

# The effect and mechanism of action of metformin on in vitro FaDu cell proliferation

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## Abstract

**Objective:** To investigate the effect and mechanism of action of metformin on proliferation of a human hypopharyngeal carcinoma cell line (FaDu).

**Methods:** FaDu cells were treated with metformin (25–125 mmol/l). Cell proliferation was evaluated via CCK-8 assay. Real-time quantitative reverse transcription–polymerase chain reaction was used to evaluate microRNA (miR)-21-5p and PDCD4 (programmed cell death 4) expression. PDCD4 protein was quantified by Western blot.

**Results:** Metformin significantly inhibited FaDu cell proliferation in a dose- (25–100 mmol/l) and time-dependent manner (12 h–36 h), significantly downregulated miR-21-5p, and upregulated PDCD4 mRNA and protein expression.

**Conclusions:** Metformin significantly inhibited FaDu cell proliferation, possibly via down-regulation of miR-21-5p and upregulation of PDCD4.

## Keywords

Hypopharyngeal carcinoma, metformin, FaDu cells, miR-21-5p, PDCD4, Real-time PCR, Western blot assay

Date received: 23 October 2015; accepted: 11 March 2016

## Introduction

Hypopharyngeal carcinoma is one of the most malignant tumours of the upper aerodigestive tract and is difficult to diagnose early due to occult lesion location.<sup>1</sup> Despite progress in medical and surgical therapy, the 5-year survival rate of this cancer has not improved over the past decade.<sup>2</sup> Indeed, hypopharyngeal carcinoma has the worst prognosis among head and neck cancers.<sup>3–5</sup>

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The insulin sensitizer metformin is the first line drug for the treatment of type 2 diabetes,<sup>6</sup> and has been shown to reduce the incidence of several tumours in diabetic patients.<sup>7-10</sup> Metformin has been shown to inhibit the growth and proliferation of cancer cells in vitro (including liver, prostate, cervical and ovarian cancers),<sup>11-14</sup> but its effect on hypopharyngeal carcinoma is unclear.

Abnormal expression of the microRNA, miR-21-5p, and its downstream target gene, programmed cell death 4 (*PDCD4*) has been shown in multiple tumours,<sup>15</sup> and miR-21-5p is significantly upregulated in hypopharyngeal carcinoma.<sup>16</sup> To date, there is little information regarding the effects of metformin on the expression of miR-21-5p and *PDCD4* in hypopharyngeal tumour cells. The aim of this study, therefore, was to investigate the effects of metformin on cell proliferation in the human hypopharyngeal carcinoma cell line, FaDu, as well as the expression of miR-21-5p and *PDCD4* in these cells.

## Materials and methods

### Cell culture

The human hypopharyngeal carcinoma cell line (FaDu) was purchased from the American Type Culture Collection (ATCC<sup>®</sup>, Manassas, VA, USA) and stored in liquid nitrogen until use. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% carbon dioxide in air. After three consecutive passages, cells were divided into groups: untreated (control); 25 mmol/l metformin; 50 mmol/l metformin; 75 mmol/l metformin; 100 mmol/l metformin; and 125 mmol/l metformin (Sigma, St Louis, MO, USA).

### Cell proliferation assay

Cells were plated at  $2 \times 10^4$ /ml/well in 96-well plates, cultured for 36 h, washed

twice with phosphate buffered saline (PBS), then cultured as follows (one plate/timepoint; three wells/group): untreated control; 25 mmol/l metformin; 50 mmol/l metformin; 75 mmol/l metformin; 100 mmol/l metformin; and 125 mmol/l metformin. Cells in each group were cultured for 12 h, 24 h and 36 h.

Cell proliferation was quantified via CCK-8 assay, according to the manufacturer's instructions (Dojindo, Kunamoto, Japan). Optical density (OD) was measured three times at 450 nm, and growth inhibiting rate was calculated as: (OD experimental group – OD blank)/(OD control group – OD blank) × 100%.

### Real time qRT-PCR

Cells were incubated with metformin (25 mmol/l, 50 mmol/l, 75 mmol/l and 100 mmol/l) for 24 h. Total RNA was extracted using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions, and cDNA was synthesized using the poly-A tailing method.<sup>17</sup> PCR was performed for miR-21-5p with U6 RNA as internal reference, using a miRNA qRT-PCR detection kit (FulenGen Corporation, Guangzhou, China) Primer sequences were: miR-21 forward 5'-GAAATGCCTCACAGCTATCGT-3' and reverse 5'-CCTCCACAAAGAGCCACC-3'; U6 forward 5'-ATCATGTTGAGACCTTCAACA-3' and reverse 5'-CATCTCTTGCTCGAAGTCCA-3' (Sangon Biotech Corporation, Shanghai, China). Cycling conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. PCR for *PDCD4* was performed using a SYBR<sup>®</sup> Green I real time qPCR (Roche Corporation, Basel, Switzerland) with β-actin as internal reference. Primer sequences were: *PDCD4* forward, 5'-CCC GAGGGATTCTGAAGGAAG-3' and reverse, 5'-TCACCGAAAAGAGAGAGTCAC-3'; β-actin forward, 5'-AGTTGCG

TTACACCCTTTC-3' and reverse, 5'-CCTTCACCGTTCCAGTTT-3' (Sangon Biotech Corporation, Shanghai, China). Cycling conditions were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, and 55°C for 30 s. All PCR experiments were performed in triplicate. Expression levels of PCR targets in experimental groups were determined relative to control cultures using the  $2^{-\Delta\Delta Ct}$  method.

### Western blot

Cells were incubated with metformin (25 mmol/l, 50 mmol/l, 75 mmol/l and 100 mmol/l) for 24 h, washed twice with PBS, and lysed with RIPA buffer (Beyotime, Jiangsu, China) on ice for 30 min. Lysates were centrifuged at  $12\,000 \times g$  for 5 min at 4°C, and the protein concentration of the supernatant was determined using a BCA assay kit (Beyotime). Total protein (40 µg) was separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and blocked with 5% skimmed milk at room temperature for 2 h. Membranes were incubated overnight at 4°C with mouse monoclonal antihuman-β-actin antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal antihuman PDCD4 antibody (1:2000 dilution; Abcam Corporation,

Cambridge, UK), washed three times with tris buffered saline–Tween 20, then incubated at room temperature for 1 h with horseradish peroxidase labelled goat antirabbit or goat antimouse secondary antibody (1:5000; Santa Cruz Biotechnology).

Immunoreactive protein bands were detected using an enhanced chemiluminescence (ECL) kit, according to the manufacturer's instructions (Beyotime) and quantified via densitometric analysis using software Image J (Image Processing and Analysis in Java). All assays were performed in duplicate.

### Statistical analyses

Data were presented as mean ± SD. Between group differences were analysed using one-way analysis of variance (ANOVA) for multiple group comparisons and Student–Newman–Keuls *q*-test for paired comparison. Statistical analyses were performed using SPSS® version 17.0 (SPSS Inc, Chicago, IL, USA) for Windows®. A *P*-value < 0.05 was considered statistically significant.

## Results

Data regarding the effect of metformin on FaDu cell proliferation are shown in Table 1. Metformin significantly inhibited

**Table 1.** Inhibition of cell proliferation in the human hypopharyngeal carcinoma cell line FaDu, treated with metformin (25–125 mmol/l) for 12 h, 24 h and 36 h, relative to untreated control cultures (%).

Timepoint	Metformin concentration (mmol/l)					
	0	25	50	75	100	125
12 h	0.0 <sup>a</sup>	10.8 ± 7.8	17.0 ± 6.7 <sup>b</sup>	35.4 ± 10.4 <sup>c</sup>	45.5 ± 14.5 <sup>d</sup>	45.7 ± 9.8
24 h	0.0 <sup>a</sup>	14.4 ± 5.1	21.3 ± 7.4 <sup>b</sup>	49.3 ± 7.1 <sup>c</sup>	65.2 ± 12.4 <sup>d</sup>	66.5 ± 6.7
36 h	0.0 <sup>a</sup>	18.4 ± 7.6	25.7 ± 5.7 <sup>b</sup>	57.6 ± 10.3 <sup>c</sup>	72.9 ± 11.3 <sup>d</sup>	74.0 ± 7.9

Data presented as mean ± SD (*n* = 3 for each experimental group)

<sup>a</sup>*P* < 0.05 vs all other concentrations at same timepoint; <sup>b</sup>*P* < 0.05 vs 25 mmol/l metformin at same timepoint; <sup>c</sup>*P* < 0.05 vs 50 mmol/l metformin at same timepoint; <sup>d</sup>*P* < 0.05 vs 75 mmol/l metformin at same timepoint; one-way analysis of variance and Student–Newman–Keuls *q*-test.

**Table 2.** Levels of micro (m)RNA-21-5p, and PDCD4 (programmed cell death 4) mRNA and protein in the human hypopharyngeal carcinoma cell line FaDu, treated with metformin for 24 h.

Parameter	Metformin concentration (mmol/l)				
	0	25	50	75	100
miR-21-5 p RNA	1.00 ± 0.15	0.60 ± 0.06*	0.47 ± 0.07*	0.38 ± 0.11*	0.34 ± 0.05*
PDCD4 RNA	1.01 ± 0.13	3.45 ± 0.78**	6.62 ± 0.72**	9.79 ± 0.84**	10.59 ± 1.63**
PDCD4 protein	1.00 ± 0.15	2.41 ± 0.41*	4.37 ± 0.57*	4.49 ± 1.36*	4.93 ± 0.67*

Data presented as mean ± SD of triplicate experiments.

\* $P < 0.05$ , \*\* $P < 0.01$  vs 0 mmol/l; Student's *t*-test.

cell proliferation in a dose- ( $P < 0.05$ ) and time-dependent manner ( $P < 0.05$ ). There was no significant difference in cell proliferation between 100 mmol/l and 125 mmol/l metformin at any timepoint.

Incubation with metformin for 24 h significantly inhibited miR-21-5p expression (each dose  $P < 0.05$  vs control; Table 2). Levels of PDCD4 mRNA (each dose  $P < 0.01$  vs control; Table 2) and protein (each dose  $P < 0.05$  vs control; Table 2) were significantly upregulated by incubation with metformin.

## Discussion

Metformin has been shown to activate the adenosine monophosphate activated protein kinase (AMPK) signalling pathway and inhibit the activity of the downstream related enzyme *in vivo*, thereby regulating the metabolism of glucose and lipids.<sup>18</sup> Studies have showed that metformin can significantly inhibit the growth and proliferation of a variety of tumour cells *in vitro*.<sup>11–14</sup> It has been reported that metformin slows bladder cancer progression by inhibiting stem cell repopulation through the COX2/PGE2/STAT3 axis in both animal and cell models.<sup>19</sup> The drug may also be used as an adjuvant in combination with antiproliferative modalities to improve the outcome of patients with obesity-activated thyroid cancer.<sup>20</sup> In one study, low doses of metformin were able to reduce

proliferation of certain glioblastoma cells,<sup>21</sup> and it has been postulated that it may have a role in the prevention and management of urological malignancies.<sup>22</sup> In addition, metformin has been shown to inhibit prostate cancer cell proliferation, migration, invasion and tumour growth mediated by upregulation of pigment epithelium-derived factor (PEDF) expression.<sup>23</sup> Furthermore, metformin had an effect on progenitor/stem cells in a chemoprevention setting, and is being investigated as a potential early intervention in patients with chronic liver disease at high risk of hepatocellular carcinoma.<sup>24</sup> Another study has reported that metformin inhibits the proliferation of human keratinocytes by regulating the MAPK and Akt signalling pathways.<sup>25</sup> The results of our study showed that 25–100 mmol/l metformin significantly suppressed the proliferation of human hypopharyngeal carcinoma cells (FaDu) in a dose- and time-dependent manner over 12 to 36 hours.

MicroRNAs (miRNAs) are a class of endogenous, single-stranded non-protein-coding RNAs (14–24 nt) that regulate the expression of target genes at a posttranscriptional level, and play a vital role in cell differentiation, biological development and disease occurrence and progression.<sup>26–28</sup> Several miRNAs are located in tumour susceptibility loci, and their abnormal expression has a close relationship with cell cancerization. For example, miR-21-5p is

thought to be a cancer-causing gene as it is upregulated in a variety of tumours and participates in the abnormal differentiation, proliferation and apoptosis of tumour cells.<sup>15</sup> Studies have reported that there are many downstream target genes of miR-21-5p, including PDCD4,<sup>29</sup> a proapoptotic factor and tumour suppressor gene.<sup>30,31</sup> The abnormal expression of miR-21-5p and PDCD4 has been shown to play a vital role in the pathogenesis, progression and metastasis of a variety of tumours, such as renal cancer, multiple myeloma and gastric cancer.<sup>32–34</sup>

Levels of miR-21-5p RNA and PDCD4 mRNA and protein in FaDu cells were significantly affected by treatment with metformin in the present study. Metformin significantly downregulated miR-21-5p, and upregulated PDCD4 mRNA and protein, while significantly inhibiting cell proliferation. A limitation of this study is that these in vitro findings may not be representative of the in vivo environment. Further in vivo studies are therefore required to validate our findings.

In conclusion, metformin inhibited the proliferation of FaDu cells in vitro. This may be associated with the downregulation of miR-21-5p expression and the upregulation of PDCD4 expression.

### Declaration of conflicting interests

The authors declare that there is no conflict of interest.

### Funding

This work was supported by Shandong Provincial Natural Science Foundation, China (#ZR2013HQ055)

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