

miR-218 inhibits glucose metabolism in non-small cell lung cancer via the NF- κ B signaling pathway

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Abstract. High glucose metabolism is recognized as one of the hallmarks of cancer and increased expression levels of several key factors involved in glucose metabolism have been reported in non-small cell lung cancer (NSCLC). Previous studies showed that microRNA (miR)-218 is reduced in NSCLC, but its function in glucose metabolism in NSCLC is not fully understood. The present study aimed to investigate the effect of miR-218 on glucose metabolism in NSCLC cell lines and the underlying molecular mechanism. The present results suggested that miR-218 reduced glucose consumption, the mechanism of glycolysis and activity in the pentose phosphate pathway. In addition, glucose transporter 1 (GLUT1) was identified to be a direct target of miR-218, while overexpression of GLUT1 did not abolish the effect of miR-218 on glucose metabolism. The present results indicated that phosphorylation of NF- κ B p65 was significantly decreased by miR-218 in NSCLC cells and that activation of NF- κ B led to the inhibition of miR-218 regulation of glucose metabolism. In conclusion, the present results suggested that miR-218 downregulated glucose metabolism in NSCLC not only by directly targeting GLUT1, but also via the NF- κ B signaling pathway.

Introduction

Lung cancer is a cause of cancer mortality in patients. In total, >two-thirds of all lung cancer types are a part of the subtype

of non-small cell lung cancer (NSCLC) (1). In NSCLC, high expression levels of glucose transporter 1 (GLUT1) and hexokinase 2 (HK2) facilitate glucose uptake and are the initial steps of glucose metabolism (2,3). Previous studies have revealed high glucose metabolism as one of the hallmarks of numerous cancers, in which glycolysis provides ATP rapidly and the pentose phosphate pathway (PPP) generates pentose phosphates for ribonucleotide synthesis and NADPH to meet the demand of rapid proliferation (4-6). Previous studies have reported that inhibition of glycolysis and PPP influences the growth of NSCLC cells (7-9).

MicroRNAs (miRNA/miRs) are non-coding RNA molecules of 19-25 nucleotides that negatively regulate gene expression by interacting with 3'untranslated regions (3'UTRs) of the target genes (10). Several miRNAs have been reported to be associated with the progression of NSCLC, serving as either oncogenes or tumor suppressors (11-13). Previous studies have suggested that miR-218 expression levels are reduced in multiple cancer types (14,15), including NSCLC (16,17). Previous studies have also revealed that miR-218 can regulate the metastasis and chemosensitivity of NSCLC (18-20). GLUT1 is indicated to be a direct target of miR-218 in bladder cancer (21), however the function of miR-218 in glucose metabolism in NSCLC remains unclear and the underlying molecular mechanism is still unknown.

The present study aimed to investigate the effect of miR-218 on the glycolysis and PPP pathways and assess the impact of miR-218 on the expression levels of related key enzymes in NSCLC cell lines. Furthermore, the potential mechanisms associated with these effects were investigated.

Materials and methods

Cell culture and treatment. NCI-H23 and A549 human NSCLC cells were purchased from Shanghai Cell Biology Institute of Chinese Academy of Sciences. Cells were maintained in RPMI-1640 medium (cat. no. 22400089; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. 10437028; Gibco; Thermo Fisher Scientific, Inc.). NCI-H23 and A549 cells (2×10^5 cells/well) were seeded into 6-well plates and transiently transfected with 50 nM

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miR-218 mimics (cat. no. B02003) or mimics control (cat. no. B04002; both Shanghai GenePharma Co., Ltd.) using Lipofectamine 2000 reagent (cat. no. 11668019; Thermo Fisher Scientific, Inc.). After 48 h of transfection the subsequent experimentations were performed.

Preparation of lentivirus and establishment of stable cell lines. The GLUT1 gene sequence was amplified from the cDNA of A549 cell line by PCR and the following primers: 5'-CCGTCTAGAGCCACCATGGAGCCCAGCAGCAAGAAG-3' (forward); 5'-GGCGGATCCTCACACTTGGGAATCAGCCC-3' (reverse) were inserted into the plasmid pCDH1-CMV-MSC-EF1-Puro (System Biosciences) at the *Xba*I and *Bam*HI sites. The acquired vector was termed 'pCDH-GLUT1'. A sequence containing the U6 promoter and a template of inhibitor of κ B (I κ B α) short hairpin RNA (shRNA), shown in Table SI, were synthesized, inserted in pCDH-GLUT1 (30 ng/ μ l) by *Bam*HI and *Not*I restriction enzymes (New England Biolabs, Ltd.), and the acquired vector was termed 'pCDH-GLUT1-shI κ B α '. The package method of lentivirus preparation was performed as previously described (22). A549 cells were transfected with lentivirus for 48 h and with 30 μ g/ml puromycin (cat. no. P8230; Beijing Solarbio Science & Technology Co., Ltd.) for 2 weeks.

Total RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells or tissue samples using TRIzol[®] reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (1 μ g) was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) at 42°C, according to the manufacturer's protocol. qPCR was subsequently performed using the SYBR[®] Premix Ex Taq[™] kit (cat. no. RR420L; Takara Biotechnology Co., Ltd.). The thermocycling protocol for the reaction was as follows: Initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Gene expression was quantified using the 2^{- $\Delta\Delta$ C_q} method (23). The primers used in the research were as follows: miR-218 forward, 5'-TTGCGGATGGTTCGGTCAAGCA-3'; miR-218 reverse, 5'-ATCCAGTGCAGGGTCCGAGG-3'; U6 forward, 5'-CGCTTCGGCAGCACATATAC-3'; U6 reverse, 5'-AAAATATGGAACGCTTCACGA-3'. U6 was used as a reference gene.

Measurement of glucose, lactate and NADPH/NADP⁺. NCI-H23 and A549 cells were seeded into 6-well plates (2 \times 10⁵ cells/well), followed by culturing till 90% confluence was reached, replaced with fresh medium and then cultured for 6 h at 37°C. The supernatant was collected, in which the contents of glucose and lactate were determined by using a glucose assay kit and a lactic acid assay kit produced by Nanjing Jiancheng Bioengineering Institute Co., Ltd. (cat. no. F006-1-1 and cat. no. A019-2-1). Glucose consumption was calculated by subtracting the final concentration from the initial concentration of the medium. The NADPH/NADP⁺ ratios in cellular lysates were determined using an NADP/NADPH assay kit (cat. no. ab65349; Abcam) at an optical density of 450 nm. All these tests were performed according to the manufacturer's protocol.

Protein extraction and western blotting. Expression levels of target proteins and GLUT1 membrane localization were examined as previously described (24). Whole-cell proteins and membrane proteins were extracted using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) and a membrane protein extraction kit (Sangon Biotech Co., Ltd.), respectively. Protein concentration was quantified by bicinchoninic acid protein assay kit (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.) and proteins (40 μ g per lane) were separated in 12% polyacrylamide gel. After electrophoresis, the proteins were transferred into PVDF membrane (cat. no. IPVH00010; EMD Millipore), followed by blocking with 5% BSA (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) in TBST (with 0.1% Tween-20) at room temperature for 3 h and then incubating with primary antibodies (1:1,000) at 4°C overnight. Next, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (cat. no. SE12; 1:2,000; Beijing Solarbio Science & Technology Co., Ltd.) or anti-rabbit (cat. no. SE13; 1:2,000; Beijing Solarbio Science & Technology Co., Ltd.) secondary antibodies for 1 h at room temperature. The protein bands were visualized by BioVision ECL western blotting substrate kit (cat. no. K820-500; BioVision, Inc.). ImageJ software (version 1.5; National Institutes of Health) was used to analyze the intensities of the band signals obtained. The antibodies of GLUT1 (cat. no. ab115730), HK2 (cat. no. ab104836), glucose-6-phosphate dehydrogenase (G6PD; cat. no. ab993), NF- κ B p65 (cat. no. ab32536), and phosphorylated (p)-NF- κ B p65 (cat. no. ab86299) were obtained from Abcam. The antibodies of phosphofruktokinase-1 (PFK1; cat. no. sc-377346), ERK1/2 (cat. no. sc-514302), p-ERK1/2 (cat. no. sc-81492), JNK1/2 (cat. no. sc-137019) and p-JNK1/2 (cat. no. sc-293136) were obtained from Santa Cruz Biotechnology, Inc. The antibodies for Akt (cat. no. 9272) and p-Akt (cat. no. 4060) were produced by Cell Signaling Technology, Inc..

Luciferase reporter assay. The 3'UTRs of GLUT1, HK2, FPK1 and G6PD were synthesized and inserted into psiCHECK-2 plasmid (Promega Corporation) at the restriction enzyme sites of *Xho*I and *Not*I. Sequences are shown in Table SI. The acquired vectors were termed as 'psiGLUT1', 'psiHK2', 'psiFPK1' and 'psiG6PD'. Then cancer cells at a density of 4 \times 10⁵/well were seeded into 6-well plates and cotransfected with either 50 nm miR-218 mimics or miRNA negative control (NC) and 2 μ g above vectors, using Lipofectamine 2000 according to the manufacturer's protocol. The cells were lysed and then assayed for luciferase activity at 48 h post-transfection using a Dual-luciferase[®] reporter assay kit (Promega Corporation). The assays were repeated independently >3 times. Firefly luciferase was used as a reference for normalization.

Statistical analysis. All data were obtained from 3-5 independent experiments and are presented as mean \pm SD. Two-sided, unpaired Student's t-test was used for comparing between groups. One-way ANOVA was used to compare categorical variables. Significant differences between experimental groups were assessed using Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference. The analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.).

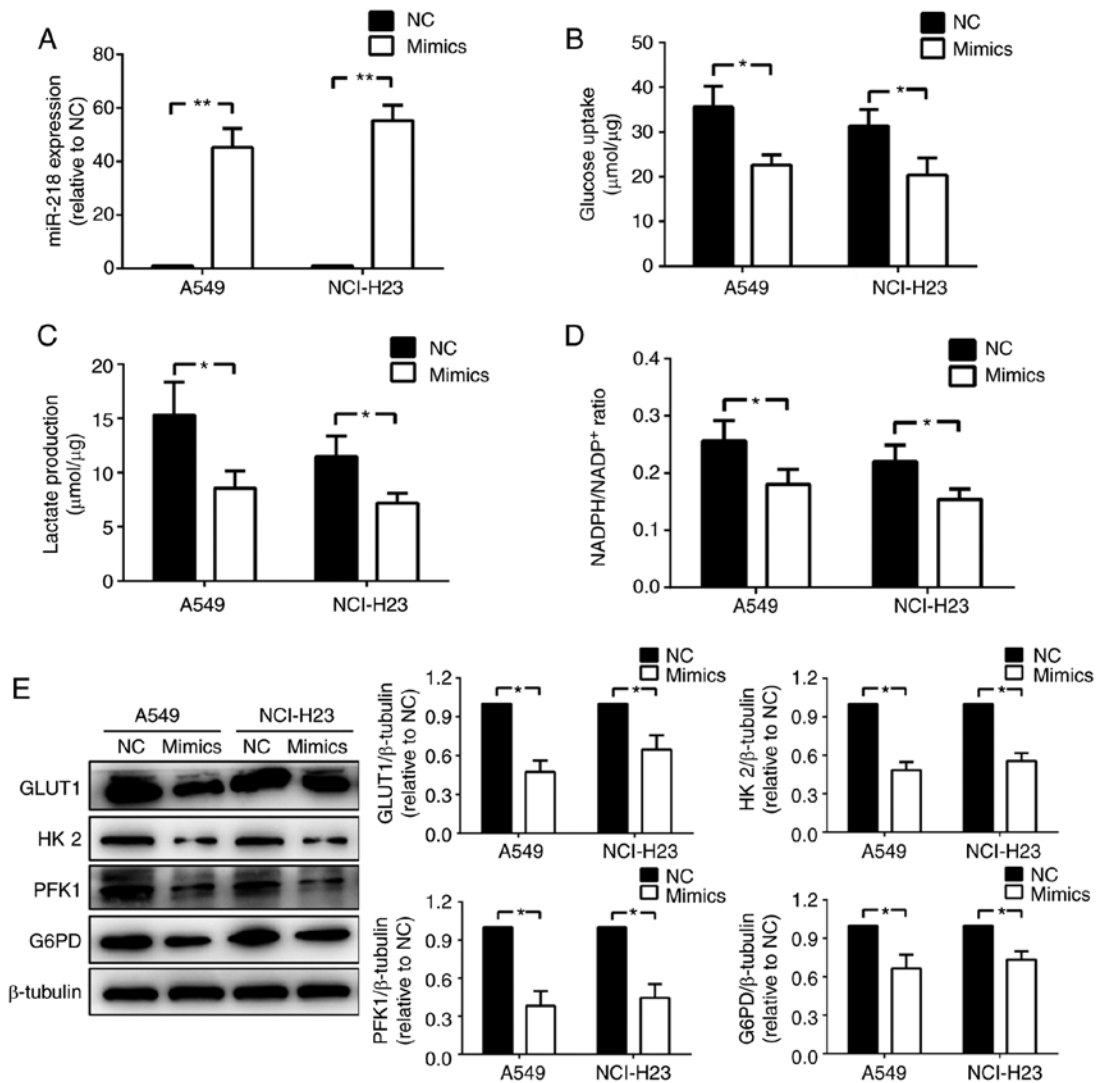


Figure 1. miR-218 reduces glucose consumption and activities of glycolysis and pentose phosphate pathway. (A) Expression levels of miR-218 were measured in NCI-H23 and A549 cells after transfection with NC or miR-218 mimics, n=3. Then (B) glucose consumption, (C) lactate production and (D) ratio of NADPH to NADP⁺ were investigated, n=5. (E) Western blotting was performed to determine the protein expression levels of key enzymes involved in glucose metabolism, n=5. *P<0.05 and **P<0.01. Data are presented as mean ± SD. NC, negative control; miR-218, microRNA-218; GLUT1, glucose transporter 1; HK2, hexokinase 2; PFK1, phosphofructokinase-1; G6PD, glucose-6-phosphate dehydrogenase.

Results

miR-218 reduces glucose consumption and activities of glycolysis and PPP. To study the effect of miR-218 on glucose metabolism in NSCLC cell lines, miR-218 mimics or NC were transfected into NCI-H23 and A549 cells. miR-218 expression levels were significantly increased in the two cell lines after transfected with mimics compared with the NC group (P<0.01; Fig. 1A). Then the levels of glucose and lactate in the supernatant, as well as the NADPH/NADP⁺ ratio in cellular lysates, were measured. Glucose uptake was significantly reduced by transfection with miR-218 mimics in both NSCLC cell lines (Fig. 1B), as well as lactate production, which represents the activity of glycolysis (Fig. 1C). Similarly, the ratio of NADPH, which is a product of PPP, to NADP⁺ in miR-218 mimic transfection group showed a significant decrease compared with the NC group (P<0.05; Fig. 1D). The present results indicated that miR-218 could decrease glucose uptake and the activities of glycolysis and PPP in NSCLC cells. In addition, the expression

levels of key enzymes involved in glucose uptake, glycolysis and PPP in the transfected NCI-H23 and A549 cells were investigated by western blotting. The present results suggested that GLUT1, HK2, PFK1 and G6PD expression levels were significantly decreased by miR-218 in both cell lines (Fig. 1E).

miR-218 inhibits glucose metabolism by downregulating GLUT1. To investigate whether miR-218 regulated the expression of GLUT1, HK2, PFK1 and G6PD directly, the 3'UTRs of these were inserted into luciferase reporter vectors. Then the luciferase reporter assay was used for detection after cotransfection with reporter vector and mimics or NC in A549 cells. The present results suggested a significant decrease in activity only by 3'UTRs of GLUT1 (Fig. 2A). Then A549-GLUT1, a cell line that overexpressed GLUT1 stably, was used to explore whether there are any other mechanisms involved in mediating glucose metabolism inhibition of miR-218 except for the downregulation of GLUT1 directly and was identified by western blotting (Fig. 2B). The present results indicated that glucose

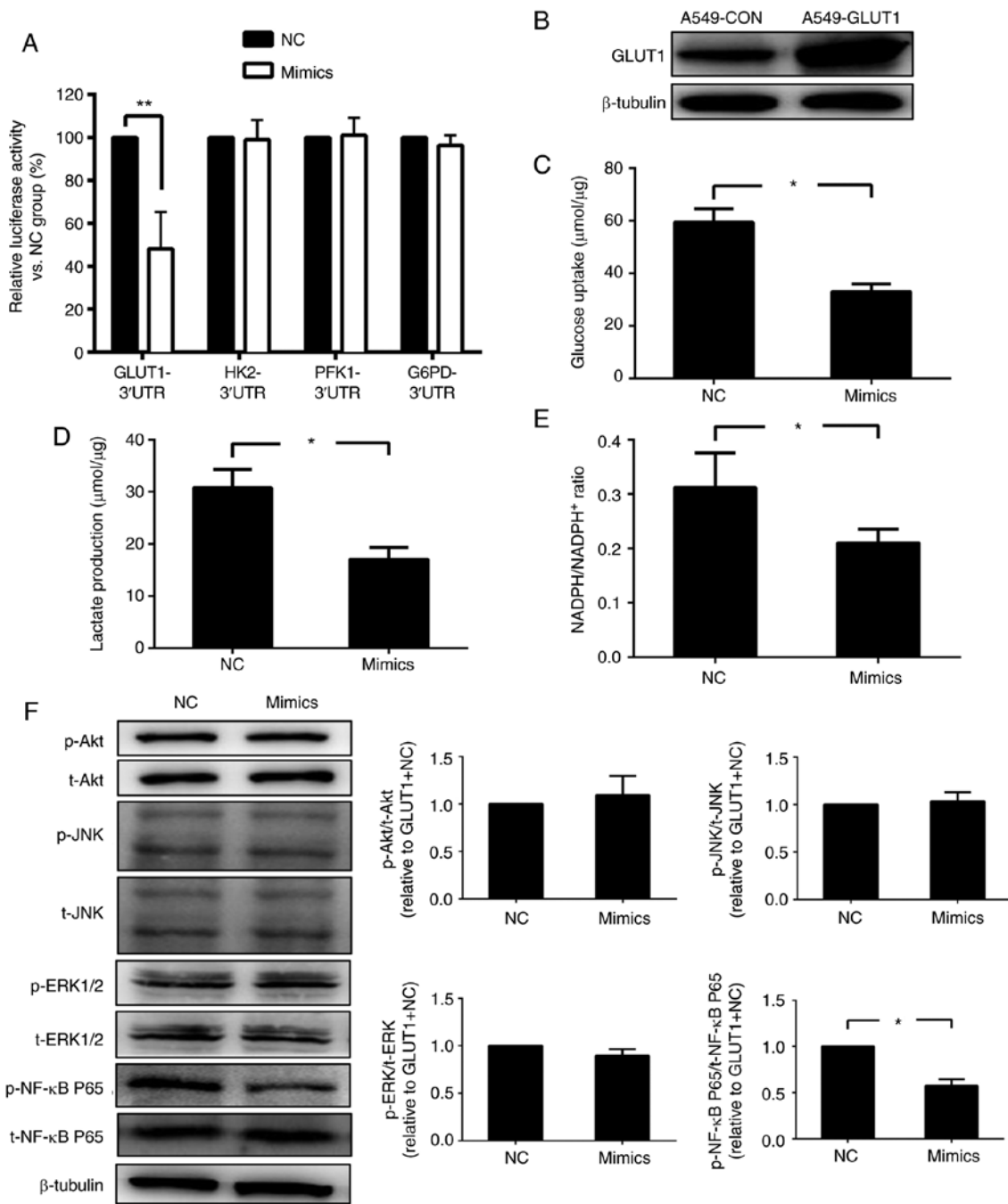


Figure 2. miR-218 inhibits glucose metabolism via downregulation of GLUT1. (A) A luciferase reporter assay was conducted to investigate whether key enzymes involved in glucose metabolism are direct targets of miR-218, $n=5$. (B) A549-GLUT1, a stable cell line that overexpressed GLUT1 was constructed and identified by western blotting. (C) Glucose consumption, (D) lactate production and (E) the ratio of NADPH to NADPH⁺ were measured after transfection with NC or miR-218 mimics, $n=5$. (F) Expression levels of several factors related to cell proliferation and survival were detected in A549-GLUT1 cells in both NC and mimics groups, $n=5$. * $P<0.05$ and ** $P<0.01$. Data are presented as mean \pm SD. NC, negative control; miR-218, microRNA-218; GLUT1, glucose transporter 1; HK2, hexokinase 2; PFK1, phosphofructokinase-1; G6PD, glucose-6-phosphate dehydrogenase; p-, phosphorylated; t-, total; UTR, untranslated region.

uptake, lactate production and NADPH/NADPH⁺ were reduced after transfection with miR-218 mimics in A549-GLUT1 (Fig. 2C-E), suggesting the possibility of other mechanisms.

To explore the signaling pathway involved in glucose metabolism inhibition of miR-218, phosphorylation levels of Akt, ERK1/2, JNK1/2 and NF-κB p65 were detected in A549-GLUT1 transfected with mimic or NC. The present results indicated that the expression level of p-NF-κB p65, but not the other three proteins, was significantly decreased

after transfection with the mimic (Fig. 2F). Therefore, the present results suggested that miR-218 may regulate glucose metabolism by modulating the activity of NF-κB.

miR-218 inhibits glucose metabolism via the NF-κB signaling pathway. To investigate whether the NF-κB pathway mediated glucose metabolism inhibition induced by miR-218, an inhibitor of NF-κB IκBα was stably knocked down by shRNA in A549-GLUT1 cells (Fig. 3A). The acquired cell line was termed

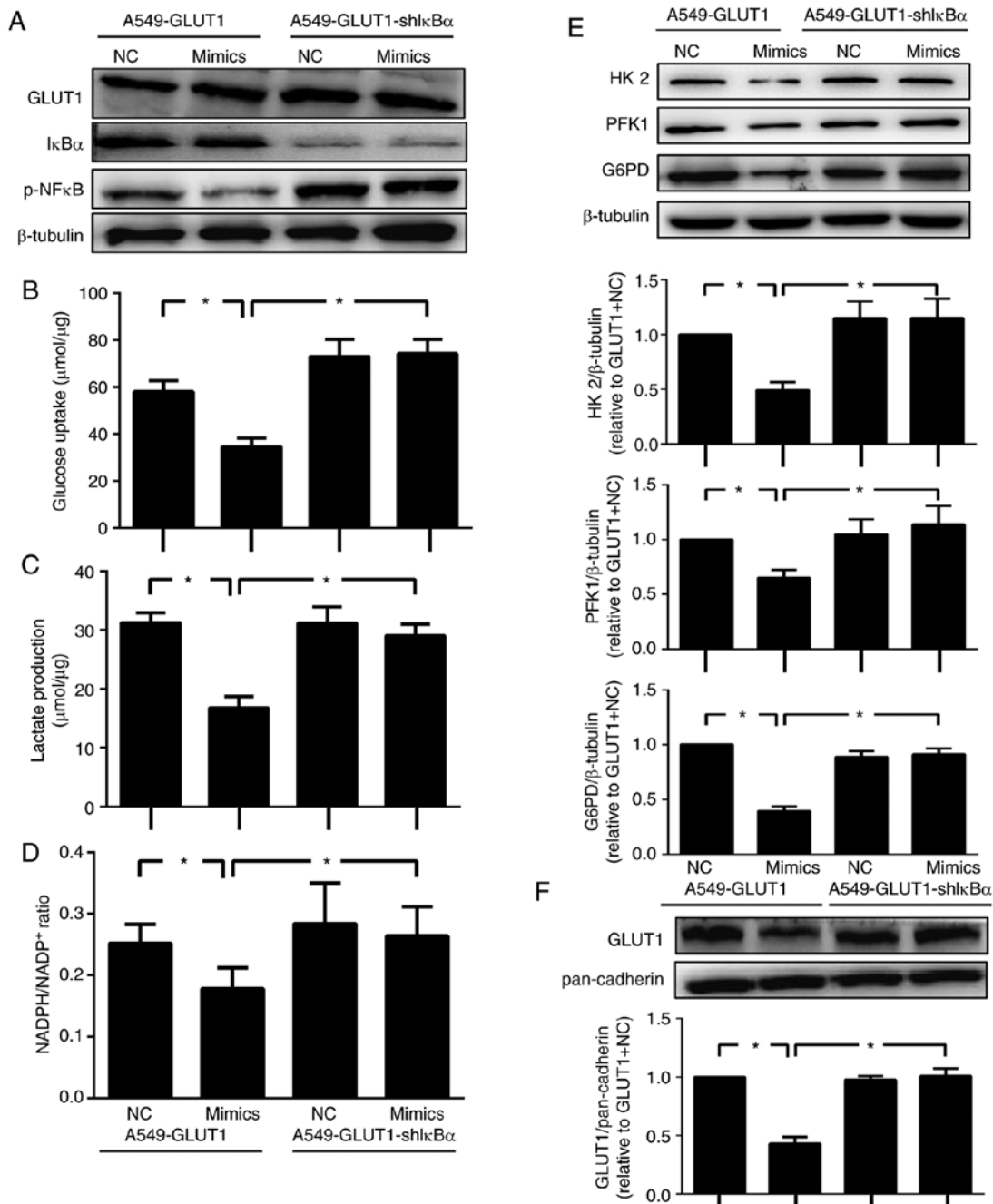


Figure 3. miR-218 inhibits glucose metabolism via the NF- κ B signaling pathway. (A) A549-GLUT1-shI κ B α , a stable GLUT1 overexpressing and I κ B α silencing cell line was constructed and identified by western blotting. (B) Glucose consumption, (C) lactate production and (D) the ratio of NADPH to NADP⁺ were examined in A549-GLUT1 and A549-GLUT1-shI κ B α cells after transfection with NC or miR-218 mimics, n=5. (E) Western blotting was performed to determine the protein expression levels of key enzymes involved in glucose metabolism and (F) the location of GLUT1 on cytomembrane, n=5. *P<0.05. Data are presented as mean \pm SD. NC, negative control; miR-218, microRNA-218; shRNA; short hairpin RNA; shI κ B α , short hairpin I κ B α RNA; GLUT1, glucose transporter 1; p-, phosphorylated; HK2, hexokinase 2; PFK1, phosphofructokinase-1; G6PD, glucose-6-phosphate dehydrogenase; I κ B α , inhibitor of κ B.

'A549-GLUT1-shI κ B α '. After transfection with miR-218 mimics, the A549-GLUT1 cells showed reduced glucose uptake, lactate production and NADPH/NADP⁺ ratio, and vice versa in the A549-GLUT1-shI κ B α (Fig. 3B-D). Similarly, HK-2, PFK1 and G6PD expression levels were decreased by miR-218 in A549-GLUT1 cells, but this effect was diminished in A549-GLUT1-shI κ B α cells (Fig. 3E). Moreover, this was in accordance with results reported in a previous study (24), in which the plasma membrane translocation of GLUT1 was reduced in mimic transfected A549-GLUT1 cells. No

difference was identified between A549-GLUT1-shI κ B α transfected with NC or mimics (Fig. 3F). The present results indicated that glucose metabolism inhibition of miR-218 may be reversed by the activation of the NF- κ B signaling pathway.

The proposed mechanisms of miR-218 regulation of glucose metabolism are shown in Fig. 4. miR-218 reduced the expression levels of GLUT1 directly, but also affected the membrane translocation of GLUT1 and expression levels of key enzymes in glycolysis and PPP pathways indirectly by inhibiting the NF- κ B signaling pathway.

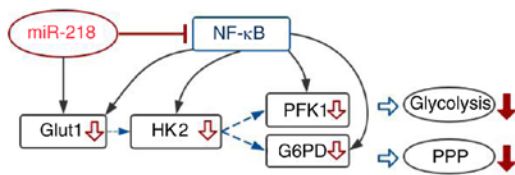


Figure 4. Potential mechanisms of miR-218 inhibition of glucose metabolism. The inhibitory action of miR-218 on glucose metabolism was via reducing the levels of GLUT1 directly and also inactivating the NF- κ B signaling pathway that affects membrane translocation or expression levels of the key enzymes involved in glucose metabolism indirectly. miR-218, microRNA-218; GLUT1, glucose transporter 1; HK2, hexokinase 2; PFK1, phosphofructokinase-1; G6PD, glucose-6-phosphate dehydrogenase; PPP, pentose phosphate pathway.

Discussion

Cancer cells have aberrant expression of miRNAs that are involved in modulating numerous cell signaling pathways (25). Previous studies have shown that miR-218 expression level is significantly reduced in NSCLC (16,17). Recently, miR-218 has been reported as a tumor suppressor in lung cancer cells by regulating the interleukin-6/STAT3 pathway (26). The present study investigated the effect of miR-218 on glucose metabolism and the possible underlying molecular mechanisms in NSCLC cells.

The present results suggested that miR-218 inhibited glucose uptake, glycolysis and PPP. Several rate-limiting enzymes such as GLUT1, HK2, PFK1 and G6PD play crucial roles during these glucose metabolism processes (27). Previous studies have shown that these enzymes are regulated directly or indirectly by miRNAs (28-32). The present results indicated that the expression levels of these enzymes were decrease by miR-218 in NSCLC cell lines. Subsequently, to identify whether the four rate-limiting enzymes were the direct targets of miR-218, a luciferase reporter assay was performed. The present results suggested that GLUT1 was directly modulated by miR-218, but the other enzymes were not. The miR-218 mimics had suppressive effects on glucose uptake, glycolysis and PPP. The present results suggested the presence of an indirect mechanism during the expression of rate-limiting enzymes mediated by miR-218. Previous studies have indicated that Akt, JNK, ERK and NF- κ B signaling pathways play important roles in glucose metabolism regulation (33-40). Therefore, in the present study the phosphorylation of these proteins was detected in A549-GLUT1 cells after transfection with miR-218 mimics. The present results suggested there were no significant differences in the phosphorylation levels of Akt, JNK and ERK, however p-NF- κ B p65 was reduced by miR-218 mimics. The present results indicated that NF- κ B may be responsible for the role of miR-218 in glucose metabolism. To investigate this effect, changes on glucose metabolism in A549-GLUT1-shI κ B α cells, A549-shI κ B α cells and A549-GLUT1 cells were studied. The present results suggested that glucose uptake, lactate production and NADPH/NADP⁺ ratio in A549-GLUT1-shI κ B α cells and A549-shI κ B α cells had no significant differences (data not shown). In addition, the present results showed that reduction of I κ B α , whose absence results in the activation of NF- κ B (41), inhibited the curb of miR-218 on glucose metabolism. The decreasing effects of miR-218 on membrane translocation of

GLUT1 and expression levels of HK2, PFK1 and G6PD were all reversed by the activation of NF- κ B.

In the present study the hypothesis was investigated *in vitro*, however metabolism in long-term cultivated cell lines may not fully reflect the situation in growing tumors, thus *in vivo* experiments are required to further investigate the effect of miR-218.

In the present study glycolysis and PPP were affected by miR-218 in NSCLC cell lines, thus similar effects based on the present results are expected *in vivo*. Although the present results suggested miR-218 inhibited glucose metabolism by inactivating the NF- κ B signaling pathway in NSCLC cells, the details of the underlying regulatory mechanism still remain unclear. Therefore, the targets of miR-218 in NF- κ B pathway should be investigated in future studies. The reasons for miR-218 expression level change in NSCLC, especially whether it was affected due to low glucose metabolism caused by itself, should be focused on.

In conclusion, the present results suggested miR-218 may inhibit glucose uptake, glycolysis and PPP in NSCLC cells by reducing the expression or membrane translocation of rate-limiting enzymes involved in glucose metabolism, in which NF- κ B played a critical role.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XY and QC designed the study. WT, XY and YS performed the experiments. JZ and HW analyzed the data. LW and DL contributed to constructing the engineering A549 cell lines and writing the original draft. WT and QC revised and edited the manuscript. All authors read and approved the final version of the manuscript for publication.

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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