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Original Article

Study on inhibitory effect of metformin in bladder cancer and its mechanism

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Abstract: Objective: To investigate the inhibitory effect of metformin on bladder cancer cell and its mechanism. Methods: In this study, metformin with different concentrations (0, 2, 5, 10, 20 mM) was used for the intervention on bladder cancer cell lines 5637 and T24 in vitro. Its influence on the growth and proliferation of cells was detected by MTT assay; the impact on cell colony formation was quantitatively analyzed by Quantity One software; the influence on cell apoptosis was examined by Annexin V/double staining assay. Bladder cancer cell xenograft was constructed in nude mice, and the effect of metformin on the volume and weight of xenograft was observed. Results: After 48 hours' intervention by metformin with different concentration, by comparing with control group, the cell activity of 5637 cells and T24 cells was obviously reduced, and a significant reduction was observed in colony formation of bladder cancer cells after the intervention of metformin. Metformiin with high concentration had no significant induction on the apoptosis of 5637 cells and T24 cells; compared with the control group, the difference was no statistically significant. In vivo experiments showed that intraperitoneal injection of metformin could obviously inhibit the growth of tumor xenograft. Conclusion: metformin can significantly inhibit the ability of proliferation and colony formation of bladder cancer cells in vitro, this inhibition may not be achieved by inducing apoptosis of bladder cancer cells.

Keywords: Metformin, bladder cancer, proliferation, colony formation, apoptosis

Introduction

Bladder cancer is one of the most common malignant tumors of urinary system, and it shows an increasing trend in recent years [1]. Currently, patients with bladder cancer that consulted at clinic are mostly with non-muscle invasive bladder cancer, and transurethral resection of bladder tumor (TUR-Bt) [2] is the main method to treat the disease. However, this method has a problem of high recurrence rate after surgery [3]. There are studies reported that the recurrence rate could reach as high as 60%-70% 5 years after TUR-Bt, and approximately 25% of the patients will develop into invasive bladder cancer, which is the main reason for poor prognosis [4]. At present, the most effective way that recognized to prevent recurrence and progression of bladder cancer is adjuvant intravesical chemotherapy or immunosuppressive medication after TUR-Bt. Although recently the intravesical chemotherapy has certain effect, it has different adverse reacts such as allergic reactions, bladder irritation, bone marrow suppression and so on for those commonly used perfusion drugs. Therefore, exploring an effective and safe medication to treat bladder cancer has become a hot topic of research.

Currently, metformin is used widely in clinic as an oral hypoglycemic drug. Since the coming out of metformin in the 1950s, it is widely used in the treatment of patients with type 2 diabetes [5]. In recent years, a number of clinical trials found that metformin may have certain degree of inhibitory effect on malignant tumors, and can significantly enhance the anti-tumor effect of chemotherapy drugs [6, 7]. There are studies reported that the recurrence rate of patients with bladder cancer, who took metformin to reduce blood glucose, was significantly

lower than those who didn't. But now, the mechanism of metformin on inhibiting bladder cancer is not clear. Thus, we investigated the effect of metformin on the proliferation, colony formation and apoptosis of bladder cancer cells by *in vitro* experiment and subcutaneous xenograft experiment; the research can provide experimental evidence for the feasibility of metformin used as an adjunct therapy to treat bladder cancer.

Material and methods

Experimental materials

Cells lines and cell culture: Human bladder cancer cell lines (5637 and T24) were purchased from American type culture collection (ATCC). Cells were seeded in RPMI-1640 culture medium containing 10% fetal bovine serum and double antibody (100 mg/L streptomycin and 1×10^5 U/L penicillin) and cultured under the condition of 37°C and 5% CO_2, cells at logarithmic phase were collected for subsequent experiments.

Main reagents and materials

RPMI-1640 medium, DMEM medium and fetal bovine serum (FBS) were purchased from Gibco Company (USA); trypsin and EDTA were purchased from Gibco Company (USA); metformin and MTT were purchased from Sigma-Aldrich Corporation (USA); Annexin V-FITC/PI apoptosis kit was purchased from BD Biosciences Company (USA).

Experimental method

The effect of metformin on the proliferation of bladder cancer cells in vitro was detected by MTT method: 5637 cells and T24 cells at logarithmic phase were used to prepare single cell suspension by conventional digestion, centrifugation, and suspension. The cells were inoculated in the 96-hole culture plate at a density of 5×10^3 cells/hole, and incubated in an incubator for overnight at 37°C and 5% CO_2. The next day, remove the medium in the hole and replenish with culture medium containing 100 μL metformin at different concentrations (2, 5, 10 and 20 mM). After 72 hours of routine culture, 20 μL MTT (5 mg/mL) was added into each hole, 4 hours later, remove the supernatant and add 150 μL DMSO solution, then vibrate for 10 minutes in dark environment. The 96-hole culture plate was implanted into enzyme-labeled instrument to examine the absorbance value of each hole at wave length of 490 nm. The cell survival rate (%) = OD value of drug group / OD value of control group ×100%.

Colony formation test: 5637 cells and T24 cells at logarithmic phase were inoculated into 6-hole culture plate at a density of 1×10^3 cells/hole and cultured in an incubator for overnight at 37°C and 5% CO_2. The next day, remove the medium in the hole and replenish with culture medium containing 100 μL metformin at different concentrations (2, 5, 10 and 20 mM) to continue the incubation. Two weeks later, remove the medium in the hole; after fixed with 4% formaldehyde for 15 minutes, the cells were rinsed and then stained with 0.1% crystal violet for 5 minutes. Then, the cells were washed with double distilled water to colorless, photographed and recorded. Cell clone was quantatively analyzed using Quantity One software. The clone formation rate (%) = the clone number of the drug group / the clone number of control group ×100%.

Cell apoptosis was detected by flow cytometry: Bladder cancer cell lines at logarithmic phase were inoculated into 6-hole cell culture plate at a density of 2×10^5 cells/hole for overnight. On the following day, medium containing metformin at different concentrations (2, 5, 10, and 20 mM) was added into the culture, after 24 hours incubation, the cells were digested with trypsin, and centrifuged and collected for the preparation of single cell suspension. The cells were washed twice with pre cooled PBS, resuspended using 250 μL binding buffer and adjusted to the concentration of about 1×10^6 cells/mL. 100 μL cell suspension was moved out and added with 5 μL Annexin V-FITC and 10 μL PI solution in sequence. After mixing, the mixture was incubated in dark environment at room temperature for 15 minutes, later, 400 μL PBS was added in and evenly mixed, then flow cytometry analysis was performed to examine the cell apoptosis.

Subcutaneously transplanted xenograft in nude mice: The bladder cancer cell line was collected and the cell density was adjusted to 2×10^7/m, 0.1 ml cell suspension was subcutaneously inoculated on the left side of back leg

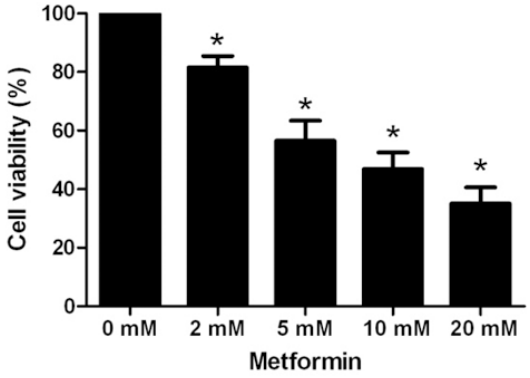

Figure 1. Measurement of cell activity of 5637 cells after the treatment of metformin at different concentration by MTT method. *P<0.05 *vs.* control group.

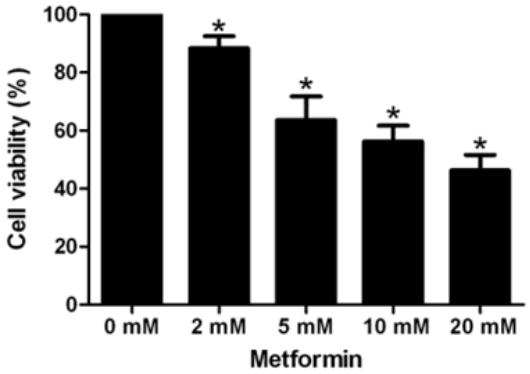

Figure 2. Measurement of cell activity of T24 cells after the treatment of metformin at different concentration by MTT method. *P<0.05 vs control group.

of nude mice; when the diameter of xenograft was about 6 mm, the mice were divided into two groups: drug group and control group. Nude mice of drug group were intr-aperitoneally injected of 100 mg/kg metformin solution every day, and nude mice of control group were intra-peritoneally injected of the same amount of sterile saline. Changes of body weight and volume of subcutaneous tumors in two groups of mice were observed.

Statistical analysis

The data were statistically analyzed by SPSS 17.0 software, the measurement data were expressed by mean ± standard deviation, and the statistical comparison was carried on using one-way ANOVA. *P*<0.05, the difference was statistically significant.

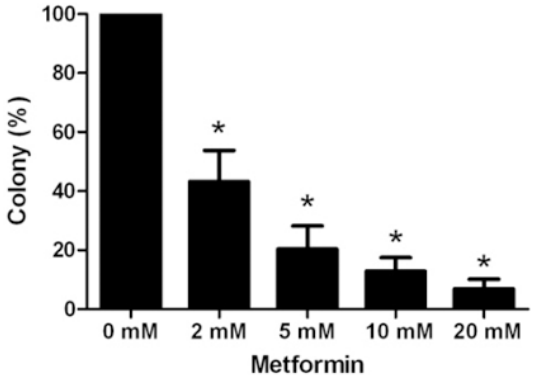

Figure 3. Effects of metformin at different concentration on colony formation of 5637 cells. *P<0.05 *vs.* control group.

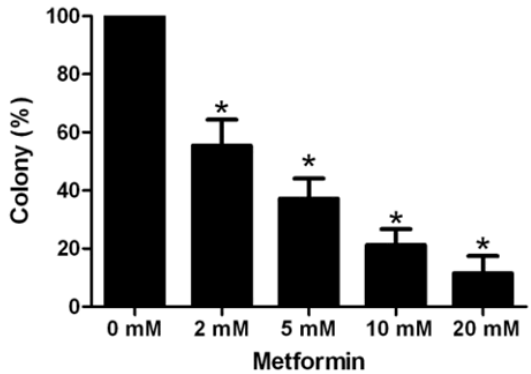

Figure 4. Effects of metformin at different concentration on colony formation of T24 cells. *P<0.05 *vs.* control group.

Results

Inhibitory effect of metformin on the proliferation of bladder cancer cells

Bladder cancer cells were treated with different concentrations of metformin in this study, the survival rate of bladder cancer cell lines 5637 and T24 were detected by MTT method. After the intervention with different concentrations (2, 5, 10 and 20 mM) of metformin for 48 hours, compared with the control group, the cell viability of 5637 cells ((81.51 ± 3.95)%, (56.52 ± 6.85)%, (46.84 ± 5.72)%, (35.14 ± 5.46)%) and T24 cells ((88.35 ± 4.22)%, (63.62 ± 8.15)%, (56.32 ± 5.37)%, (46.25 ± 5.35)%) in drug group were statistically inhibited, as shown in **Figures 1** and **2**. The results showed that metformin significantly inhibited the activity of

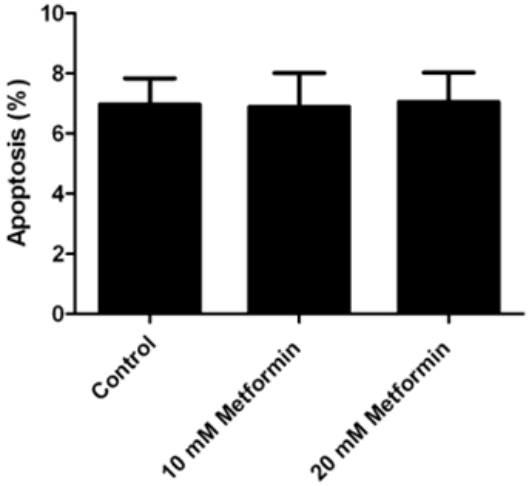

Figure 5. Apoptotic rate of 5637 cells was detected by flow cytometry after treated with metformin at concentrations of 10 mM and 20 mM for 24 h.

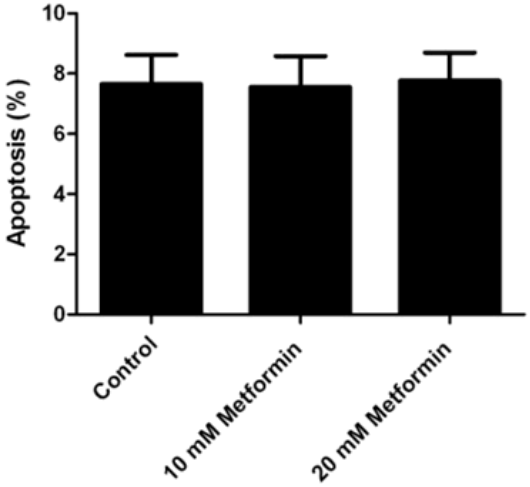

Figure 6. Apoptotic rate of T24 cells was detected by flow cytometry after treated with metformin at concentrations of 10 mM and 20 mM for 24 h.

bladder cancer cells, and the inhibition was significantly dose-dependent.

Inhibitory effect of metformin on the colony formation of bladder cancer cells

After the intervention with different concentrations of metformin for two weeks, compared with the control group, the clone number of 5637 cell was significantly reduced. Under the effect of metformin at the concentration of 2 mM, the clone number of 5637 cell was (43.21 ± 10.55)% ($P<0.05$). With the increase of concentration of metformin, the clone number of 5637 cells decreased, which showed significant dose dependence, as shown in **Figure 3**. After treatment with different concentrations of metformin, the clone number of T24 cells was significantly decreased by comparing with the control group. The clone number of T24 cell was (21.32 ± 5.37)% and (55.47 ± 8.87)% under the intervention of metformin at concentrations of 10 mM and 2 mM, respectively, which also showed significant dose dependence, as shown in **Figure 4**. The results showed that metformin could inhibit the colony formation of bladder cancer cells in a dose-dependent manner.

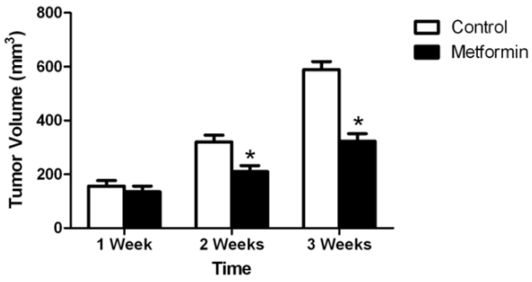

Figure 7. Effect of metformin on the volume of xenograft in nude mice. *$P<0.05$ *vs.* control group.

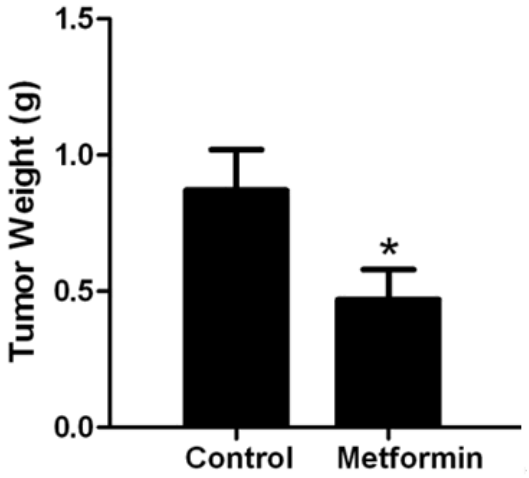

Figure 8. Effect of metformin on the weight of xenograft in nude mice. *$P<0.05$ *vs.* control group.

Effect of metformin on apoptosis of bladder cancer cells

Annexin V-FITC/PI staining was used to label apoptotic and necrotic cells after the treatment of metformin at different concentrations for 24

h, the results showed that apoptosis rate of 5637 cells was (6.89 ± 1.12)% and (7.05 ± 0.98)% at the concentration of 10 mM and 20 mM, respectively, and the apoptosis rate of T24 cells was (7.55 ± 1.03)% and (7.76 ± 0.94)%, respectively. Compared with the control group, there was no statistical difference, as shown in **Figures 5** and **6**. The results showed that metformin was not able to induce apoptosis of bladder cancer cells.

Effects of metformin on the general situation, tumor weight and volume of xenogragt in nude mice

The nude mice in both drug group and control group showed no abnormities like restlessness and listlessness etc. and there was no death in both groups. There were no significant differences in body weight and food intake between the two groups, indicating that metformin had no obvious influence on the general condition, body weight and food intake of nude mice, which expressed low toxicity and relative safety of metformin.

Compared with the control group, the growth rate of subcutaneous xenograft in nude mice was significantly inhibited; after 14 days of drug intervention, the volume of xenograft in drug group was obviously smaller than that in control group. 3 weeks later, the nude mice were sacrificed, and the weight of xenograft in drug group was significantly lighter than that of control group, see **Figures 7**, **8**. Obviously, metformin has the inhibitory effect on the growth of bladder cancer cell xenograft.

Discussion

As one of the most widely used oral hypoglycemic agents in clinical application, metformin has the characteristics of rapid hypoglycemic effect and fewer side effects; it is mainly used for the treatment of type 2 diabetes [8]. A number of animal experiments and clinical trials showed that metformin can significantly inhibit the proliferation of a variety of tumor cells, including renal carcinoma, breast cancer, prostate cancer, gastric cancer and ovarian carcinoma and so on [9, 10]. Recent clinical trials were conducted on patients with bladder cancer and combined with type 2 diabetes, and the result showed that among them, the patients who orally took metformin showed significantly reduced tumor recurrence while blood glucose was reduced. This suggests that metformin may have a certain inhibitory effect on bladder cancer cells [11, 12]. In view of insulin or other types of oral hypoglycemic agents may increase the risk of bladder cancer, the protective effect of metformin on patients with bladder cancer complicated with type 2 diabetes has drawn more and more attention of scholars [13, 14]. However, the exact inhibitory effect of metformin on bladder cancer cells and its mechanism are still not clear.

5637 cells representing high risk of superficial bladder cancer and T24 cells representing infiltration bladder cancer were selected as cell models for *in vitro* experiment, and were treated with different concentrations of metformin. The results of the research showed that after the treatment with different concentrations of metformin (2, 5, 10 and 20 mM) for 48 hours, the cell activity of 5637 cells was (81.51 ± 3.95)%, (56.52 ± 6.85%), (46.84 ± 5.72%) and (35.14 ± 5.46)% respectively, and the cell viability of T24 cells was (88.35 ± 4.22)%, (63.62 ± 8.15%), (56.32 ± 5.37%) and (46.25 ± 5.35)% respectively, compared with the control group, the differences were statistically significant (all $P<0.05$). This study shows that metformin can inhibit the extracorporeal proliferation of bladder cancer cells in a dose-dependent manner. The inhibitory effect of metformin in this study is essentially consistent with the results from other studies that conducted on other types of tumor cells [15, 16].

In addition, this study further investigated the effect of metformin on the colony formation ability of bladder cancer cells. After two weeks of treatment with different concentrations of metformin, the clone number of 5637 cell and T24 cell in drug group was significantly lower than those in control group. After the treatment of 2 mM concentration of metformin, the clone number of 5637 cell and T24 cell was (43.21 ± 10.55)% and (55.47 ± 8.87)%, respectively ($P<0.05$). With the increase in the concentration of metformin, the clone number of 5637 cell and T24 cell both decreased gradually in a dose-dependent manner. These results showed that metformin could significantly inhibit the ability of colony formation of bladder cancer cells.

The inhibition of tumor cell proliferation is often closely related to cell apoptosis. At present, there still are controversies about whether metformin can induce tumor cell apoptosis. The study of prostate cancer cells showed that metformin mainly inhibited the proliferation of tumor cells by inducing cell apoptosis. In this study, flow cytometry was used to detect the effect of metformin on the apoptosis of bladder cancer cells, and we found there was no significant difference in apoptosis ratio between drug group and control group. It is different from the recently approved mechanism of metformin inducing apoptosis of many kinds of tumor cells such as prostate cancer cell, renal cancer cell, breast cancer cell and gastric cancer cell [17, 18]. Some studies have reported that metformin can selectively induce the apoptosis of colon cancer cells with absence of P53 [19, 20]. In the subcutaneous bladder cancer xenograft experiment of this study, the results showed that metformin significantly inhibited the growth of subcutaneous xenograft. The results of all these studies suggest that, for tumors cells with different genetic background and different tissue sources and growth state, the mechanism of metformin inhibiting tumor cells is not exactly the same. The inhibitory effect of metformin on bladder cancer may be achieved through other mechanisms, which need to be further studied.

In conclusion, this study found that metformin can significantly inhibit the proliferation and colony formation ability of bladder cancer cells, but this inhibition is not achieved by inducing apoptosis of bladder cancer cells. And we believe that therapy of metformin will become a new method in adjuvant therapy for bladder cancer patients, and provides a new way for the prevention and treatment of malignant tumors.

Disclosure of conflict of interest

None.

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