (MS2209), following the manufacturer's instructions using a luminometer (Berthold Technologies).

2.6. RNA extraction

RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and qualified with Agarose gel electrophoresis. Unqualified RNA samples were excluded in subsequent experiments.

2.7. miRNA real-time PCR analysis

To detect the cellular level of miRNA-182, the reverse transcription was carried out using High Capacity RNA-to-cDNA kit (Takara) with no more than 500 ng RNA per 10 μL PCR system as template. qRT-PCR was then taken using TaqMan probe (Roche). MiRNA-182 level was carried out using the TaqMan probe and Mater Mix (Thermo Fisher Scientific) in an ABI 7900 thermocycler. MiR-182 level was presented relative to U6 gene.

2.8. Real-time PCR for mRNA expression

The reverse transcription was carried out using High Capacity RNA-to-cDNA kit (Takara) with no more than 500 ng RNA per 10 μL PCR system as template. Real-time PCR to measure the mRNAs expressions of alpha-enolase (ENO1), GLUT1, HIF-1α, HK1, HK2, LDHA, PDK1 were carried out using SYBR green detection and standard techniques. cDNA was transcribed from total RNA using random 9 primers. PCR primers as follows:

ENO1-F	CGCGGATCCATGTCTATTCTCAAG
ENO1-R	TGTCGACTGCCCACAGCTTACTTG
GLUT1-F	GATTGGCTCCTTCTCTGTGG
GLUT1-R	TCAAAGGACTTGCCCAGTTT
HIF-1α-F	CTGATCATCTGACCAAAACTC
HIF-1α-R	GTTTCAACCCAGACATATCCAC
HK1-F	TCTCCAGAATCATGGACCA
HK1-R	GATCCTGCTCTTAGGCGTTC
HK2-F	GAGCCACCACTCACCCTACT
HK2-R	ACCCAAAGCACACACGGAAGTT
LDHA-F	TTGACCTACGTGGCTTGGAAG
LDHA-R	GGTAACGGAATCGGGCTGAAT
PDK1-F	CTGTGATACGGATCAGAAACCG
PDK1-R	TCCACCAAACAATAAAGAGTGCT

qPCR was performed with gene specific primers at 95 °C for 10 s, 60 °C for 60 s (40 cycles) in 10 μl reaction mix containing 3 μl cDNA, 2 μl primers and 5 μl SYBR Green master mix (Applied Biosystems) using an ABI 7900 thermocycler. Beta-actin was used as an internal control. Quantification was performed with the △△Ct method.

2.9. Statistical analysis

All statistical tests were performed by utilizing SPSS 16.0 software. The significance of differences between groups in terms of proliferation, metabolites, mRNA expression and Luciferase activity was determined using Welch's *t*-test. In all analyses, a P value of less than 0.05 was considered significant.

3. Results

3.1. The function of differentially expressed miR-182 on cell proliferation

Two NSCLC cell lines differentially expressed miR-182 were used in this study. One is A549, with relatively lower expression of miR-182. Another one is 95C, with relatively higher expression of miR-

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