Metformin inhibits HaCaT cell viability via the miR-21/PTEN/Akt signaling pathway

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Abstract. Substantial preclinical evidence has indicated out a direct anti-proliferation effect of metformin on various solid tumors; however, further and more detailed exploration into its molecular mechanism remains to be performed. The present study aimed to investigate the effect of metformin on cell viability and its underlying mechanism, in the cultured human skin keratinocyte cell line, HaCaT. In addition, it aimed to clarify the role of the microRNA-21(miR-21)/phosphatase and tensin homolog (PTEN)/AKT serine/threonine kinase 1 (Akt) signaling pathway, which has been hypothesized to be involved in the molecular mechanism of this drug. Cell Counting Kit-8 assays were used to assess the impact of metformin on cell viability; reverse transcription-quantitative polymerase chain reaction was used to quantify the expression of miR-21; western blotting was used to monitor the expression level of PTEN and Akt proteins. In addition, miR-21 expression levels were artificially manipulated in HaCaT cells using a miR-21 inhibitor in order to observe the subsequent expression changes of miR-21 targets and alterations in cell viability. The results indicated that metformin suppressed HaCaT cell growth in a dose- and time-dependent manner ($P<0.05$). Metformin treatment downregulated miR-21 expression ($t=-8.903$, $P<0.05$). Following transfection with the miR-21 inhibitor, HaCaT cell growth was significantly slower than in the control groups ($P<0.05$). In addition, reduced miR-21 levels results in significantly increased PTEN protein expression levels and reduced Akt protein expression levels compared with control ($P<0.05$). Metformin was, therefore, concluded to inhibit HaCaT cell growth in a time-and dose-dependent manner, and the miR-21/PTEN/Akt signaling pathway may serve a crucial role in the molecular mechanism of metformin's effect on HaCaT cells. Therefore the present study presents an advanced insight into the potential inhibitory effect of metformin on tumor cells.

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Introduction

HaCaT cells are an immortalized, non-tumorigenic cell line derived from keratinocytes of normal adult skin (1). Pathologically excessive proliferation of these cells reportedly leads to various skin diseases, such as psoriatic lesions and various skin cancers (2). Thus, HaCaT cells are extensively employed as an extrinsic cell instrument in anti-neoplastic medicine experiments (3). Metformin, a biguanide insulin sensitizer widely prescribed to type 2 diabetes (4), has gained increasing focus due to its newly discovered anti-neoplastic capacity in a broad spectrum of malignant tumors, including prostate, pancreatic, ovarian and gastric cancers (5-8). However, the anti-growth ability of metformin seems to have tumor type dependence (9) and the mechanism whereby metformin affects various cancers is still not completely understood (10).

MicroRNAs (miRNAs or miRs) are a class of small modulatory non-coding RNA molecules that can either bring about instability or translational efficiency repression through base-pairing to target mRNAs (11), effectively resulting in the repression of molecules that take part in cell cycle processes including cell proliferation, differentiation and apoptosis (12). miR-21 is reportedly overexpressed in the vast majority of solid neoplasms (13). Among the target molecules of miR-21, phosphatase and tensin homolog (PTEN) and AKT serine/threonine kinase 1 (Akt) are of particular interest (14). PTEN protein has been identified as a potent neoplasm inhibitor, while Akt is found positioned at the intersection of multiple signaling pathways, and is a verified sensing node that is upregulated in most tumors and can be suppressed by reversing the phosphorylation of the phosphoinositide 3-kinase (15).

Therefore, the present study aimed to explore the *in vitro* proliferation inhibition effect of metformin on HaCaT cells and to investigate the mechanism which might be involved in the miR-21/PTEN/Akt signaling pathway.

Materials and methods

Cell culture and metformin treatment. HaCaT cells were purchased from American Type Culture Collection

(cat. no. PCS-200-011; American Type Culture Collection, Manassas, VA, USA). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, cat. no. 12491-015; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; cat. no. 26170043; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2. The medium was changed every 2 days. Metformin hydrochloride with 98.8% purity was obtained from Shouguang Fukang Pharmaceutical Co., Ltd. (cat. no. 1115-70-4; Shouguang, China) and was diluted in phosphate-buffered saline (PBS) to a concentration of 1 M. It was used at increasing concentrations (0-100 mM) and different action durations (up to 72 h) in serum-free media.

Cell Counting Kit-8 (CCK-8) cell viability assay. HaCaT cells were seeded into 96-well plates at density of 1,500 cells per well in 100 μl common media overnight. The next day, cells were washed and incubated with 20, 40, 60, 80 and 100 mM metformin in serum-free DMEM for 24, 48 and 72 h. Following addition of 10 μl colorimetric water-soluble CCK-8 tetrazolium salt solution to each well (cat. no. CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), cells were cultured at 37˚C for 1 h. A quantitative automatic microplate reader (model no. 2010; Anthos Labtec Instruments GmbH, Salzburg, Austria) was used to measure the absorbance of reactions at 450 nm (A_{450}). Growth inhibition rates of HaCaT cells at various treatment times and metformin concentrations were calculated using the formula: Cell growth inhibition rate (%)=(A_{450} control-A_{450} metformin)/A_{450} control x100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol® reagent (cat. no. 15596-018; Invitrogen; Thermo Fisher Scientific, Inc.) was used for total RNA isolation. The amount and purity of RNA was assessed spectrophotometrically. Extracted RNA was reverse-transcribed into cDNA using a miRNA cDNA synthesis kit (cat. no. 203301; Universal cDNA Synthesis kit II; Takara Bio, Inc., Otsu, Japan) in accordance with the manufacturer's specification. qPCR was performed with the primers listed in Table I and ExiLENT SYBR® Green master mix (cat. no. 203403; Takara Bio, Inc.), as outlined in Table II. An ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used. The amplification conditions of the PCR were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 1 min, the ramp-rate was 1.6˚C/sec. A total of three experimental repeats were performed. The $2^{-\Delta\Delta Cq}$ method was employed for quantitation, with miR-21 expression levels normalized to the U6 RNA control (16).

Western blotting analysis. Following 48 h of treatment with 30 mM metformin, total protein was isolated from cells by incubation in radioimmunoprecipitation assay buffer (cat. no. P0013C; Beyotime Institute of Biotechnology, Haimen, China) containing 1 mM phenylmethane sulfonyl on ice for 30 min, followed by centrifugation at 13,690 x g, 4˚C for 30 min. Protein concentration was evaluated by bicinchoninic acid assay (cat. no. P0010; Beyotime Institute of Biotechnology). Proteins (50 μg per lane) were separated by 10% SDS-PAGE, then transferred to polyvinylidene difluoride membranes. Non-specific binding was blocked by incubation in 5% skimmed milk in Tris-buffered saline with 0.05% Tween-20 (TBST) buffer for 2 h at room temperature. Membranes were subsequently incubated at 4˚C overnight in the following primary antibodies: rabbit monoclonal anti-PTEN (1:1,000; cat. no. 9188; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit monoclonal anti-Akt1/2/3 (1:1,000; cat. no. 4685; Cell Signaling Technology, Inc.) or rabbit monoclonal anti-GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) as an internal reference. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. The membrane was developed using enhanced chemiluminescence (cat. no. 05-1327; EMD Millipore, Billerica, MA, USA) and scanned with an electrophoresis gel imaging analysis system (Tanon-5000R; Tanon Science & Technology Co., Ltd, Shanghai, China). Gray value of each band was quantified using ImageJ 1.46b (National Institutes of Health, Bethesda, MD, USA) relative to the GAPDH control.

Transfection with miR-21-inhibiting oligonucleotides. HaCaT cells were divided into three groups: IN group, transfected miR-21-inhibitor (5'-TCAACATCAGTCTGATAAGCTA-3'; Ambion; Thermo Fisher Scientific, Inc.); NC group, transfected

Table I. Primer sequences. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Target	Primer sequence
miR21	RT-5'-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTCAACA-3'
	F-5'-GTGCAGGGTCCGAGGT-3'
	R-5'-GCCGCTAGCTTATCAGACTGATGT-3'
U6	RT-5'-CGCTTCACGAATTTGCGTGTCAT-3'
	F-5'-GCTTCGGCAGCACATATACTAAAAT-3'
	R-5'-CGCTTCACGAATTTGCGTGTCAT-3'

Table II. Quantitative PCR reaction setup, as instructed by the manufacturer.

Reaction compound	Volume (μl)	Final concentration
SYBR premix Ex Tap™ II	10.0	1X
Template	2.0	------
F primer	0.8	0.4 μM
R primer	0.8	0.4 μM
ROX	0.4	------
RNase free H_2O	6.0	------
Total	20 μl	

Table III. Expression of miR-21 following treatment with 30 mM metformin. Levels of miR-21 expression were evaluated by quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation.

	Experimental group expression	Control group expression	t value
miR-21	1.14±0.18	2.67±0.23	-8.903[a]

[a]P<0.05. miR, microRNA.

Table IV. Expression levels of miR-21 and PTEN and Akt proteins were detected by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively.

	MOCK	NC	IN
miR-21	1.03±0.09	1.09±0.17	0.38±0.11
PTEN protein	1.05±0.07	1.11±0.07	2.29±0.08
Akt protein	1.03±0.09	1.10±0.07	0.47±0.05

Data are presented as the mean ± standard deviation. miR, microRNA; PTEN, phosphatase and tensin homolog; Akt, AKT serine/threonine kinase 1; MOCK, group mock-transfected with PBS; NC, group transfected with miR-21 inhibitor negative control; IN, group transfected miR-21-inhibitor.

with miR-21 inhibitor negative control (5'-CATTAATGTCGG ACAACTCAAT-3'; Ambion; Thermo Fisher Scientific, Inc.); and MOCK group, mock-transfected with PBS. Transfections were performed by incubating 1 μl Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), 100 μl serum-free RPMI-1640 (cat. no. 11875085; Gibco; Thermo Fisher Scientific, Inc.), and 20 pM miR-21 inhibitor or miR-21 inhibitor negative control at room temperature for 20 min, then adding to HaCaT cells and culturing at 37˚C for 6 h. Serum-free culture media was then substituted with complete RPMI-1640, and cells were harvested 48 h later.

Statistical analysis. Data are presented as the mean ± standard deviation unless otherwise stated. Statistical Program for Social Science software v.19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Two independent samples t-tests were applied to compare the difference between experimental and control groups, while Mantel Haenszel χ^2 tests were used to analyze the results of CCK-8 assays. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of metformin on HaCaT cell viability. CCK-8 assays demonstrated that increasing concentrations and durations of metformin treatment resulted in attenuation of HaCaT cell viability (χ^2=3.974; P<0.05; Fig. 1). This result indicated a time- and dosage-dependent inhibitory action of metformin on the viability of HaCaT cells.

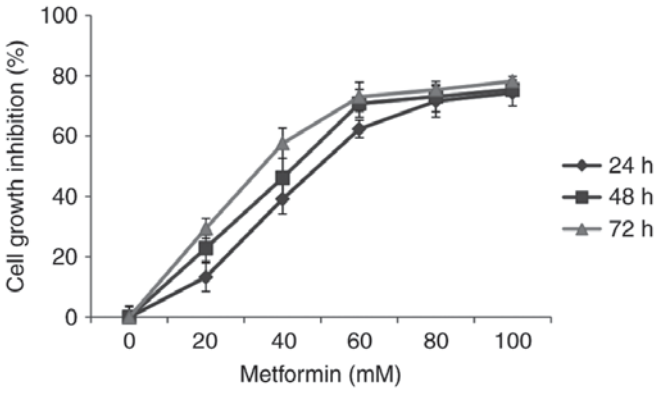

Figure 1. Growth inhibitory effect of metformin on HaCaT cells. HaCaT cells were incubated with 20, 40, 60, 80 and 100 mM metformin for 24, 48 and 72 h. Cell viability was assessed by Cell Counting Kit-8 assay. Cell growth inhibition (%)=(A_{450} control-A_{450} metformin)/A_{450} control x100%. Data are presented as the mean ± standard deviation and were statistically analyzed using the Mantel Haenszel χ^2-test. A_{450}, absorbance at 450 nm.

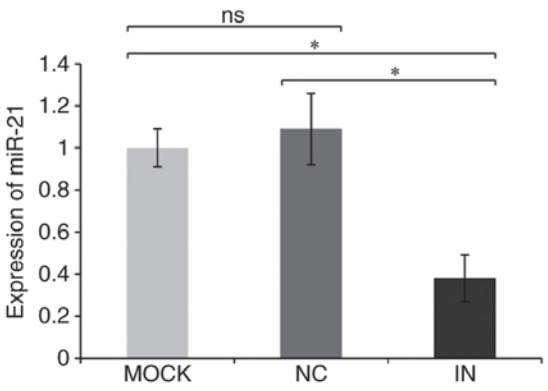

Figure 2. Transfection results. Reverse transcription-quantitative polymerase chain reaction was used to detect miR-21 expression levels following transfection with either miR-21-inhibitor, miR-21 inhibitor negative control or PBS. Data are presented as the mean ± standard deviation. *P<0.05 and nsP>0.05, as indicated by brackets. miR, microRNA; MOCK, group mock-transfected with PBS; NC, group transfected with miR-21 inhibitor negative control; IN, group transfected miR-21-inhibitor.

Expression of miR-21 in HaCaT cells following metformin treatment. Assessment of the effect of metformin treatment on the expression of miR-21 was a primary consideration. As demonstrated in Table III, miR-21 levels in the experimental and control groups were 1.14±0.18 and 2.67±0.23, respectively (P<0.05). Therefore, the levels of miR-21 expression were significantly reduced by metformin treatment.

Transfection results. To acquire a better understanding of the function of miR-21, a miR-21 inhibitor was transfected into HaCaT cells. RT-qPCR was then used to confirm the efficacy of miR-21 inhibition. Relative miR-21 quantitation data in the IN, NC and MOCK groups were 0.38±0.11, 1.09±0.17, and 1.03±0.09, respectively (Table IV and Fig. 2). miR-21 expression was, therefore, significantly reduced in the IN group compared with the NC and MOCK groups (both P<0.05), indicating that the miR-21 inhibitor was effective at reducing miR-21 expression.

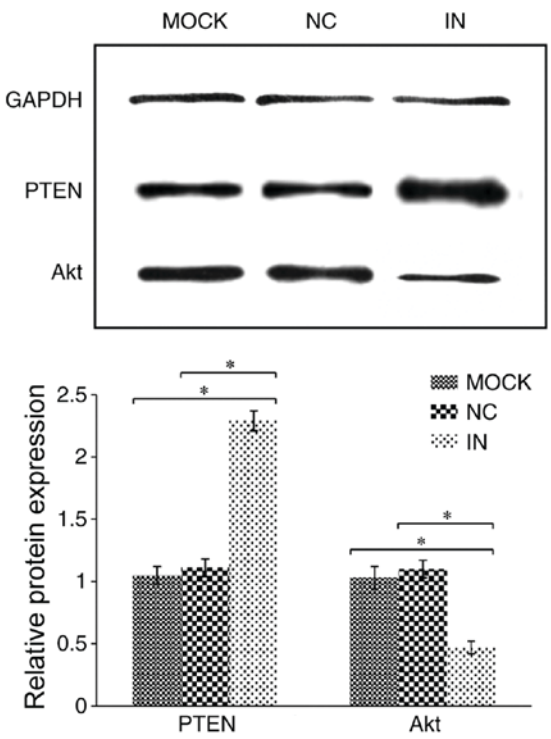

Figure 3. Expression of PTEN and Akt proteins following inhibition of miR-21, detected by western blotting analysis, with quantification relative to GAPDH. Data are presented as the mean ± standard deviation. *P<0.05, as indicated by brackets. PTEN, phosphatase and tensin homolog; Akt, AKT serine/threonine kinase 1; miR, microRNA; MOCK, group mock-transfected with PBS; NC, group transfected with miR-21 inhibitor negative control; IN, group transfected miR-21-inhibitor.

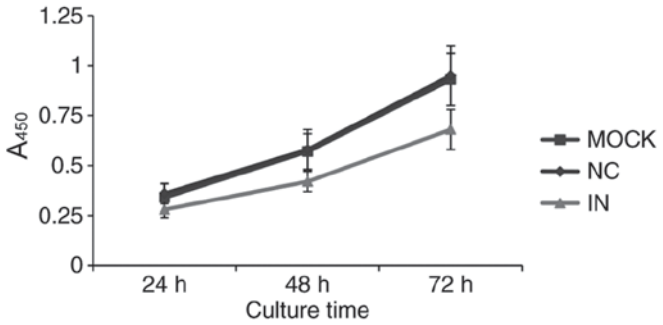

Figure 4. miR-21 inhibition reduces HaCaT cell viability. Following inhibition of miR-21, cell viability was assessed by Cell Counting Kit-8 assay. Data are presented as the mean ± standard deviation and were statistically analyzed using the Mantel Haenszel χ^2-test. Cell viability was significantly reduced in the IN group compared with MOCK and NC groups (P<0.05). miR, microRNA; MOCK, group mock-transfected with PBS; NC, group transfected with miR-21 inhibitor negative control; IN, group transfected miR-21-inhibitor; A_{450}, absorbance at 450 nm.

Inhibition of miR-21 enhances PTEN protein expression but reduces Akt protein expression. PTEN protein expression was demonstrated to be significantly upregulated in cells in the IN group compared with the NC and MOCK groups (both P<0.05; Table IV and Fig. 3). By contrast, Akt protein expression was demonstrated to be significantly downregulated in cells in the IN group compared with the NC and MOCK groups (both P<0.05; Table IV and Fig. 3).

Inhibition of miR-21 inhibits HaCaT cell viability. Since miR-21 is overexpressed in most solid tumors (17), miR-21 is hypothesized to promote tumor proliferation. Therefore, cell viability was examined by CCK-8 assay in cells with artificially lowered miR-21 levels. Cell growth was demonstrated to be downregulated in cells in the IN group compared with the NC and MOCK groups (Fig. 4). This result indicates that reduced miR-21 expression inhibits HaCaT cell viability.

Discussion

To the best of the authors' knowledge, the present study is the first to demonstrate a duration- and dosage-dependent inhibitory effect of the anti-diabetic drug metformin on HaCaT cell viability. Furthermore, the miR-21 expression levels were observed to be significantly reduced following metformin treatment. Therefore, the present study conjectured that miR-21 serves a pivotal role in adjusting the proliferative activity of HaCaT cells. To investigate the suspected vital function of miR-21 in the anti-viability effect of metformin, miR-21 inhibitor transfection was used to artificially reduce miR-21 expression in HaCaT cells, which resulted in reduced growth compared with control cells.

Therefore, a tentative conclusion was drawn that miR-21 might be at the core of the basic mechanism by which metformin inhibits HaCaT cell growth. In order to further examine the function of miR-21, the effect of reduced miR-21 expression on 2 functional downstream targets of miR-21, PTEN and Akt, was examined (18,19). Significantly increased PTEN expression and decreased Akt expression was observed in cells transfected with the miR-21 inhibitor compared with cells transfected with the scrambled-sequence oligonucleotide or PBS (20). Combined with the previously reported critical role of PTEN and Akt in regulating cellular biological procedures (21,22), an intricate modulatory network including miR-21 and PTEN/Akt was hypothesized to be involved in metformin's anti-proliferation effect. Yang *et al* (23) observed a high level of homology between the sequence of miR-21 and the 3'untranslated region (UTR) of PTEN mRNA, suggesting that miR-21 would bind to the 3'UTR of PTEN mRNA. This structural similarity between miR-21 and PTEN is a powerful mechanism explanation of miR-21's down-regulatory effect on PTEN protein expression. A negative association has previously been demonstrated between PTEN and Akt activation (24). However, some limitations and unsolved problems remain in the present study, such as the detailed mechanism of metformin's inhibitory effect on miR-21 and whether other signaling pathways are also involved in this effect. Further studies will examine these questions in the future.

Therefore, the present study concluded that metformin inhibits HaCaT cell viability via the miR-21/PTEN/Akt signaling pathway. The miR-21/PTEN/Akt signaling pathway may therefore be considered as a potential target to further investigate the molecular mechanism of metformin's action. This conclusion may consolidate certain theoretical foundations for associated research (25) and promote the application of metformin in skin cancer remedy (26).

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