Metformin mediated microRNA-7 upregulation inhibits growth, migration, and invasion of non-small cell lung cancer A549 cells

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Metformin, a medication widely used in the treatment of type 2 diabetes mellitus, has a possible antitumor effect in type 2 diabetes mellitus patients. MicroRNA-7 is a significant microRNA in non-small cell lung cancer. Metformin has an inhibitory effect on lung cancer and regulates the expression of certain microRNAs, but there is no report connecting metformin with microRNA-7 in lung cancer. Thus, we used qPCR to measure microRNA-7 expression in A549 non-small cell lung cancer cells treated with metformin. We used CCK8, cell scratch, and Transwell assays to test the growth, migration, and invasion of A549 cells. Western blotting was used to measure the expression level of relevant proteins in A549 cells. We found that microRNA-7 was dramatically upregulated by metformin via AMPK in a dose- and time-dependent manner. Both metformin and microRNA-7 mimic reduced A549 cell growth, migration, and invasion. Metformin downregulated the levels of p-NF-κB p65, p-Erk1/2, p-AKT, and p-mTOR proteins. The treatment with the microRNA-7 mimic had the same result. The decrease of these proteins caused the inhibition of A549 cell growth, migration, and invasion. Our discovery revealed that metformin, via increasing the expression of microRNA-7 mediated by AMPK, regulates the AKT/mTOR, MAPK/Erk, and NF-κB signaling pathways, thereby suppressing A549 cell growth, migration, and invasion. *Anti-Cancer Drugs* 31:345–352 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

Introduction

MicroRNAs (miRNA), with lengths of approximately 22 nucleotides, are a class of noncoding RNA molecules encoded by endogenous genes that degrade or inhibit the translation of the target mRNA 3′ untranslated regions by complete or incomplete base pairing. MicroRNA-7 (miR-7) was identified as a potential tumor suppressor targeting the epidermal growth factor receptor in glioblastoma in 2008 [1]. miR-7 plays a key role in both normal development [2] and human diseases, such as diabetes [3], and in several types of solid tumors [1,4,5]. It has been shown that miR-7 may act as a tumor suppressor that can regulate the occurrence and development of lung cancer by targeting PI3KR3/AKT, Bcl-2, IGF-1R, and other signaling pathways.

Metformin is a first-line medication for type 2 diabetes mellitus (T2DM) treatment. Metformin can lower blood glucose concentration by reducing hepatic gluconeogenesis and increasing glucose uptake in skeletal muscles; however, it does not cause hypoglycemia and rarely causes lactic acidosis [6]. Research interest in metformin's anticancer effects began in 2005 with Evans *et al.* [7], who showed a lower risk of cancer in T2DM patients after metformin therapy. In subsequent studies, metformin also reduced the risk for pancreatic cancer [8], lung cancer [9], endometrial cancer [10], and so on. The downstream effect of metformin is the phosphorylation of AMP-dependent kinase (AMPK), an important sensor of cellular energy charge; its activation inhibits AKT and mTOR signaling.

Growing reports have shown that metformin's anticancer effects are related to miRNA regulation. However, metformin has different effects on miRNAs in different types of cancers. Metformin induced upregulation of miR-193 family members, thus killing triple-negative breast cancer cells [11]. In renal cell carcinoma, metformin resulted in growth inhibition depending on miR-21-mediated PTEN expression [12].

Though many studies have focused on the effects of metformin on microRNAs, there is no report related to metformin with miR-7 in non-small cell lung cancer

(NSCLC). Therefore, we aimed to explore the tumor-inhibitory effects of metformin and the connection between metformin and miR-7 in the A549 cell line *in vitro* and to analyze the possible signaling pathways involved. Our results provide insight into the anti-lung cancer mechanism of metformin through miR-7, which may aid in the development of new therapeutic strategies for NSCLC patients develop.

Materials and methods

Cell culture
A549 cells were obtained from the American type culture collection and cultured in F-12 culture medium (Gibco, Grand Island, New York, USA) with 10% fetal bovine serum (FBS) (BI, Kibbutz Beit-Haemek, Israel) and 1% penicillin-streptomycin (HyClone, Logan, Utah, USA).

Transfection
Metformin (MET) was bought from Sigma (Darmstadt, Germany) and dissolved in phosphate buffer saline. A549 cells were inoculated in six-well cell culture plates with complete culture medium, and metformin (5–50 mM) was added –o the medium for 24 or 48 hours when the cells grew to a density of 70%–80%.

The miRNA mimic for hsa-miR-7-5p (MI0000263) and its negative control (mimic NC) were bought from RiboBio (Shanghai, China). The mimic and NC mimic were transfected by using Lipofectamine 2000 (Invitrogen, Calsbad, California, USA) at a final concentration of 50 nM. The miRNAs were added to A549 cells and incubated with complete medium for 24 or 48 hours.

A549 cells were treated with dorsomorphin (10 µM), an inhibitor of AMPK, for 24 hours.

CCK8 assay
A549 cells were plated in 96-well plates at a cell density of 5×10^4 cells/well in 100 µl complete medium. After 24 hours of incubation, mimic-, mimic NC- or metformin-treated A549 cells were incubated for 24 or 48 hours. Then, 10 µl of CCK8 reagent (DOJINDO, Tokyo, Japan) was added to each well and cultured for 2 hours at 37°C. The absorbance of cells in each well was observed at 450 nm.

Transwell assay
The Transwell assay used 24-well Transwell chambers (Corning, Kennebunk, Maine, USA). For the Transwell migration assay, 1.5×10^4/ml A549 cells were added to the upper chambers with serum-free F12 medium. For the Transwell invasion assay, 1.5×10^4/ml A549 cells were added to the upper chambers coated with Matrigel (BD Biosciences, New York, USA) at a density of 1.5×10^4 cells/ml in serum-free F12 medium.

The lower chamber contained 600 µl of medium containing 20% FBS. After culturing for 24 or 48 hours, unmigrated cells were removed from the upper chamber with cotton swabs, and migrated cells were fixed with methanol for 15 minutes and stained with 0.1% crystal violet for 15 minutes. To calculate the mean number of migrated cells, five independent visual fields were chosen for observation and photographed with a light microscope.

Cell wound scratch assay
A549 cells were inoculated in 6-well plates in serum-free F12 medium at a density of 4×10^5 cells/ml. After 24 hours, a sterile 10 µl pipette tip was used to create two wounds in the well. The cells were continuously cultured for 48 hours with different treatments. The scratch width was observed and photographed at the beginning of the assay and at 24 hours and 48 hours after wounding by a light microscope. The wound closure ratio was calculated as the percentage between the migrated distance and the initial wound distance.

qRT-PCR
The total RNA of A549 cells was extracted with TRIzol reagent (Invitrogen). The total RNA was detected using nanophotometer, and the 260/280 nm ratio of the RNA was between 1.9 and 2.1. The Mir-X miRNA First-Strand Synthesis Kit (Takara Biotechnology, Shiga, Japan) was used to reverse transcribe total RNA into cDNA. qRT-PCR was performed under the following conditions: 95°C for 30 seconds, 39 cycles of 95°C for 5 seconds, and 57.9°C for 30 seconds. The fold change of gene expression was calculated via the $2^{-\Delta\Delta}$ Ct method: the formula was $\Delta Ct = Ct_{gene} - Ct_{reference}$. Experiments were performed in triplicate.

Primer sequences: miR-7-5P F, 5′-GGCTGGAAGACT AGTGATTTTGTTG-3′ (The reverse primer was the universal primer in the kit); U6 (reference gene) F: 5′-GGAACGATACAGAGAAGATTAGC-3′; U6 R: 5′-TGGAACGCTTCACGAATTTGCG-3′.

Western blot analysis
The A549 cells were lysed with RIPA lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS) and 1% phenylmethylsulfonyl fluoride and a BCA Kit was used to quantify the protein concentrations. The protein samples were resuspended in 5× loading buffer and denatured for 10 minutes at 100°C. Proteins were separated in 6% or 10% SDS-PAGE and transferred onto NC membranes by electro-elution (100 V, 50–120 minutes, moist electrotransfer). The membranes were blocked at room temperature for 1 hour using 5% blocking solution and then incubated at 4°C overnight with a 1:1000 dilution of anti-GAPDH, anti-NF-κB p65, anti-Erk1 + 2 (Abcam, Cambridge, UK), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-mTOR, anti-phospho-mTOR (Ser2448), anti-phospho-NF-κB p65 (Ser536) (Cell Signaling Technology, Beverly, Massachusetts, USA), anti-AKT, and anti-phospho-AKT (Ser473) (Abclonal, Wuhan, China). The

membranes were washed with Tris buffer containing 0.1% Tween-20 (TBST) three times, then incubated with secondary antibody (1:5000; Signalway Antibody LLC, Maryland, USA) for 1 hour at room temperature. After several washes with TBST, electrochemiluminescence was used to detect the immunoreactive proteins.

Statistical analysis

GraphPad Prism 7.0 was used for statistical analysis. All experiments were repeated three times. The parametric independent *t*-test was used to perform the statistical comparisons between groups, and a result of $P < 0.05$ was considered significant.

Results

Either metformin or microRNA-7 can inhibit the growth, migration, and invasion of A549 cells

We used the CCK8 assay to test cell growth after metformin treatment. Increasing concentrations of metformin inhibited A549 cell growth at both 24 and 48 hours

Fig. 1
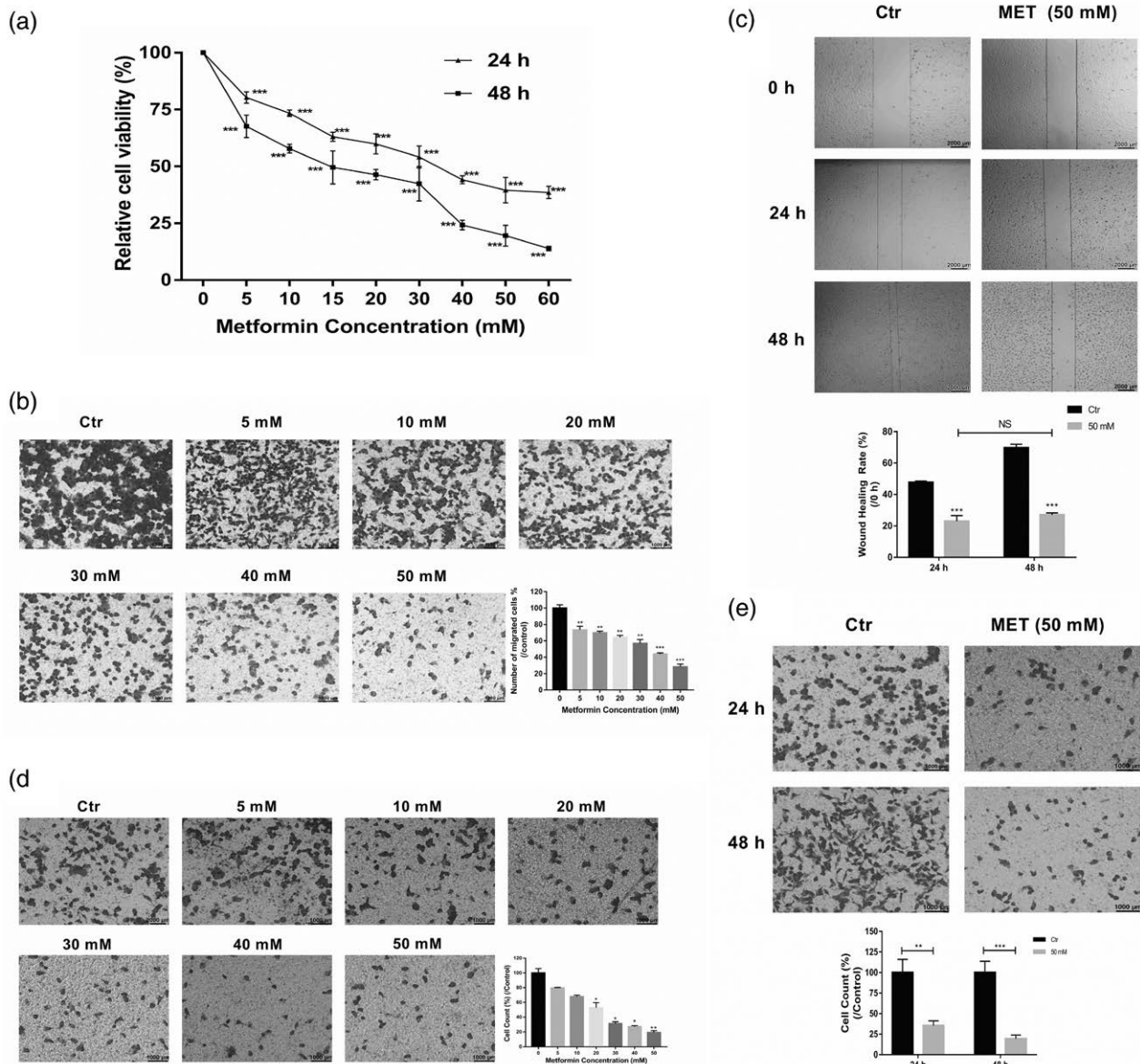
Testing the effects of MET or miR-7 on A549 cell growth, migration, and invasion. (a) The cell viability of A549 cells was measured after exposure to increasing doses of metformin for 24 and 48 hours by the CCK8 assay. (b) We used a Transwell migration assay to measure the migration of A549 cells after treatment with various concentrations of MET for 24 hours (magnification: ×100). (c) The cell wound scratch assay measured the effect of metformin on A549 cell migration for 24 and 48 hours (magnification: ×40). (d) Transwell assays were used to test A549 cell invasion. A549 cells seeded in the upper chambers were treated with MET for 24 hours (magnification: ×100). (e) Invasion of A549 cells incubated with MET (50 mM) for 24 and 48 hours (magnification: ×100). miR-7, microRNA-7.

(Fig. 1a). The IC_{50} values of metformin in A549 cells were 32.11 and 13.65 mM for 24 and 48 hours, respectively. These results revealed that metformin can inhibit A549 cell growth with time- and concentration-dependent effects. The Transwell migration assay results indicated that metformin can reduce A549 cell migration after transfection for 24 hours, and the inhibitory effect rose with increasing metformin concentration (Fig. 1b). We used a cell wound scratch assay to test the migration of A549 cells at various time points. The results implied that MET can reduce the migration of A549 cells in a time-dependent manner (Fig. 1c). We used a Transwell invasion assay to measure the invasion ability of A549 cells. After 24 hours of treatment with various concentrations of metformin,

Fig. 2
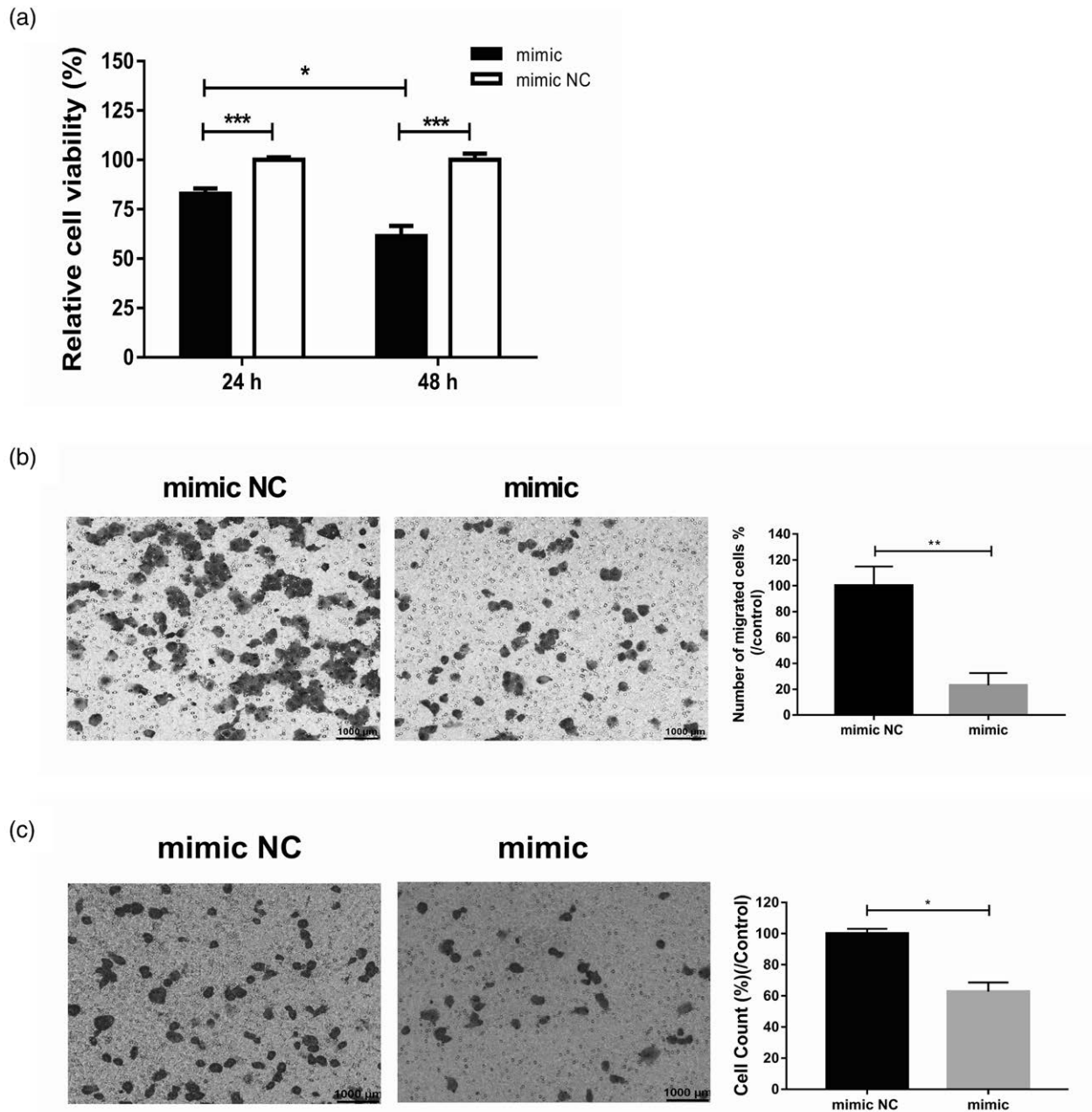
(a) A549 cells were transfected with miR-7 mimic or NC mimic for 24 and 48 hours. (b) A549 cells transfected with miR-7 mimic or NC mimic, and cell migration was analyzed 24 hours post-transfection (magnification: ×100). (c) A549 cells were transfected with miR-7 mimic and NC mimic, and cell invasion was analyzed 24 hours post-transfection (magnification: ×100). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the control group. miR-7, microRNA-7.

Fig. 3
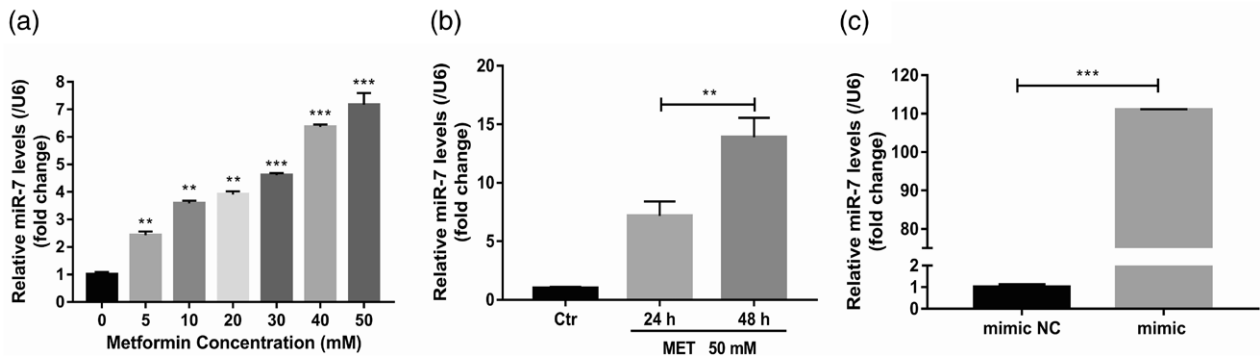
Metformin can upregulate the level of miR-7 in A549 cells. (a) A549 cells were incubated with different concentrations of MET for 24 hours. (b) Effects of metformin (50 mM) on the relative expression of miR-7 in A549 cells. (c) miR-7 mimic or mimic NC were transfected into A549 cells for 24 hours, and then the miR-7 level was detected. miR-7, microRNA-7.

Fig. 4
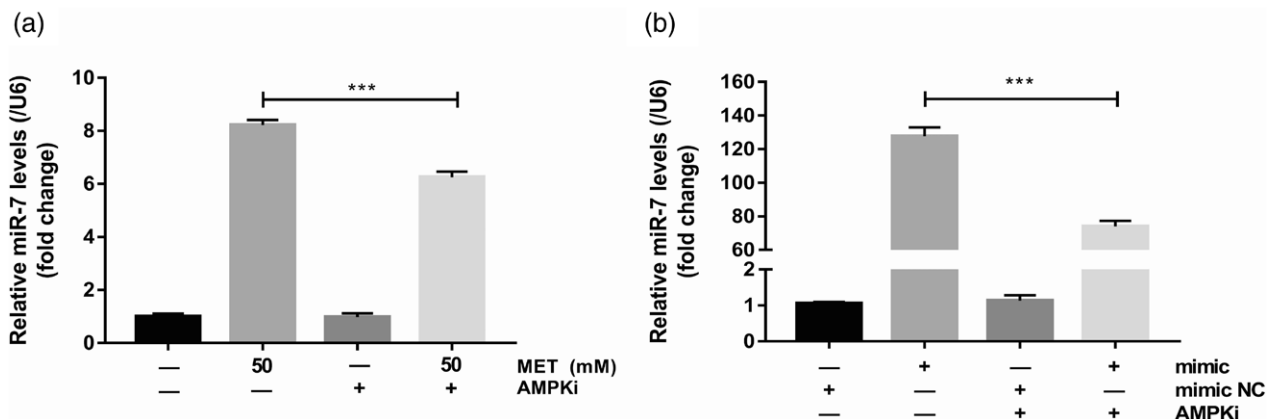
Metformin regulates the content of miR-7 via AMPK in A549 cells. (a) The expression level of miR-7 in the MET plus AMPKi group was markedly lower than that in the MET group. (b) The level of miR-7 in the miR-7 mimic plus AMPKi group was markedly lower than that in the miR-7 mimic group. ***$P < 0.001$. miR-7, microRNA-7.

the number of invasive A549 cells was significantly lower (Fig. 1d). When treated with 50 mM MET, the numbers of invasive cells were 35.6% and 19.4% lower than those in the control groups at 24 and 48 hours, respectively, implying time-dependent inhibition of invasion (Fig. 1e).

The CCK8 assays showed that, compared with the viability of the mimic NC group, the viability of A549 cells were markedly lower in the miR-7 mimic group (Fig. 2a), and the inhibitory effect was more significant at 48 hours. The Transwell migration assay indicated that A549 cell migration decreased markedly after miR-7 transfection compared with the mimic NC transfection (Fig. 2b). The Transwell migration assay revealed that after transfecting the miR-7 mimic, the number of A549 cells that passed through the membrane was 40% lower than that in the

mimic NC group (Fig. 2c). miR-7 reduced the growth, migration, and invasion of A549 cells.

Metformin can stimulate the level of microRNA-7 expression in A549 cells

To observe the effects of metformin on the level of miR-7 expression, we treated A549 cells with differential concentrations of metformin for different lengths of time and used qPCR to measure the miR-7 level. We discovered that the miR-7 level was upregulated markedly by MET in a dose- and time-dependent manner (Fig. 3a and b). miR-7 expression in the mimic group was more than 100 times higher than that in the mimic NC group, which proved that the transfection efficiency was high (Fig. 3c). The results illustrated that in A549 cells, metformin can upregulate the level of miR-7.

Fig. 5
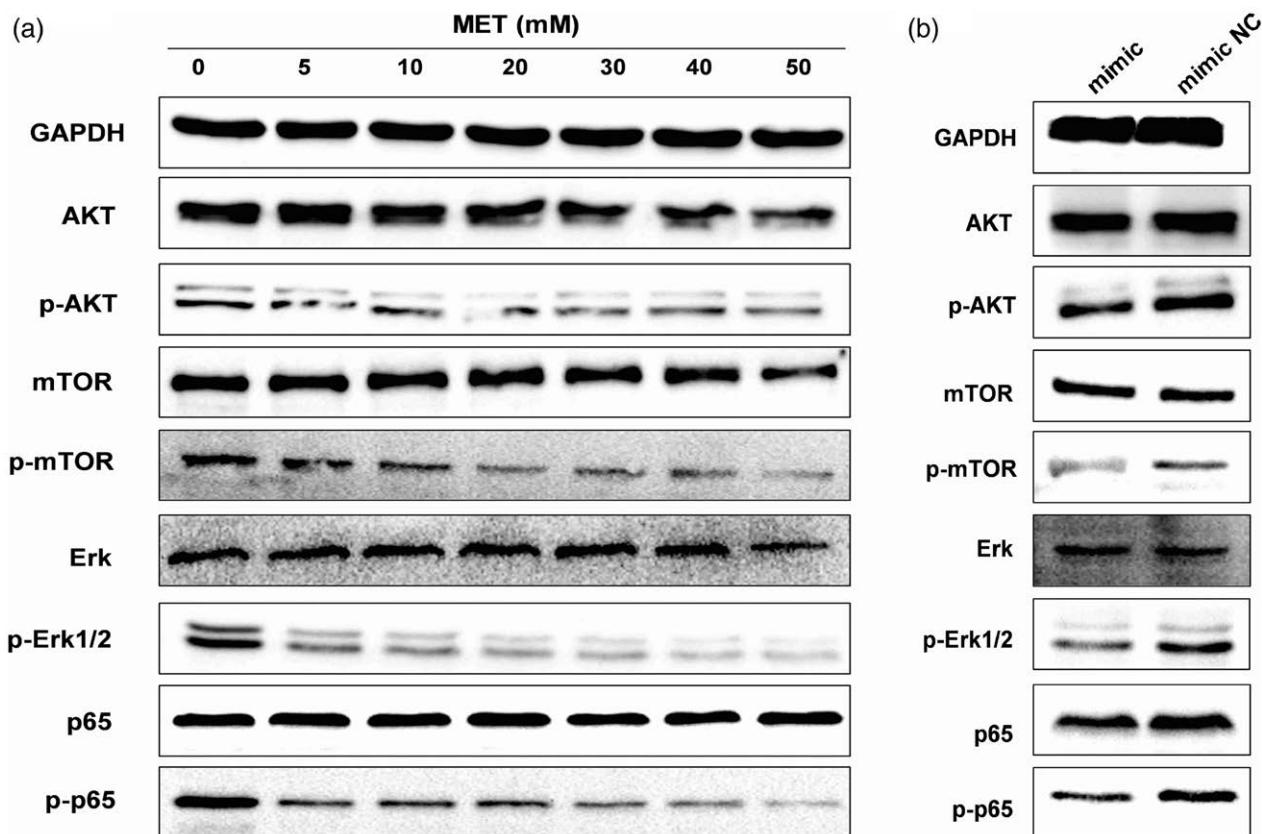
The protein expression of NF-κB, MAPK/Erk, and AKT/mTOR signaling pathway proteins in A549 cells. (a) Metformin can inhibit the levels of p-p65, p-Erk1/2, p-AKT, and p-mTOR proteins in A549 cells. (b) The levels of p-NF-κB p65, p-Erk1/2, p-AKT, and p-mTOR proteins were reduced in the miR-7 mimic group compared with those in the mimic NC group. miR-7, microRNA-7.

Metformin increases microRNA-7 expression through AMP-dependent kinase in A549 cells

To explore whether metformin regulates miR-7 through AMPK-dependent pathways, we inhibited AMPK with an AMPK inhibitor in MET-treated A549 cells and used qPCR to detect the expression level of miR-7. The results revealed that the addition of an AMPK inhibitor significantly decreased the miR-7 level in the 50 mM MET treatment group (Fig. 4a). Interestingly, the level of miR-7 was also decreased by AMPK inhibitor in the miR-7 mimic transfection group, indicating that suppression of AMPK-dependent pathways alone can decrease the miR-7 levels (Fig. 4b). These results show that metformin regulates miR-7 expression through AMPK.

Metformin and microRNA-7 regulate the protein expression of NF-κB, MAPK/Erk and AKT/mTOR signaling pathways in A549 cells

The western blot assay revealed that, compared with the control treatment, various concentrations of metformin can significantly attenuate the level of p-p65, p-Erk1/2, p-AKT, and p-mTOR, and higher concentrations of metformin, yielded more obvious inhibitory effects (Fig. 5a). Compared with those in the mimic NC group, these protein levels were markedly decreased in the miR-7 mimic group (Fig. 5b). The results demonstrated that both metformin and miR-7 mimic can downregulate the protein expression of p-p65, p-Erk1/2, p-AKT, and p-mTOR.

Discussion

Metformin has been used as a primary treatment for T2DM for a long time. In 2005, Evans *et al*. [7] found that MET can reduce the risk of cancer in T2DM patients, and since then, the possible antitumor effect of metformin has attracted wide attention. Several preclinical studies have reported that metformin can suppress malignant tumor growth in mice [13,14] and inhibit the growth of human malignant tumor cells [15,16]. In addition, some studies have shown that some microRNAs regulate the growth, migration, and invasion of lung cancer cells. In our research, we found that MET can promote the expression of miR-7 in a dose- and time-dependent manner mediated by AMPK in A549 cells. Furthermore,

both metformin and miR-7 can inhibit A549 cell growth, migration, and invasion by decreasing the expression of proteins in the AKT/mTOR, MAPK/Erk, and NF-κB signaling pathways.

In the present study, we discovered that metformin treatment for 24 hours strongly reduces A549 cell growth in a dose-independent manner, and this effect is enhanced at 48 hours. As shown in Fig. 1a, metformin (5 mM) treatment of A549 cells lead to a significant decrease in proliferation. However, the inhibitory effect on A549 cells has no plateau period, even when we set the maximum concentration of metformin to 60 mM (which is already a relatively high concentration); the same phenomenon occurred with the inhibition of migration and invasion in A549 cells, which implies that A549 is sensitive to both high and low concentrations of metformin.

miR-7 has been characterized as both an oncogene and a tumor suppressor in NSCLC. Studies by Xiong *et al.* and Liu *et al.* [4,17] reported a tumor-suppressive role for miR-7. These results are in direct contrast with those of others [18,19]. In our research, we found that miR-7 overexpression markedly attenuated the growth, migration, and invasion of A549 cells. Based on these results, we wondered whether there is a correlation between metformin and miR-7 in A549 cells. Therefore, we used qPCR to test the expression of miR-7 after transfecting different concentrations of metformin into A549 cells, and we found that MET can upregulate the expression of miR-7 in A549 cells.

Evidence accumulated from in-vitro and in-vivo studies supports the fact that metformin has a direct anti-cancer effect on cancer cells, partially through activating AMPK [20]. To verify whether metformin regulates miR-7 through AMPK-dependent pathways, we treated A549 cells with 50 mM metformin and AMPK inhibitors together. The results revealed that the content of miR-7 was decreased markedly when AMPK was inhibited. This phenomenon also occurred in the miR-7 mimic and AMPKi cotransfection groups. Interestingly, the expression of miR-7 was not completely inhibited with the addition of AMPK inhibitors, so we speculated that there may be two reasons: one is that the dosage of AMPKi is not high enough, such that AMPK was not completely inhibited; the second reason was that metformin may promote the expression of miR-7 through other pathways, so we will do more in-depth research on this problem in the future.

To research the potential mechanism of metformin and miR-7 in inhibiting the growth, migration, and invasion of A549 cells, we focused on three signaling pathways that can regulate the growth of tumor cells. These pathways, MAPK, NF-κB, and AKT/mTOR, play important roles in the formation, growth, apoptosis, invasion, metastasis, and angiogenesis of lung cancer. Activated AKT/mTOR,

Erk, and NF-κB can activate a variety of transcription factors to upregulate cyclin D1 and CDK4 to promote the progression of the cell cycle (G1/S phase), accordingly promoting cell growth [21–23]. AKT/mTOR, Erk, and NF-κB signaling pathways can increase the invasion and metastasis of NSCLC by activating MMP-2 and -9 (matrix metalloproteinases-2 and -9), and activated AKT can increase the transcriptional activity of NF-κB and increase the migration ability of tumor cells [24–26]. PicTar (http://pictar.mdc-berlin.de/) and MicroRNA.org (http://www.microrna.org) predicted that mTOR, Erk, and NF-κB p65 are potential targets of miR-7. In our study, both metformin and miR-7 significantly inhibited these three signaling pathways, indicating that miR-7-mediated inhibition of the AKT/mTOR, Erk, and NF-κB signaling pathways plays a role in the suppression of A549 cell growth, migration and invasion. According to all the above results, we concluded that MET can upregulate the expression of miR-7 by mediating AMPK signaling in NSCLC A549 cells and then by inhibiting the AKT/mTOR, Erk, and NF-κB signaling pathways to suppress the growth, migration, and invasion of A549 cells.

However, although the levels of miR-7 increased much more dramatically in the mimic transfection group compared with the metformin treatment group (>100 times versus only 2–8 times), the inhibitory effect of metformin, even at low concentrations, on the growth, migration, and invasion of A549 cells was markedly higher than that of mimic transfection. Similarly, the inhibition of p-AKT, p-mTOR, p-Erk, and p-p65 proteins by metformin was more significant than that in the mimic group. These results suggest that in addition to metformin promoting the expression of miR-7, there are other downstream mechanisms by which metformin inhibits A549 cell growth, migration, and invasion.

In conclusion, we found that metformin, via increasing the content of miR-7 mediated by AMPK, regulates the AKT/mTOR, MAPK/Erk, and NF-κB signaling pathways, thereby suppressing the growth, migration, and invasion of A549 cells. These results provide a new explanation for the mechanism of metformin's anticancer effect and lay the foundation for further preclinical studies on the combined use of metformin and miR-7 mimics as therapeutic strategies to suppress lung cancer *in vivo*.

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