## ORIGINAL ARTICLE

# Effect of metformin on the proliferation and apoptosis of the renal cancer cell line 786-O and the underlying mechanisms

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## Summary

**Purpose:** *To investigate the effects of metformin (Met) on the proliferation and apoptosis of a renal carcinoma cell line and study the underlying mechanisms.*

**Methods:** *Renal cell carcinoma (RCC) line 786-O was cultured with media containing different concentrations of Met. The proliferation and apoptosis of 786-O cells were detected by the MTT method and flow cytometry, respectively. The invasion of 786-O cells was detected by scratch test, and the expression of pro-apoptotic proteins was determined by Western blot assay.*

**Results:** *The proliferation rates of 786-O cells with Met concentrations of 10, 15, and 20 mM were decreased by 62.8, 61.7, and 65.1%, respectively, with no significant difference among these concentrations (p>0.05). Twenty-four*

*hrs after the scratch assay, the mean migration index in the control group and Met treatment group was 51.6% ± 5.9 and 28.1% ± 4.3, and that after 48 hrs was 92.2% ± 6.4 and 68.0% ± 4.9, respectively (p<0.05). At low serum concentration, the percentage of apoptotic cells in the Met treatment group (17.6%) was significantly higher (p<0.05) than that in the control group (5.2%). Met (10 mM) treatment significantly increased the expression of Caspase-3 and Bax proteins in 786-O cells (p<0.05).*

**Conclusions:** *Metformin may be a potential drug of choice in the treatment of metastatic RCC.*

**Key words:** *apoptosis, metformin, proliferation, renal carcinoma*

## Introduction

RCC accounts for 80–90% of all renal malignant tumors, and is one of the most common tumors in the urinary system, accounting for about 2–3% of all adult malignancies, with approximately 210,000 new cases worldwide . Of all urinary tract tumors, RCC is the most lethal and epidemiological data show that more than 40% of RCC patients die of the disease, with up to 100,000 RCC-related deaths each year worldwide [1,2].

Surgery is the main method to cure RCC . More than 90% of patients with focal RCC who underwent radical nephrectomy or nephron-preserving surgery achieve 5-year disease-free survival [3]. However, metastasis is discovered in about 1/3 of the RCC patients at initial diagnosis, making these patients not eligible for radical surgery. In addition, about 20-30% of focal RCC patients develop recurrence or metastasis after sur-

gery [4]. Metastatic RCC (mRCC) has a very poor prognosis, with a median survival of only 6–12 months, and the 5-year overall survival rate is less than 10%. Therefore, the treatment of mRCC remains a major clinical challenge [5].

Metformin (Met) is widely used clinically as a biguanide antidiabetic drug, mainly in the treatment of type 2 diabetes [6]. Recent studies have found that the risk of prostate cancer, breast cancer, liver cancer, and other tumors was reduced in diabetic patients who used Met, and the cancer-related mortality rate was also reduced [7-10]. Furthermore, an experimental study showed that Met could suppress the proliferation of *in vitro*-cultured prostate cancer cells, breast cancer cells, pancreatic cancer cells, and melanoma cells, and *in vivo* experiments also confirmed that Met could inhibit the growth of the tumors listed above [11-14]. These studies suggested that Met might be a useful agent for tumor prevention. However, very little research has been reported on the effects of Met in RCC. Given the current difficulties in the treatment of mRCC, studying the possible effects of Met as an inexpensive and safe drug on the growth of RCC might be valuable, and the results would provide a meaningful foundation for progress in the clinical treatment of mRCC.

In this study, an *in vitro*-cultured RCC cell line (786-O) was established, the impact of Met on the growth and apoptosis of 786-O cells was evaluated, and the possible mechanisms of Met activity were explored to provide new evidence of Met's anti-tumor effects, and propose new ideas and options for the clinical treatment of mRCC.

## Methods

### Detection of 786-O cell proliferation

The RCC cell line 786-O (Cell Bank of the Chinese Academy of Sciences, Beijing, China) was cultured using conventional procedures; the well-growing third-generation 786-O cells were seeded in a 96-well plate at a concentration of $2 \times 10^3$ cells/well, with four parallel wells for each well. Twenty-four hrs later, when the cells adhered to the wall, they were transferred to Dulbecco's modified Eagle's medium (GIBCO Corp.; Carlsbad, CA, USA) containing different concentrations of Met (0, 1, 5, 10, 15, and 20 mM; Sigma Aldrich Corp.; St. Louis, MO, USA), and cultured for another 48 hrs MTT solution (20 μl; 2 mg/ml) was then added into each well, and the absorbance value was measured at 490 nm. The proportion of viable cells (% of control) was analyzed for the different Met concentration groups for the selection of the optimal Met concentration to serve as the treatment group in subsequent experiments.
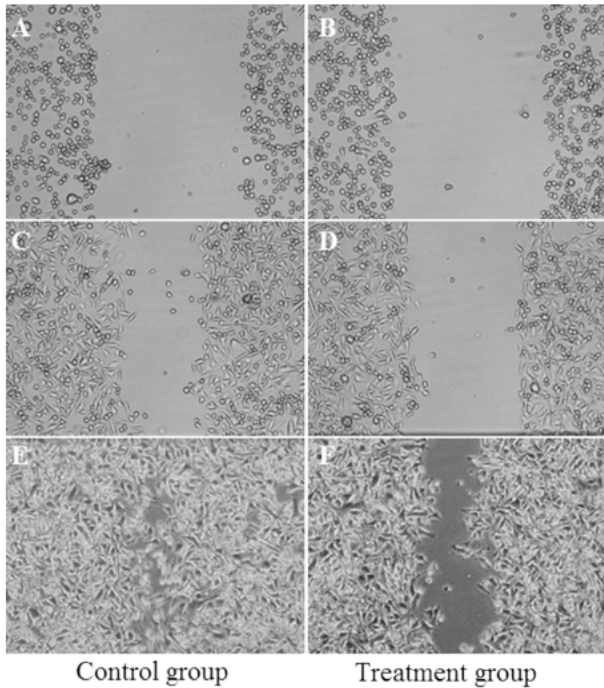
### Scratch test of 786-O cells

The well-growing third-generation 786-O cells were cultured in a 6-well plate for 24 hrs, and then a straight-line scratch was made on the culture plate with a 1-ml pipette tip to form a cell-free region. Medium containing 10 mM Met (the experimental group) or that without Met were added for further cultivation (37ºC, 5% CO₂). Images at 0, 24, and 48 hrs of incubation were collected using IX73 inverted microscope (Olympus Corp., Tokyo, Japan). The width of the "naked" region of the cells around the upper, middle and lower part of scratch in image was measured, and the average value was calculated. The migration index (MI) was used to indicate the speed of cell migration.

### Flow cytometry

The well-growing third-generation 786-O cells were inoculated into a 6-well plate for 24-hr incubation, and then the medium was replaced with 1% or 10% fetal bovine serum (FBS) (GIBCO Corp., CA, USA) and 10 mM Met. The cells were then cultured for another 24 hrs. The cells were collected and stained with an Annexin-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining apoptosis detection kit (Invitrogen Corp., CA, USA), and apoptosis detection was conducted by flow cytometry (FACSCalibur flow cytometer; BD Biosciences, CA, USA)

### Western blot

The 786-O cells were conventionally cultured to the third generation, and then cultured for a further 24 hrs until the cells adhered to the wall of the plate. The medium was then replaced with 10 mM Met and 1% FBS for continuous culture. The total cell proteins in each group were extracted by cell lysis buffer at different culture time points (0, 12, 24, and 48 hrs), and the BCA protein assay kit (Sigma Aldrich Corp., CA, USA) was used to detect the protein concentrations of each group. A 5% stacking gel and 8% separating gel were prepared for polyacrylamide gel electrophoresis at 60 V for 30 min and 150 V for 1 h, respectively. After electrophoresis, the separating gel was removed, washed three times, and placed in the PVDF membrane (Advantec MFS, Inc., CA, USA) for transfer at 100 mA for 30 min. After membrane transfer, horseradish peroxidase-labeled rabbit anti-human monoclonal antibody (Caspase 3, Bax) (Santa Cruz Biotechnology, Inc., CA, USA) was added and swing-incubated (1:1500) at 4°C overnight. The membrane was washed and goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) was added to the developer solution (Santa Cruz Biotechnology, Inc., CA, USA) for incubation for 30 min at room temperature in the dark. The system was washed three times with the eluent to terminate the staining, and the ChemiDoc XRS gel imaging system (Bio-Rad Laboratories, Inc., CA, USA) was used to analyze the optical density (OD) values of the

**Figure 1.** Effects of Met on the migration ability of 786-O cells. The cell recovery at different time points after scratch was observed under the microscope. The 786-O cells were seeded in 6-well plates and cultured for 24 h, then 1 ml pipette tip was used to make a straight line scratch, forming a cell-free region; then images were acquired after incubation at different time periods: 0 h (**A**, **B**), 24 hrs (**C**, **D**) and 48 hrs (**E**, **F**). p values: 24 hrs: 0.047, 48 hrs: 0.029

membrane strips and the internal reference. The experiment was repeated three times for calculation of the relative OD value.

*Statistics*

Statistical analyses were perfomed using the SPSS 19.0 software for Windows (SPSS, Chicago, IL, USA). Data were expressed as mean±standard deviation (x±s), and the differences among groups were compared using the one-way analysis of variance (one-way ANO-VA). A p value <0.05 was considered as statistically significant.

## Results

*Proliferation of 786-O cells*

To study the effect of Met on the proliferation of 786-O cells, Met was added into the culture medium of 786-O cells at different concentrations (0, 1, 5, 10, 15, and 20 mM). After 48-h cultivation, the MTT assay was performed, which showed that Met could significantly inhibit the proliferation of 786-O cells in a dose-dependent manner up to 10
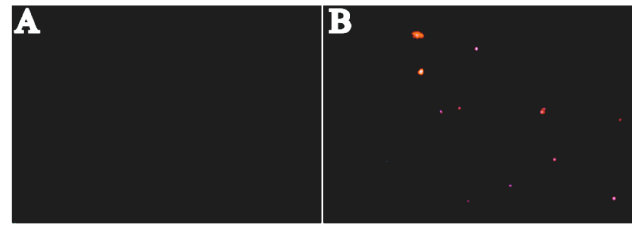
**Figure 2.** Detection of 786-O cell apoptosis by Annexin V-FITC/PI staining. (**A**) Met+10% FBS; (**B**) Met+1% FBS; the red-stained apoptotic cells could be observed under fluorescence microscopy in B, while could not in A (A vs B, p=0.038).

mM. When the Met concentration was increased up to 10, 15, and 20 mM, the 786-O proliferation rate was reduced by 62.8, 61.7, and 65.1%, respectively, with no significant difference among these concentrations (p>0.05). To comply with the principle of "minimum dose and maximum efficacy," we decided that the optimal proliferation inhibition concentration of Met was 10 mM.

*Migration of 786-O cells*

We used the cell scratch test to observe the impact of Met on 786-O cell migration. The rate of 786-O cell recovery was determined at different time points after the scratch. At 24 hrs after the scratch, the MIs of the control group and the Met treatment group were 51.6% ± 5.9% and 28.1% ± 4.3%, respectively, representing a statistically significant difference (p=0.047). At 48 hrs after the scratch, the MIs of the control group and the Met treatment group were 92.2% ± 6.4% and 68.0% ± 4.9%, respectively, which was also statistically significant (p=0.029). These results indicated that Met could decrease the MI of 786-O cells, and therefore reduce the invasive ability of RCC (Figure 1).

*Apoptosis of 786-O cells in the presence of low serum concentration*

To detect the ability of Met to induce apoptosis in 786-O cells, the Annexin V-FITC/PI staining method was performed to measure the proportion of dead 786-O cells. In the 10% FBS culture medium, the cells in the experimental and control groups were morphologically similar, exhibiting no red-stained dead cells under the fluorescence microscope; when the medium was replaced with 1% FBS culture medium, some cells showed rounding, wrinkling, and other changes, and red-stained apoptotic cells were observed under the fluorescence microscope (p=0.038 ; Figure 2).
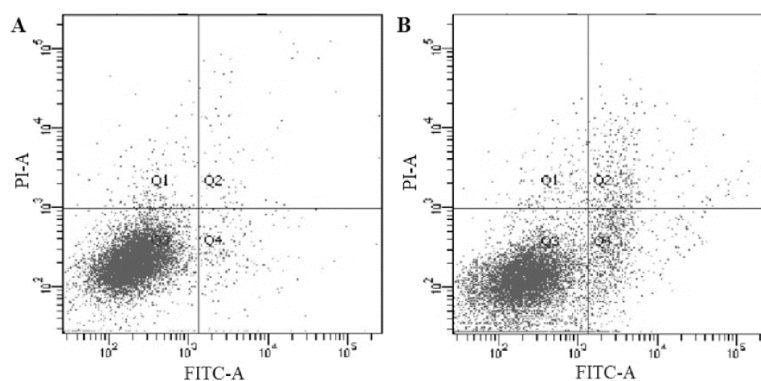
**Figure 3.** Detection of the apoptotic cells by Annexin V-FITC/PI staining and flow cytometry. In the flow scatterplot, Q3 indicated the living cells, while Q1, Q2 and Q4 indicated the apoptotic cells; the apoptotic cell ratio (dead cells (%) was calculated as follows:dead cells (%)=(Q1+Q2+Q4)/(Q1+ Q2+Q3+Q4)×100%; (**A**): 10% FBS+Met; (**B**): Met+1% FBS (p<0.034).

Flow cytometry was used to quantify the Met-induced effects on cell death, which showed no significant difference between the experimental and control group in the 10% FBS medium, whereas in the low serum concentration (1% FBS), the percentage of apoptotic cells in the experimental group was 17.6%, which was significantly higher (p=0.034) than that in the control group (5.2%). This result suggested that Met could not induce apoptosis of 786-O cells under a normal serum concentration, but could induce apoptosis under a low serum concentration (Figure 3).

*Expression of pro-apoptotic proteins in 786-O cells*

We further explored the possible mechanism through which Met induced the apoptosis of 786-O cells. The western blot results showed that a 10 mM Met concentration (in 1% FBS medium) significantly increased the expression of Caspase-3 and Bax proteins in 786-O cells after different incubation periods, with significant differences among the different concentration groups (p=0.042, p=0.039, p=0.040, p=0.031, respectively). Expression levels also increased in a time-dependent manner, suggesting that Caspase-3/Bax-mediated apoptosis is an important mechanism contributing to the induction of apoptosis of 786-O cells by Met (Figures 4 and 5).

## Discussion

Recent studies have found that the widely used hypoglycemic agent Met might reduce cancer risks in diabetic patients [15]. Although reports about the potential for Met to reduce the risk of RCC are rare and show inconsistent results, some studies have shown an increasing trend of RCC risk in patients with obesity and diabetes who have not used Met before. This suggests that Met might exhibit a certain inhibitory effect toward the occurrence of RCC in patients with diabetes [16]. One study found that Met could improve the disease remission rate in patients with diabetes and breast cancer receiving neoadjuvant endocrine therapy [17], suggesting that Met could not only help to reduce blood sugar but might also play a role as an anti-cancer drug. These findings have motivated research interest of the anticancer effects of Met.

Currently, Met is considered to inhibit cell proliferation in prostate cancer, breast cancer, pancreatic cancer, and melanoma [18]. In this study, it was found that Met could inhibit the *in vitro* proliferation of 786-O cells; 1 mM Met decreased the proliferation of 786-O cells by nearly 20%, and 5 mM Met reduced proliferation by about 30%. Met was also previously shown to inhibit the proliferation of breast cancer cells; 1 mM Met could reduce the proliferation of BT20 breast cancer cells by about 21% [14]. Therefore, the effects of Met on the 786-O cells shown in our study are consistent with the literature; the inhibitory effect of Met on RCC cell proliferation in patients who had not used Met befor was concentration-dependent up to 10 mM. At Met concentrations of 10, 15, and 20 mM, the 786-O proliferation rates were reduced by 62.8%, 61.7%, and 65.1%, respectively, with no statistically significant difference among these concentrations (p>0.05).

The invasion and migration of tumor cells are crucial components of tumor progression, play-
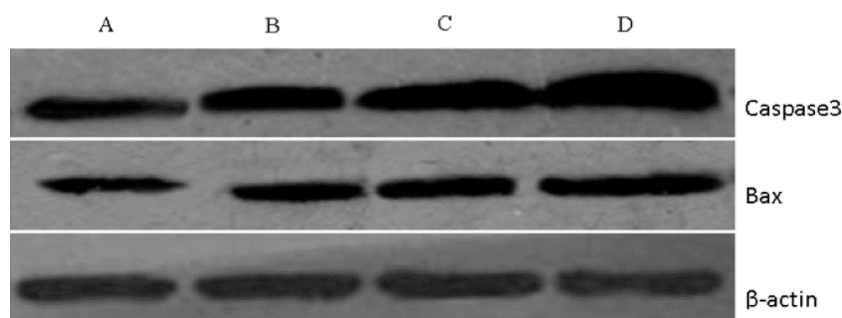
**Figure 4.** Detection of apoptotic protein expressions of 786-O in different time periods by Western blot. (**A**) 0 hrs; (**B**) 12 hrs; (**C**) 24 hrs; (**D**) 48 hrs. p values: **A**: 0.042, **B**: 0.039, **C**: 0.040, **D**: 0.031.
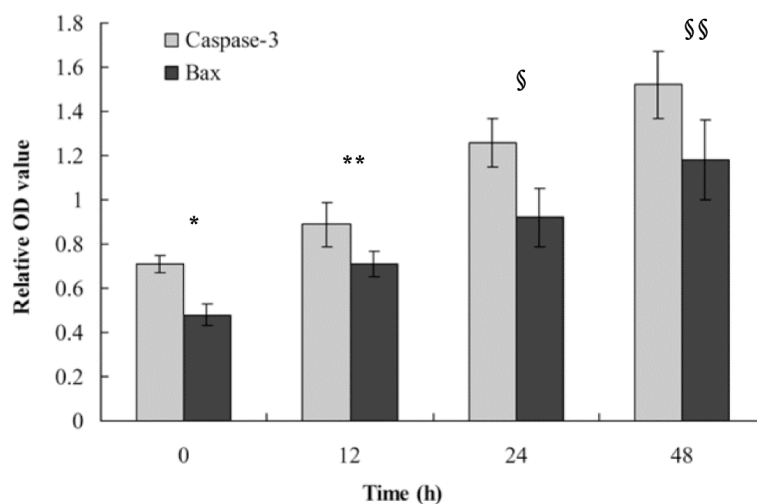


**Figure 5.** Relative OD values of the apoptotic protein expressions at different time periods. p values: *0.042, **0.039, $^{§}$0.040, $^{§§}$0.031.

ing extremely important roles in the invasion and distal metastasis of tumor cells [19-22]. Our study showed that Met could decrease the MI of 786-O cells by nearly 30%, and could inhibit the scratch-healing ability of 786-O cells. These results suggest that Met has important effects on tumor cell migration abilities, and could thus inhibit the distant invasion and migration of RCC.

To test whether Met could induce the apoptosis of 786-O cells, we used the Annexin V-FITC/PI staining method to observe 786-O cell death. The results showed that in the normal serum concentration (10% FBS), Met could not induce the apoptosis of 786-O cells, while in an environment of low serum concentration (1% FBS), Met could induce apoptosis. These results indicate that Met could induce apopto-

sis of RCC cells in a condition of low nutritional status, which also suggests that Met might help to enhance the lethal effects of traditional chemotherapy drugs towards RCC.

We further explored the possible mechanism through which Met could induce the apoptosis of 786-O cells. Western blot results showed that 10 mM Met (in 1% FBS medium) could significantly increase the expression of Caspase-3 and Bax proteins in 786-O cells at different treatment times, indicating that Caspase-3/Bax-mediated apoptosis is one of the important mechanisms underlying the ability of Met to induce apoptosis.

In conclusion, this study explored the impacts and molecular mechanism of Met on the growth of 786-O cells, at both the cellular and

molecular levels. These results provide new evidence for the anti-tumor effects of Met, and new ideas and treatment options for the clinical treatment of mRCC, suggesting that Met might become a potential drug of choice in the treatment of mRCC.

## Acknowledgements

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