Original Article

Effects of antidiabetic drug metformin on the migration and invasion abilities of human pulmonary adenocarcinoma A549 cell line *in vitro*

Ning Wu, Hong-Jun Gu, Qiang Li

Department of Respiratory Medicine (Drs Wu and Li), Changhai Hospital, Second Military Medical University, Shanghai 200433; Department of Internal Medicine (Dr Gu), Soochow Medical Center of People's Liberation Army 101 Hospital, Soochow 215007, PR China

ABSTRACT

Background and purpose: There is growing evidence that metformin, a clinically widely used drug in the treatment of type II diabetes, may impede the growth of human tumors. However in a recent study it was found that metformin treatment might result in promotion of the angiogenic phenotype and promote early tumorigenic progression. In order to evaluate the relevance between metformin and tumor metastases, we investigated the effects of metformin on the migration and invasion abilities of human pulmonary adenocarcinoma cell line A549 *in vitro* and explored the possible underlying mechanisms.

Methods: A549 cells were treated with 0.5 mmol/L, 2 mmol/L and 8 mmol/L metformin for 72h. The laterad-migration and invasion abilities of the cells in vitro were evaluated by scratch assay and Boyden-Chamber assay, respectively. Expressions of MMP2 and MMP9 mRNA of the cells before and after metformin treatment were measured by Real-Time PCR.

Results: The migration rate of A549 cells was increased after metformin treatment at the concentration of 8mmol/L. The invasion ability was also significantly increased from 37.4 ± 4.6 to 59.8 ± 7.2($P<0.05$) by 8mmol/L metformin treatment. No significant difference of the migration and invasion abilities was observed between the Group 0.5mmol/L, 2mmol/L and the Control. The expressions of MMP2 and MMP9 mRNA were both up-regulated after metformin treatment, while in the 8mmol/L Group the genes changes were the most significant.

Conclusions: Metformin can increase the migration speed and enhance invasion abilities of A549 cells *in vitro*, which may be attributed to the up-regulation of MMP2 and MMP9.

Key Words: pulmonary adenocarcionma; metformin; migration; invasion ability; MMP2; MMP9

Introduction

As a euglycemic agent, metformin can reduce the hepatic gluconeogenesis and increase peripheral tissues' sugar intake and utilization, so that blood sugar would be reduced efficiently. From the 1950s, it has been widely applied for treating type II diabetes (1). Through researches in the recent years, it was found that metformin might be efficient on anti-tumor, which had been verified in treating some animentary and reproductive system tumors. However, a recent animal trial about breast cancer showed that though metformin treatment could slow down the growing of transplanted tumor, vessels in tumor were significantly increased, which implied that metformin might promote neoangiogenesis (2). If this presumption was right, then, metformin would lead to a risk of promoting tumorous metastases. This research studied the effect of metformin on the migration and invasion of human pulmonary adenocarcinoma cell line A549 in vitro; moreover, it evaluated the related mechanisms

Materials and Methods

Materials

Pulmonary adenocarcinoma A549 cell lines were obtained from cell bank, Chinese Academy of Sciences. Fetal bovine serum, double-antibody and RPMI1640 medium were obtained from Hyclone Australia.

Metformin was purchased from Sigma Corporation of America. Millicell cell culture inserts were from Millipore Corp (United States). Matrigel Basement Membrane Matrix was from BD Bioscience, USA and Trizol from Invitrogen Coperation, USA. Reverse Transcriptase RNA Kit and SYRB Realtime-PCR Kit were obtained from Takara Biotechnology (Da Lian) Co., ltd. PCR premier was synthesized by Shanghai SBS Genetech Technology Co., Ltd.. Real-time PCR machine was type 7500, from Applied Biosystems Inc, USA.

Grouping and management of cells

Cells were conventionally cultured in RPMI1640 medium containing 10% fetal bovine serum, 100U/ml penicillin and 100ng/ml streptomycin, at 37℃ in 5% CO2. Experimental groups included control group, 0.5mmol/L metformin group, 2mmol/L metformin group and 8mmol/L metformin group. Cells in logarithm phrase were collected and inoculated for 24hours, after their adherence, each group would be kept in RPMI1640 medium, which contains metformin, through which, the final metformin concentration would respectively reach at 0.5mmol/L, 2mmol/L and 8mmol/L, besides, PBS in the same volume was added to control group medium.

Cell scratch test

After trypsinization and collection for the cells treated with metformin for 72h of each group, they were inoculated in a six-well plate at $5×10^5$ cells for each well, and cultured in a conventional way for 24 hours. When cells of each group had achieved 80-90% integration, the medium was abandoned. After cleaning the cells with sterile PBS for once, 200ul transferpettor tip was applied to draw a straight line along the Y direction in the center of each well in the plate. Cells were rinsed tenderly for twice with sterile PBS so as to remove scratched floating cells. Draw five parallel lines in 0.5-1cm distance with marker pen over the vertical cells in the bottom of each plate well, which were observed under microscope. Appropriate serum-free medium was added into each well for conventional culture so that to observe the cells over the scratching line to check their growth at 0h, 8h, 16h and 24h under microscope and photos were taken. For each group, 5 visual fields and 3 repeated holes were detected, and the experiment would be repeated for three times.

Evaluation of cell in vitro invasion

Boyden-Chamber assay was applied. Millicell cell culture inserts with 8um diameter PET membrane were put into a 24-well culture plate, and then cover the bottom of

Millicell cell culture inserts with Matrigel Basement Membrane Matrix 50ul/well, keep them in 37℃ for a whole night to make them become gelatinous. A549 cells, which had been treated with metformin with different concentrations for 72h, would get trypsinization and be collected. Then, RIMP1640 culture solution with 1% fetal bovine serum was applied to make cell suspension, and regulate the cell density into $5×10^5$/ml. Inoculate over insert at 100ul/well, besides, add 600ul culture medium which contains 10% fetal bovine serum to insert. There were 3 repeated holes in each cell group. After conventional culture for 30 hours, fetch Millicell and remove the cells over top of microporous membranes carefully with cotton swabs, then fix with 4% paraformaldehyde for 10minutes, stain with methyl violet for 10 minutes. Take upper, lower, left, right and central five visional fields under light microscope (×40), count lower membrane cells, and calculate the average value.

MMP2 and MMP9 mRNA expression detected by Real-Time PCR

After interfering cells with metformin for 48 hours, extract total RNA of each group by Trizol, then cDNA would be get from 500ng RNA reverse transcription, then take cDNA product as format to evaluate MMP2 and MMP9 mRNA expression by Real-Time PCR. MMP9 premier: upstream: 5'-AACTCACGCGCCAGTAGAAG-3'; downstream: 5'-GAGGTGGACCGGATGTTCC-3'; product length was 105bp, and the annealing temperature was 60℃. MMP2 premier: upstream: 5'-GCCCAAGAATAGATGCTGACTG-3'; downstream: 5'-TGAAAGGAGAAGAGCCTGAAGTG-3'; product length was 165bp, and the annealing temperature was 56℃. GAPDH premier: upstream: 5'-GCACCGTCAAGGCTGAGAAC-3'; downstream: 5'-ATGGTGGTGAAGACGCCAGT-3'; product length was 142bp, and the annealing temperature was 56℃. Amplification conditions of Realtime-PCR: 95℃ 30s; 95℃ 15s, 60℃ 15s, 72℃ 45s, there were 40 cycles in total and the experiment was repeated for three times. GAPDH was taken as internal reference, and Rotor-Gene 6000 Series Software 1.7 was employed for result analysis, the relative qualification (RQ) of target gene $=2-\Delta\Delta CT$. We would take mean value of target gene mRNA of control group as 1, and calculate mRNA relative qualification of other groups.

Statistical analysis

Experimental data was expressed as Mean±SD, and SPSS 11.5 statistical software was applied, one-factor analysis of variance was carried out by multi group comparison. *LSD-t* test was performed between two groups, we would take it to be with statistical
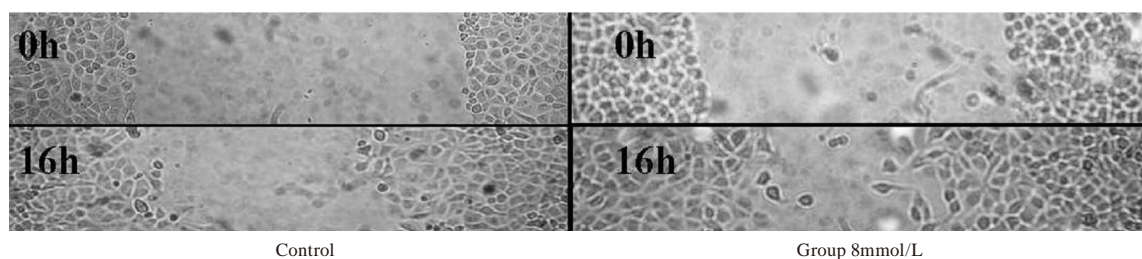
Fig 1. The lateral migration ability of A549 cells was improved after treated by metformin of high concentration
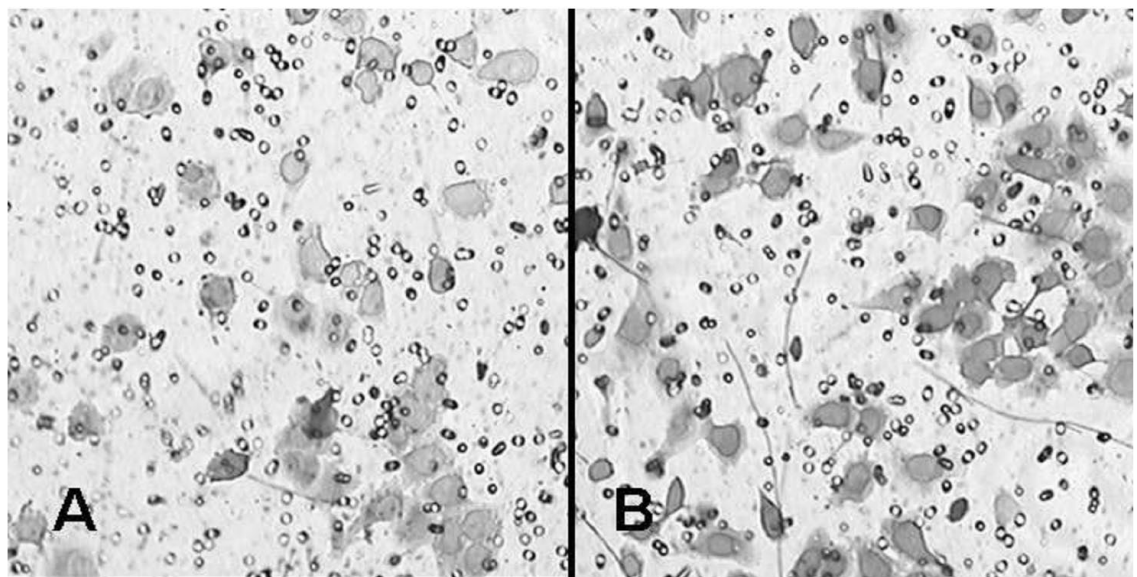

Fig 2. The invasion ability of A549 cells in vitro was increased after treated by metformin of high concentration.
(A) Control; (B) Group 8mmol/L.

significance when *P*<0.05.

Results

Effects of metformin to A549 cell lateral migration ability

After cell scratch for 16 hours, compare it with control group, A549 cell scratch of 8mmol/L metformin group was obvious narrowed, which indicated lateral migration speed of this cell group had been significantly improved (Figure 1), however, there was no big speed difference over metformin 0.5mmol/L group and 2mmol/L when comparing with control group.

Effect of metformin to A549 cell in vitro invasion

After conventional culture in Millicell cell culture inserts for 30 hours, the count of cells crossed Matrigel Basement Membrane Matrix contained membrane in control group and 8mmol/L metformin group were respectively 37.4±4.6 and 59.8±7.2, the difference between which showed statistical significance (*P<0.05*) (Figure 2). The counts of 0.5mmol/L and 2mmol/L metformin groups

were respectively 36.5±2.8 and 40.6±4.9, both of which had no obvious difference when comparing with that of control group.

Effect of metform to MMP2 and MMP9 mRNA expression in A549 cells

Realtime-PCR result showed that after metformin interference, MMP9 mRNA expression in A549 cells of each group had been heightened, which had significant statistical difference comparing with control group (*P<0.05*), besides, the mRNA expression level would increase as the concentration of metformin increases. MMP2 expression in cells of metformin 8mmol/L group was obviously higher than that of control group (*P<0.05*), however, MMP2 mRNA expression in cells of 0.5mmol/L and 2mmol/L metformin groups had no statistical difference with that of control group (Table1, Figure 3).

Discussion

Metformin is a safe and effective hypoglycemic drug

Tab 1: The impact of metformin to expression of MMP2 and MMP9 mRNA

Group	MMP9	MMP2
Control	1.00±0.088	1.00±0.048
0.5 mmol/L	8.66±3.296*	0.89±0.053
2 mmol/L	27.57±4.785*	1.08±0.076
8 mmol/L	35.14±1.55*	2.10±0.322*

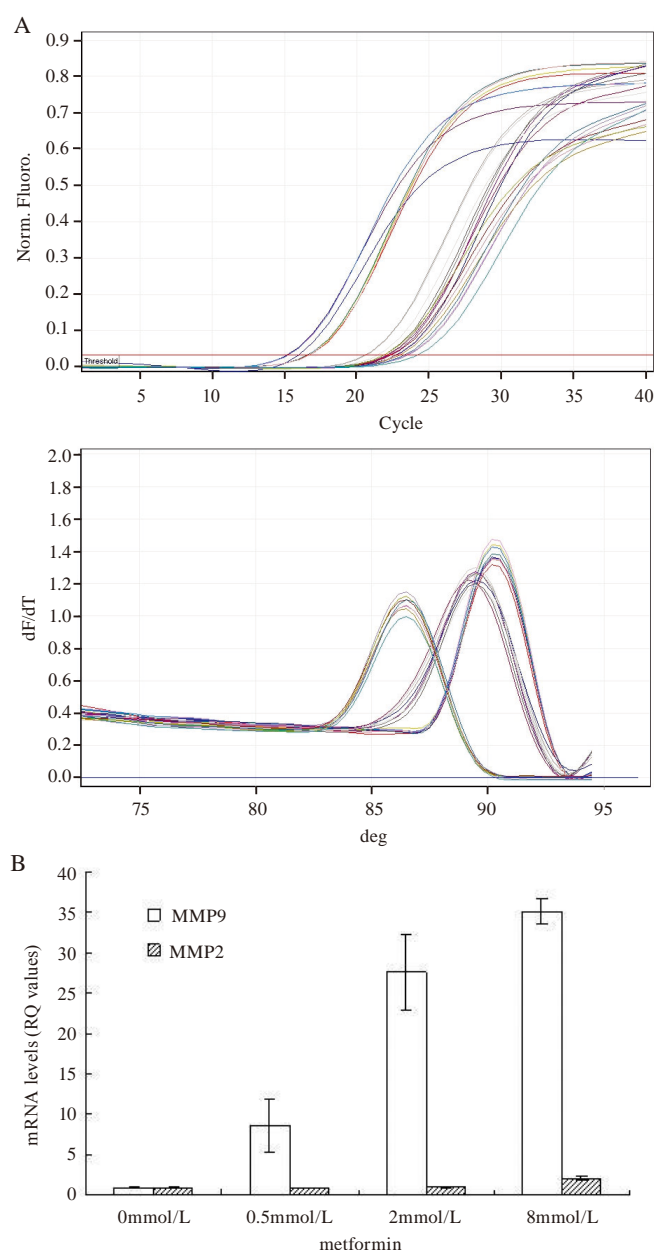The levels of mRNA expression are given as RQ values.
*$P<0.05$

Fig 3. The impacts of metformin to expression of MMP2 and MMP9 mRNA in A549 cells. (A) The amplification curves (left) and melting curves (right) of MMP2 and MMP9 genes acquired from realtime-PCR analysis; (B) The relative mRNA levels of MMP2 and MMP9 genes.

widely applied in clinical. It has been verified through research that mainly through activating AMPK (AMP-activated protein kinase) signal in cells, metformin would establish transduction pathway, so as to reduce intrahepatic gluconegenesis, meanwhile increase sugar absorption and utilization of skeletal muscle and so on, so that peripheral blood sugar would be reduced (3). In researches in recent years, it had been shown that activation of AMPK signaling pathway had significant effect on inhibiting tumor occurrence and development (4,5), as an effective AMPK activator, potential anti-tumor ability of metformin had been recognized. According to many researches, metformin has obvious inhibition to breast cancer, prostate cancer, ovarian cancer and colorectal cancer (2, 5-8). However, a recent research to animals with breast cancer has showed that though metformin could inhibit the growth of transplanted tumor in mice, it meanwhile increases VEGF (vascular endothelial growth factor) expression in estrogen receptor α negative breast cancer tissues, and it also increases intratumoral microvessel density, which indicates that metformin might lead to invasion and increased transfer activity for some tumors (2). It has been shown in our preliminary researches that metformin has a strong inhibition to human pulmonary adenocarcinoma A549 cells (9), and in this research, migration in vitro and invasive activity of A549 cells under metformin interference were detected, it had been found that after metformin interference in a certain concentration, migration in vitro and invasive activity had been increased.

Tumor metastases means tumor cells adhere and pass through extracellular matrix, survive and migrate beyond primary foci. The key step for cancer cell metastases lies in tumor cells migration and their invasion to surrounding tissues and vessels, occurrence and development of which requires the combined effect of change of cell proliferation, differentiation and locomotion related gene and their expression regulation mode and external factors for promoting cell locomotion (10). MMPs (matrix metalloproteinases), as a kind of Zn+ dependent endogenous proteinase, containing at least 25 members, can almost degrade all components of extracellular matrix except for polysaccharide, and it is involved in tumor progression, metastases and other various pathophysiological processes (11). Among MMPs secreted by tumor cells, MMP2 and MMP9 are the most important degradable collagenases, both of which have significant effect of tumor neovascularization, tumor cell invasion and the progress of metastasis formation. It has been verified by lots of studies that high expression level of MMP2 and MMP9 has a close relationship with lung cancer metastases, moreover, expression increase of these two protease would be more obvious in small cell lung

cancer with early stage metastases (12, 13). This research has compared MMP2 and MMP9 mRNA expression in A549 cells before and after metformin interference, whose result shows that after medicine treatment, both of these two gene expressions have been significantly increased, and especially for MMP9, it has been indicated that up-regulation of MMP2 and MMP9 expression might be one of the mechanisms for A549 cell migration and strengthened invasive ability after metformin interference. As to metformin's influence to angiogenesis in pulmonary adenocarcinoma tissues and its correlation with tumor metastases, experiment *in vivo* is required for further verification.

This research shows that metformin can promote human pulmonary adenocarcinoma A549 cell lines in vitro migration and the strengthening of invasive activity, it potentially promotes tumor metastases, which might be relevant to metformin's inducing expression up-regulation of MMP2 and MMP9 in tumor cells.

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