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microRNA 181a-5p Reprogramed Glucose and Lipid Metabolism in Non- Small Cell Lung Cancer

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Abstract

microRNAs play critical roles in cancer metabolisms. miRNA-181a-5p is significantly down-regulated in non-small lung cancer tissue and mesenchymal like lung cancer cells. Epithelial-mesenchymal transition mechanisms and cancer metabolism are controlled by the same signaling pathway. To assess the functional role of miR-181a-5p in cancer metabolism, we performed functional assay and searched for target genes. To define the role of endogenous miR-181a-5p in cancer metabolism, we performed LDH assay, glucose uptake assay, Oil Red O staining, and mitochondrial ATP synthase inhibitor assay. Target gene of miR-181a-5p was determined via luciferase reporter assay, qRT-PCR, and western blot analyses. Functional effect of miR-181a-5p on cancer metastasis was measured by migration and invasion assays. Our results showed that overexpression of miR-181a-5p decreased aerobic glycolysis and lipid content. Overexpression of miR-181a-5p also diminished cancer invasion and migration. SIRT1 and ACSL4 were repressed by binding of miR-181a-5p to the 3'-UTR. These findings suggest that miR-181a-5p might be involved in reprograming of cancer metabolism.

Keywords: Lung Cancer; microRNA; Aerobic Glycolysis; Lipid Metabolism; Cancer Metabolism

Introduction

Cancer cells required many nutrients for rapid cell proliferation, energy production, and increased biosynthetic activity of various cellular constituents required for cell division [1]. Reprograming of glucose metabolism and lipid metabolism within the tumor microenvironment is an effective strategic shifting for survival and invasion. Along with metabolic reprogramming, unavoidable by-products of aerobic metabolism such as reactive oxygen species may damage cells and promote DNA mutations [2]. Thus, metabolic reprogramming may trigger tumorigenesis [3]. Enhanced aerobic glycolysis and lipid metabolism provides cancer cells with avoidance of immune surveillance, survival advantage, and metastasis [4]. Genetic or epigenetic changes of oncogene or tumor suppressor gene causes disturbances to cell signaling pathways. They might also lead to reprogramming of glucose metabolism and lipid metabolism. Targeting metabolic reprogramming is a very promising and rapidly rising direction for anti-cancer therapy [5]. The importance of genetic regulation mechanisms by microRNA (miRNA) in the regulation of metabolic activity of tissues has been emphasized. Recent studies have demonstrated that miRNAs play very critical roles in glucose and lipid metabolisms [6]. In previous studies, miRNA-181a-5p has been found to be significantly down-regulated in lung cancer tissue compared to that in corresponding nonmalignant lung tissues [7-9]. We have also investigated miRNA profiles of mesenchymal-like lung cancer cell lines to determine the relationship between epithelial-mesenchymal transition mechanisms (EMT) of miRNA and non-small cell lung cancer (NSCLC) and found that miR-181a-5p is down-regulated in mesenchymal like lung cancer cell lines [10]. Recent evidence suggests that EMT-related transcriptomic alterations are correlated with metabolic reprograming in

cancer. Metabolic alterations may allow cancer to adapt to environmental stressors, supporting the irregular molecular demand for rapid proliferation of cancer cells. Moreover, it has been demonstrated that expressions of key molecules in signaling pathways of EMT are also main regulators in dysregulated metabolic alteration [11]. These findings suggest that miR-181a-5p might be involved in lung cancer metastasis and metabolism.

We hypothesize that miR-181a-5p plays a role in cancer metabolism of non-small lung cancer cells. To test whether miR-181a-5p could play a role in cancer metabolism of non-small lung cancer, we performed LDH, glucose uptake assay, and mitochondrial ATP synthase inhibitor assay [12,13]. Sirtuin 1 (SIRT1) and acyl-CoA synthetase long-chain family member 4 (ACSL4) were selected as candidate targets of miR-181a-5p based on literature search. SIRT1 and ACSL4 play a role in cancer metabolism [14-16]. To determine whether miR-181a-5p could regulate SIRT1 and ACSL4, we performed luciferase assays. We report that miR-181a-5p decreased aerobic glycolysis and lipid content. Overexpression of miR-181a-5p also diminished cancer invasion and migration. SIRT1 and ACSL4 were repressed by binding of miR-181a-5p to the 3'-UTR. Thus, miR-181a-5p is likely to be an important regulator of non-small cell metabolism and metastasis.

Materials and Methods

Cell Culture and Growth Conditions

Non-small lung cancer cells (HCC358, HCC827, HCC1438, H292, H262, H522, A549, H460 and H1299) were maintained in RPMI 1640 medium (GIBCO BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin).

Glucose-Uptake Assay

H460 and H1299 cells were transfected with miR-181a-5p mimic (Ambion) and miR-mimic-Negative control#1 (Ambion). At 48 hours after transfection, glucose uptake rate was measured using Glucose Uptake Assay Kit Colorimetric (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Lactate Dehydrogenase Assay

At 72 hours after transfection with miR-181a-5p mimic (Ambion) or miR-mimic-Negative control#1 (Ambion) in H460 and H1299 cells, CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) was performed to quantitatively measure lactate dehydrogenase (LDH) levels according to the manufacturer's instructions.

Mitochondrial ATP synthase Inhibition Assay

Following miRNA transfection on day 3, H460 and H1299 cells were treated with oligomycin A for 30 minutes. Cellular ATP changes after treatment were measured with CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions.

Measurement of extracellular acidification rate (ECAR) after treatment of miR-181a-5p

The ECAR of H460 cells were measured using the XFp extracellular flux analyzer (Agilent Technologies, Santa Clara, CA, USA) as follows: 100,000 cells were plated per well into an XFp cell culture microplate. Experiments were conducted by the manufacturer's protocol. The assay was performed in triplicate.

Lipid Staining

To observe fat droplets in lung cancer, A549 cells were stained with Oil Red O (Sigma, USA) on day 3 following transfection with miRNA mimics or siRNA. Cells were washed twice with PBS and fixed with 10% formalin for 60 minutes. Cells were stained with 0.5% Oil Red O 100% propylene glycol (Sigma) for 5 min at 60 °C and subsequently washed twice with distilled water. Cells stained with hematoxylin for 30 seconds were imaged under a light microscope (Olympus, Tokyo, Japan).

Invasion and migration assays

The invasion assay was performed in triplicates using 48-well microchemotaxis chambers (Neuro. Probe, Inc., Gaithersburg, MD, USA) and 8-μm pore membranes (Neuro. Probe, Inc., Gaithersburg, MD, USA) pre-coated with 10 μg/ml Matrigel (BD Bioscience). Cells ($1X10^4$) in 50 μl of serum-free medium were placed in the upper chamber. The lower chamber was filled with 26-27 μl of medium supplemented with 10% FBS. After incubation at 37˚C for 24 hours, cells that migrated to the lower surface of the membrane were stained with a Diff-Quick kit and counted under a microscope. The migration assay was done using the same procedure using membranes coated with 5 μg/ml collagen IV (TRAVIGEN, Gaithersburg, MD, USA).

TaqMan miRNA expression assay

qRT-PCR analysis for miRNAs was performed in triplicates using MicroRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

miRNA mimic and siRNA transfection

Cells were seeded into 6-well plates at a density of 1.2×10^5 cells/well. On the next day, cells were transfected with 30 nM miR-181a-5p mimic (Ambion Austin, TX, USA) and miR-mimic-Negative control#1 (Ambion) with Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Cells were also transfected with specific Silencer® Select siRNA for SIRT1 (Ambion), ACSL4 (Ambion), and Silencer® Select Negative Control No. 1 siRNA (Ambion) using Lipofectamine™ RNAiMAX (Invitrogen).

qRT-PCR

Total RNA was isolated with TRIzol solution (Ambion) according to the manufacturer's instructions. First strand cDNA was synthesized using oligo (dT) primer and SuperScript III First-strand Synthesis System (Invitrogen). Aliquots of the reaction mixture were used for qPCR amplification with a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories). PCR was run for 40 cycles of denaturation at 95 ˚C for 15 seconds, annealing at 56 ˚C for 15 seconds, and elongation at 72 ˚C for 15 seconds. Gene expression was quantified by the comparative CT method, with CT values normalized to that of housekeeping gene ß-actin. After amplification, melting curve analysis was performed to ensure the specificity of products.

Western blot

Cells were lysed in Pro-Prep protein extraction solution (INtRON Biotechnology, Gyeonnggi-do, Korea) at 72 h after transfection. An equal amount of proteins was resolved on 8% SDS-PAGE gels (Laemmli, 1970). Primary antibodies used for the analysis included mouse anti-SIRT1(1:1000, Abcam, Cambridge, UK), ACSL4 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and b-actin antibodies (1:2000; Santa Cruz Biotechnology).

Approach to search the potential target genes of miR-181a-5p

SIRT1 and ACSL4 were selected as candidate targets of miR-181a-5p based on literature search and in silico program (TargetScanHuman 7.2).

Luciferase Reporter Assays

To verify that miR-181a-5p could regulate SIRT1 and ASCL4 gene directly, we generated a Renilla luciferase reporter plasmid cloned downstream to a segment of SIRT1 and ASCL4 3'UTR containing putative miR-181a-5p binding sequences that were predicted to have two binding site [position 2345-2367 bp of SIRT1 3'UTR (NM_012238) and position 2399-2421 bp of ASCL4 3'UTR (NM_004458)] using TargetScanHuman 7.2. These constructs were then co-transfected into NCI-H1299 cells with miR-181a-5p mimic or mimic-Negative control and Renilla/firefly reporter plasmid. After 48h, renilla/firefly luciferase activity was measured using a Lumat LB9501 instrument (Berthold, Bad Wildbad, and Germany). Results were normalized against the activity of firefly luciferase. All experiments were performed in triplicates.

Statistical Analyses

Statistical differences between groups were analyzed using Student's t-test when two groups were compared. Statistical significance was considered when P value was less than 0.05.

Results

Function of miR-181a-5p in Aerobic Glycolysis

We first investigated the biological function of miR-181a-5p in aerobic glycolysis. Our previous studies have shown that miR-181a-5p has low expression in H460 and H1299 cell lines (mesenchymal-like lung cancer cells) [10]. Thus, experiments were carried out using H460 and H1299 cell lines. Ectopic expression of miR-181a-5p in human lung cancer cells H460 and H1299 efficiently reduced glucose consumption (Figure 1A). Consistent with the effect of miR-181a-5p on glucose consumption of cancer cells, overexpression of miR-181a-5p significantly reduced LDL production (Figure 1B). Cancer cells are known to produce ATP from both aerobic glycolysis and Oxidative phosphorylation (OXPHOS). Thus, we investigated the relative contribution of aerobic glycolysis and OXPHOS to the production of ATP with regard to miRNA-181a-5p. To ascertain the proportion of ATP generated from OXPHOS versus glycolysis, we limited OXPHOS ATP synthesis by treating lung cancer cells with oligomycin A. We found that the relative ATP production was reduced in lung cancer cells treated with miR-181a-5p and oligomycin A. This indicates that miR-181a-5p treatment could reduce a proportion of ATP from glycolysis, suggesting that miR-181a-5p could inhibit aerobic glycolysis in lung cells (Figure 1C). We next assessed glycolytic flux by measuring the extracellular acidification rate (ECAR) to confirm inhibitory effects of miR-181a-5p on the glycolysis of non-small lung cancer cell. Overexpression of miR-181a-5p significantly reduced glycolysis rate and glycolytic capacity of H460 cells.

miR-181a-5p reduces total lipid in lung cancer cell

(Figure 1D). Oil Red O staining of lung cancer cells revealed reduced lipid droplets in miR-181a-5p treated cells. ASCL4 siRNA

also induced reduction of lipid droplets in lung cancer cells compared to the negative group (Figure 1E). These results indicate that miR-181a-5p could reduce lipid metabolism in lung cancer by targeting ASCL4.

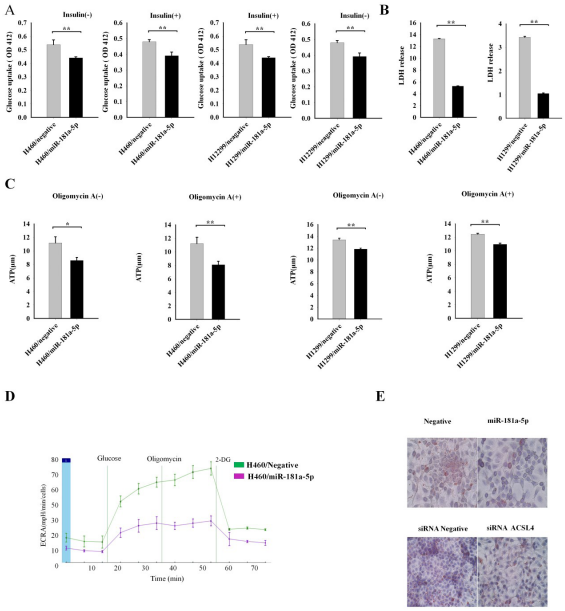

Figure 1: miR-181a-5p decreases aerobic glycosis and lipid metabolism in lung cancer cells. **A:** miR-181a-5p decreases glucose levels intracellularly in 72h. **B:** miR-181a-5p decreases Lactate dehydrogenase (LDH) release from cell death. Negative: miRNA mimic-Negative control#1. **C:** ATP levels in negative control or miR-181a-5p with or without treatment with oligomycin A. miR-181a-5p decreases ATP production from lung cancer cells. Negative: miRNA mimic-Negative control#1. D, Measurement of extracellular acidification rate (ECAR) in H460 cells with negative control or miR-181a-5p treatment. ECAR were measured followed by treatment with oxidative phosphorylation inhibitor oligomycin, and subsequently with glycolytic inhibitor 2-deoxyglucose (2-DG). miR-181a-5p treatment also exhibited decreased ECAR. E: Lipid content in A549 cells treated with miR-181a-5p and siRNA for ACSL4. Oil Red O staining showed that miR-181a-5p reduced lipid droplets (red spots) in A549 cells. SiRNA Negative: Silencer® Select Negative Control No. 1 siRNA (Ambion). Data are presented as means ± SD of three independent experiments. *P<0.05, **P<0.01 compared to the control.

miR-181a-5p inhibits lung cancer cell migration and invasion in vitro

To determine whether overexpression of miR-181a-5p might affect lung cancer progression, we performed invasion and migration assays using a lung cancer cell line. Cells transfected with miR-181a-5p mimic displayed significant reduction of invasion and migration at various degrees of H460 and H1299 cells (Figures 2A and B). To confirm that the effect of miR-181a-5p in lung cancer cells occurred through SIRT1 and ACSL4 repression, siRNA was used to transfect H1299 cells. SIRT1 and ACSL4 siRNA decreased both invasion and migration (Figures 2C and D).

Quantitative analysis of miR-181a-5p, SIRT1, and ACSL4 expression in lung cancer cell lines

Quantitative reverse transcription-PCR (qRT-PCR) was applied to detect expression levels of miRNA-181a-5p, SIRT1, and ACSL4. In H358, H292, H1438, and H827 cell lines (epithelial-like lung cancer cells), miR-181a-5p was highly expressed whereas SIRT1 and ACSL4 showed low expression [10,17]. However, miR-181a-5p exhibited low expression while SIRT1 and ACSL4 had high expression in H226, H460, H522, and H1299 cell lines (mesenchymal-like lung cancer cells) (Figure 3A) [10,17]. Thus, miR-181a-5p expression is decreased in cells with increased expression of SIRI and ACSL4 while its expression is increased in cells with reduced expression of SIRI and ACSL4, at least at cell line level.

miR-181a-5p regulates SIRT1 and ACSL4 expression at mRNA and protein levels

miRNAs can suppress the expression of target genes through translational repression or degradation of target transcripts. To assess the functional role of miR-181a-5p in downregulation of endogenous SIRT1 and ACSL4 expression, H460 and H1299 cells were transfected with miR-181a-5p mimics and expression levels of targets genes were then measured by qRT-PCR and Western blot analysis. When miR-181a-5p was overexpressed, relative expression levels of SIRT1 and ACSL4 were diminished compared to those in the control group (Figure 3).

SIRT1 and ACSL4 transcriptions are repressed by binding of miR-181a-5p to the 3'-UTR

To verify that miR-181a-5p could regulate SIRT1 and ACSL4 genes directly, we generated a Rellina luciferase reporter plasmid. It was cloned downstream to each segment of SIRT1 and ACSL4 3'UTR containing the putative miR-181a-5p binding sequence (Figure 4A). As shown in Figure 4B, significantly lower luciferase activity was generated by miR-181a-5p mimics as compared with negative miRNA ($P < 0.05$). This result indicates that miR-181a-5p can suppress SIRT1 and ACSL4 transcriptions by

directly binding to the 3'-UTR. Indirectly, relative expression levels of p53, p27, and p21 mRNA undergoing regulation by SIRT1 in H460 cell lines were increased by miR-181a-5p (Figure 4C)

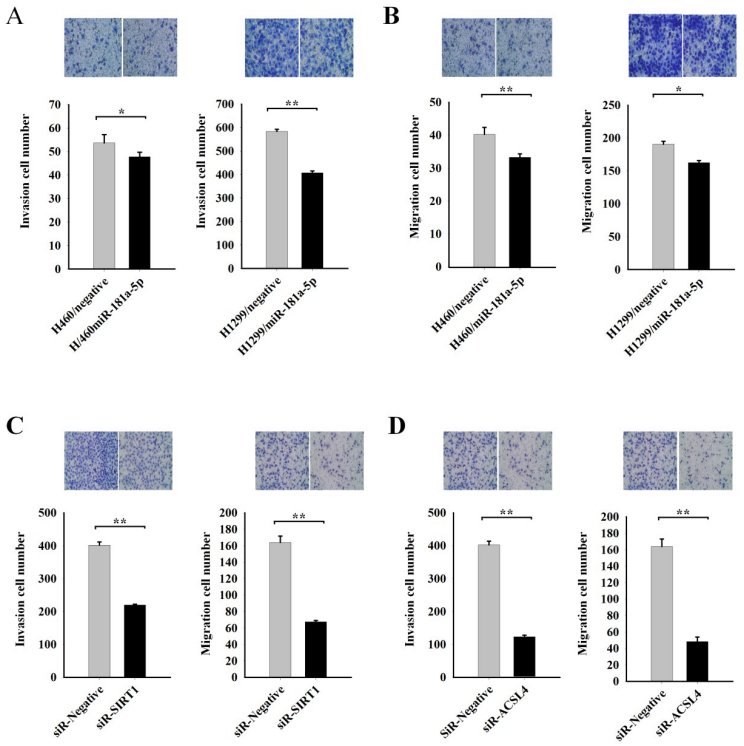

Figure 2: miR-181a-5p inhibits tumor cell invasion and migration in vitro. **A:** Microchemotaxis chamber invasion assay of H460 and H1299 cells transfected with negative and miR-181a-5p. miR-181a-5p mimic displayed significant reduction of invasion to different degree in H460 and H1299 cell lines. Negative: miRNA mimic-Negative control#1. **B:** Microchemotaxis chamber migration assay of H460 and H1299 cells transfected with negative and miR-181a-5p. miR-181a-5p mimic displayed significant reduction of migration to different degrees in H460 and H1299 cell lines. Negative: miRNA mimic-Negative control#1. **C:** Microchemotaxis chamber invasion and migraion assay of H1299 cells transfected with scramble siRNA and siRNA SIRT1. SIRI1 siRNA decreased lung cancer cell invasion and migration. SiR-Negative: Silencer® Select Negative Control No. 1 siRNA (Ambion). **D:** Microchemotaxis chamber invasion and migration assay of H1299 cells transfected with scramble siRNA and siRNA ACSL4. ACSL4 siRNA decreased lung cancer cell invasion and migration. SiR-Negative: Silencer® Select Negative Control No. 1 siRNA (Ambion). Data are presented as means ± SD of three independent experiments. *P<0.05, **P<0.01 compared to the control.

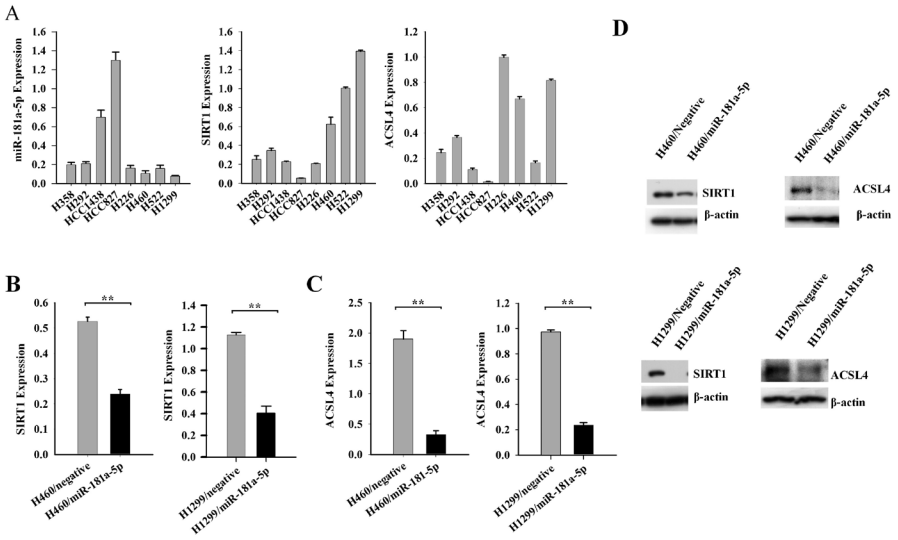

Figure 3: Relationship of SIRT1 and ACSL4 in cell lines. **A:** Real-time (RT)-PCR analysis of miR-181-5p, SIRT1, and ACSL4 expression in lung cancer cell lines. miR-181a-5p and targets genes (SIRT1 and ACSL4) showed a negative correlation tendency in lung cancer cell lines. **B:** The effect of miR-181a-5p on SIRT1 mRNA expression in lung cancer cells. SIRT1 mRNA was diminished in miR-181a-5p treated cell lines. **C:** Effect of miR-181a-5p on ACSL4 mRNA expression in lung cancer cells. ACSL4 mRNA was diminished in miR-181a-5p treated cell lines. **D:** Effect of miR-181a-5p on SIRT1 and ACSL4 protein expression in lung cancer cells. SIRT1 and ACSL4 protein levels were diminished in miR-181a-5p treated cell lines. Data are presented as means ± SD of three independent experiments. *P<0.05, **P<0.01 compared to the control.

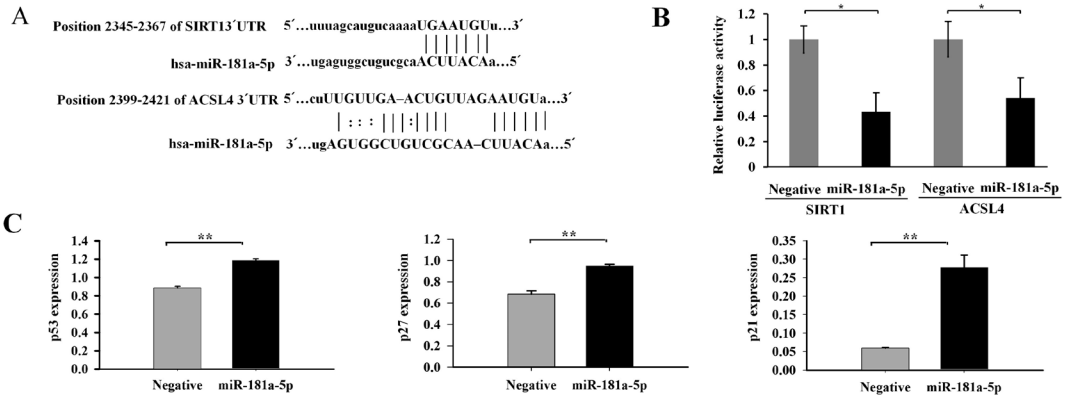

Figure 4: SIRT1 and ACSL4 are directly regulated by miR-181a-5p in lung cancer cells. **A:** Predicted miR-181a-5p binding sites within the 3'-UTR of SIRT1 and ACSL4 mRNA. **B:** The signal from a luciferase reporter was significantly decreased at one miR-181a-5p target site at the 3-UTR of SIRT1 and ACSL4. **C:** Effect of miR-181a-5p on p53, p27, and p21 expression in lung cancer cells. Relative expression levels of p53, p27, and p21 mRNA were increased in miR-181a-5p treated cell lines. Data are presented as means ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared to the control.

Discussion

Carcinogenesis occurs through complicated and multistep molecular changes of oncogenes, tumor suppressors, and metabolic pathways. Change of metabolic pathways is a type of evolution that conforms deviation to the tumor microenvironment and bioenergetic requirements to allow for uncontrolled cell growth [18]. Warburg observed that tumor cells shifted from oxidative phosphorylation to aerobic glycolysis more than 90 years ago [19]. Since then, research is at the precipice of understanding the molecular details of cancer metabolism. Recently, many research studies have been carried out to understand cellular pathways needed for energy production and synthesis of molecules to sustain the replication of cells that allows for uncontrolled cell growth. Based on these researches, cancer metabolism is now a new promising therapeutic targeting strategy [20].

Hence, we systemically investigated the role of miR-181a-5p in lung cancer metabolism in this study. First, we confirmed that miR-181a-5p regulated aerobic glycolysis and lipid metabolism in lung cancer. Second, we confirmed that miR-181a-5p suppressed migration and invasion in lung cancer cell lines. Next, we showed that miR-181a-5p directly regulated SIRT1 and ACSL4 by binding to its 3' UTR. Moreover, induced overexpression of miR-181a-5p in lung cancer reduced LDH production, glucose uptake, and glycolysis. In addition, Oil Red O staining demonstrated that miR-181a induced downstream regulation of lipid metabolism. Lastly, our study showed that miR-181a suppressed migration and invasion of lung cancer by targeting SIRT1 and ACSL4.

Metastatic disease is responsible for more than 90% of all cancer-related deaths. "Rewiring" the metabolic programming is crucial for cancer metastases to overcome nutrient and energy deficits. Aerobic metabolism of glucose to lactate is critical for increasing lactate production in tumor cells. Lactate production induces normal cell death via caspase-mediated activation of p53-dependent apoptotic pathway whereas cancer cells can export lactate by monocarboxylate transporters, resulting in acidification of microenvironment [21]. Extracellular acidification of microenvironment provides a favorable microenvironment for the activation of proteases that can induce extracellular matrix degradation and facilitate tumor cells to metastasis [22-24].

p53 directly influences various glucose metabolism pathways, including glucose uptake, glycolysis, and oxidative phosphorylation. The expression of glucose transporters GLUT1 and GLUT4 is directly downregulated by p53. p53 inhibits the glycolytic pathway by upregulating the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), an enzyme that decreases levels of glycolytic activator fructose-2,6-bisphosphate. p53 promotes oxidative phosphorylation by activating the expression of cytochrome c oxidase assembly protein (SCO2) [20,25]. p53 also inhibits pyruvate dehydrogenase kinase 2 (PDK2) which inactivates the pyruvate dehydrogenase complex (PDC) that converts pyruvate to acetyl-CoA to carry out cellular respiration. p53 is known to counteract the Warburg effect by dampening aerobic glycolysis and promoting oxidative phosphorylation through multiple mechanisms [26,27]. NAD-dependent Class III histone deacetylase SIRT1 is a protein that is critically involved in cellular metabolism by deacetylating p53 [28-30].

Cancer cells require alteration not only in glucose metabolism, but also in lipid metabolism. Lipids including triacylglycerides, phosphoglycerides, sterols, and sphingolipids play several important roles at cellular and organismal levels. Fatty acids are the major building blocks for the synthesis of triacylglycerides that are mainly used for energy storage. Phosphoglycerides, sterols, and sphingolipids are the major structural components of biological membranes [31]. Alternations of lipid metabolism in cancer affect cellular processes, including cell growth, proliferation, differentiation, and motility [32]. Many lipids are synthesized from fatty acids. However, they must be activated by acyl-CoA synthetase (ACS) enzymes to enter bioactive pools. Mammals have five ACS isoforms (ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6) [33]. ACSL4 is upregulated in some colon cancers. Overexpression of ACSL4 promotes tumor cell survival by preventing apoptosis likely through depletion of un-esterified arachidonic acid which yields a proapoptotic signal. Chemical inhibition of ACSL1, ACSL3, and ACSL4 by triacsin C

induces apoptotic cell death in lung, colon, and brain cancer cells [16,34,35]. Although tumors display enhanced metabolism compared to benign tissues, metabolism in tumor is a distinct difference between individual patients. Due to genetic and environmental parameters, cancer metabolism of NSCLC is heterogeneous. EGFR-mutated adenocarcinomas are biologically indolent with lower glucose metabolism than wild-type tumors [36]. Metabolically active lung cancer requires targeting treatment of metabolic reprogramming [37,38].

Conclusion

In summary, our study demonstrates that miR-181a-5p can reprogram cancer metabolism in NSCLC. We identified miR-181a-5p as regulator of SIRT1 and ACSL4 expression. Therefore, miR-181a-5p appears to represent a novel therapeutic candidate to alleviate cancer progression in metabolically active lung cancer.

Acknowledgments

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