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Original Article

Research of the effects of metformin on the proliferation of non-invasive bladder cancer cells in vitro

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Abstract: Objective: To study the effects and mechanisms of metformin on the proliferation of 253J non-invasive bladder cancer cell line. Methods: 253J cells were treated *in vitro* with metformin at different concentrations (5 mmoL/L, 10 mmoL/L and 20 mmoL/L). The effects of metformin on the proliferation of 253J cells, cell cycle, cell apoptosis and the expression of cell proliferation related proteins were respectively tested by CCK-8 method, flow cytometry, Annexin V-FITC/PI dual fluorescent and the Western blot method. Results: Metformin could apparently restrain the proliferation of 253J cells (P=0.004). Besides, with the increase of metformin concentration and the extension of treatment time, the survival rate of 253J cells decreased gradually. At the same time, metformin could promote 253J cell apoptosis (P=0.003) and make the cell stagnate in G0/G1 phase (P=0.002), and its function gradually increased with the increase of metformin concentration. The results of Western blot indicated that the expression levels of AMPK and p38 and their phosphorylated proteins in the experimental group were significantly higher than those in the control group, while the expression of Cyclin D1 protein was significantly decreased (P=0.001). Conclusion: Metformin can restrain the proliferation and promote the apoptosis of 253J non-invasive bladder cancer cell line, and the mechanisms may be related to the regulation of Cyclin D1 expression by AMPK signaling pathway.

Keywords: Metformin, non-invasive bladder cancer, AMPK signaling pathway

Introduction

In recent years, the incidence of bladder tumor increased year by year [1]. In clinical treatment, the pathological type of the majority of patients with bladder cancer was non-muscle invasive. Besides, it had a high recurrence rate and a low 5-year survival rate after chemotherapeutic medicine lavage and transurethral resection of bladder tumor (TURBT) [2, 3]. Obviously, the prognosis of the bladder cancer is not optimistic. Hence, finding a safe and effective drug against bladder cancer has been the focus of scholars.

Metformin is a first-line oral biguanides hypoglycemic drug, which was widely used in treatments of patients with type 2 diabetes mellitus [4]. Recent studies have shown that metformin can not only control blood glucose levels and the fatality rate of patients with diabetes, but also significantly enhance the treatment effectiveness of antitumor chemotherapeutic medicine [5]. Other studies have showed that the tumor recurrence rate was significantly lowered after taking metformin for non-muscle invasive bladder cancer patients with diabetes [6]. However, the mechanisms of metformin on bladder cancer are still unclear at present. Although some studies have shown that metformin can significantly inhibit the proliferation of high-grade bladder cancer cells (5637 cells, T24 cells), whose role in low invasive bladder cancer cell lines is still unclear [7]. Therefore, in this study, we investigated the effects of metformin on 253J non-invasive bladder cancer cell line through *in vitro* cell experiment and preliminary analyzed its molecular mechanisms, in order to offer experimental and theoretical basis to the experts to consider metformin as a new kind of adjuvant drug against non-invasive bladder cancer.

Materials and methods

Experimental materials

Cell lines and cell culture: Human 253J non-invasive bladder cancer cell line was purchased from the American type culture collection (ATCC). Cells were inoculated in the RPMI-1640 culture medium containing 10% fetal bovine serum and antibiotics (100 mg/L streptomycin and $1*10^5$ U/L penicillin), and then placed in an incubator (5% CO_2, 37°C); the cells at logarithmic phase were then used for subsequent experiments.

Main reagents and materials: RPMI-1640 culture medium, fetal bovine serum, trypsin and EDTA were all purchased from the American Gibco Company; Mouse anti-human AMPK, p38, p-AMPK, p-p38 and Cyclin D1 protein were primary antibodies and all purchased from the American Abcam Company; the secondary antibodies labeled goat anti-mouse horseradish peroxidase were purchased from the American Santa Cruz Company; metformin and ECL chemiluminescence reagent were purchased from the American Sigma Company; CCK-8 kit was purchased from DOJINDO Laboratories; Annexin V-FITC/PI Apoptosis kit was purchased from BD Biosciences Company (USA); enzyme linked immunosorbent assay instrument was from Thermo Company; flow cytometry was from BD Company (USA) and Gel imaging system was from Bio-Rad Company.

Experimental methods

The effects of metformin on the proliferation of 253J non-invasive bladder cancer cells were detected by CCK-8 method: The 253J cells were inoculated in the 96-well plate at a density of $2*10^3$ cells/well, and placed in an incubator (5% CO_2, 37°C). They were randomly divided into a control group and other experimental groups, while the experimental groups were respectively stimulated at different concentrations of metformin (5 mmoL/L, 10 mmoL/L and 20 mmoL/L) and each group had 5 repeat wells. After the cells being respectively stimulating by metformin for 24 h, 48 h and 72 h, 10 μL CCK-8 was added into each well. After 2-hour routine culture, the 96-well plate was implanted into enzyme linked immunosorbent assay instrument to examine the absorbance value (OD value) of each well at wave length of 450 nm. An empty tube with only culture liquid and CCK-8 reagent was used as the blank control. According to the OD values of each group: the cell survival rate (%) = OD value of drug group/OD value of control group *100%. Metformin was not added in the control group while other processing methods were as same as the experimental group.

The effects of metformin on the cell cycle of 253J non-invasive bladder cancer cells were detected by flow cytometry: The 253J cells were inoculated in the 6-well plate at a density of $4*10^4$ cells/well. After being stimulated by different concentrations of metformin (5 mmoL/L, 10 mmoL/L and 20 mmoL/L) for 48 h, the cells were digested and washed with pre-cooled PBS and then collected in the flow cytometry tube. Seventy percent of ice alcohol was used to re-suspended cells and placed at -20°C for one hour. The cells were washed with PBS, then centrifuged at 2000 r/min for 5 min and re-suspended by HBSS solutioncontaining 50 μg/mL PI. Finally, the cell cycle of 253J cells of each group was detected by the flow cytometry and analyzed by the ModFit software.

The effects of metformin on the cell apoptosis of 253J non-invasive bladder cancer cells were detected by flow cytometry: The 253J cells which were treated with different concentrations of metformin for 48 h, were washed with PBS and then collected in the flow cytometry tube. After double staining with 10 μL Annexin V-FITC and 5 μL PI, incubated the cells in dark environment with room temperature for 15 min. Then, the cell apoptosis of 253J cells was detected by the flow cytometry and analyzed by the ModFit software.

The expression of protein involved in the cell proliferation was detected by the western blot: The 253J cells were treated with 20 mmoL/L metformin for 48 h and washed twice with pre-cooled PBS. The cell lysates were added to the collected cell total protein and the protein concentrations were detected by the BCA protein assay method. The total protein of samples in each group was transferred to PVDF membrane after separating by SDS-PAGE gels electrophoresis, and then the membrane was blocked at room temperature for 2 h by TBS-T solution containing 5% skim milk powder. Then the primary antibodies were added and incubated at

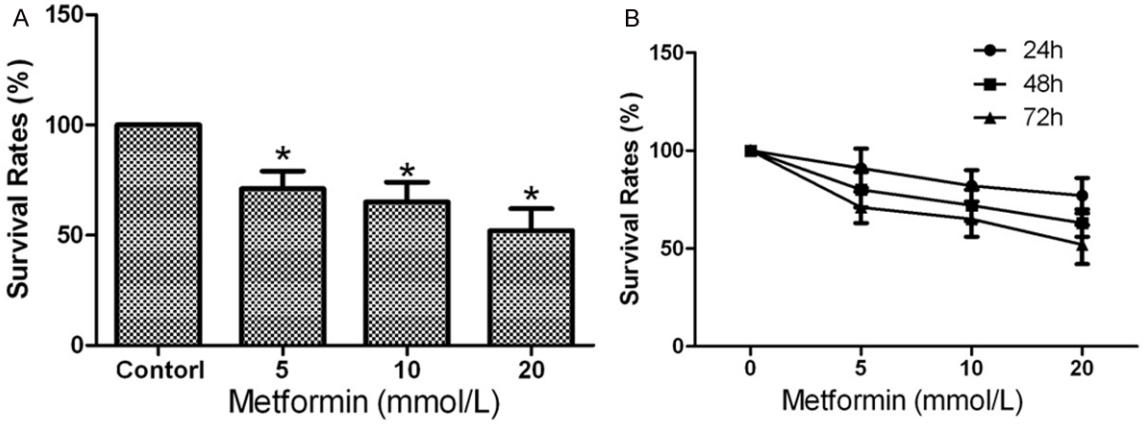

Figure 1. The effects of metformin on the proliferation ability of 253J non-invasive bladder cancer cells. *P<0.05 *vs.* control group.

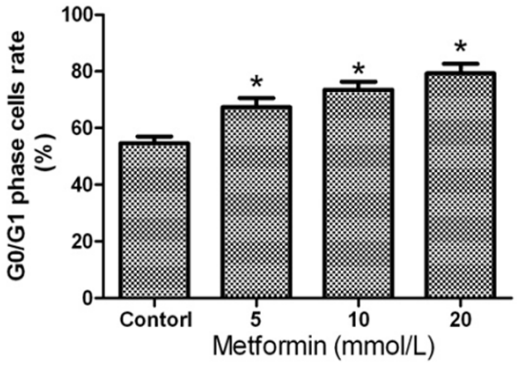

Figure 2. The effects of metformin on cell cycle of 253J cells. *Compared with the control group, P<0.05.

4°C for overnight. Next, the secondary antibodies were added after washing the membrane at room temperature with TBS-T solution containing 0.1% of Tween 20 and then incubated at room temperature for one hour. Finally, the membrane was washed again with TBS-T solution, then the protein was reacted with the ECL chemiluminescence reagent and exposed and scanned by Gel imaging system. The β-actin was taken as the internal reference.

Statistical analysis

The data were statistically analyzed by SPSS 17.0 software and the measurement data were expressed by mean ± standard deviation. The comparison among samples of each group was carried on by one-way ANOVA, and the comparison between two independent samples was carried on by t-test. P<0.05 indicated that the differences were statistically significant.

Results

The effects of metformin on the proliferation ability of 253J cells

253J non-invasive bladder cancer cells were treated with different concentrations of metformin in this study, the survival rate of 253J cells were detected by CCk-8 method. As shown in **Figure 1A**, after being treated with different concentrations of metformin (5 mmoL/L, 10 mmoL/L and 20 mmoL/L), the cell viability of 253J cells were (71.3±8.2)%, (65.4±9.1)% and (52.3±10.1)% respectively. When compared with the control group, the proliferation ability of 253J cells in the experimental group was significantly inhibited (P=0.004). With the increase of metformin concentrations and extension of stimulant time, the survival rate of 253J cells under the effects of the metformin gradually reduced, which indicated that metformin significantly inhibited the proliferation of 253J non-invasive bladder cancer cells in a time and concentration dependent manner (**Figure 1A** and **1B**).

The effects of metformin on cell cycle of 253J cells

After 253J non-invasive bladder cancer cells being treated for 48 h by different concentrations of metformin (5 mmoL/L, 10 mmoL/L and 20 mmoL/L), the changes of cell cycle were detected. The results showed that the cell ratio

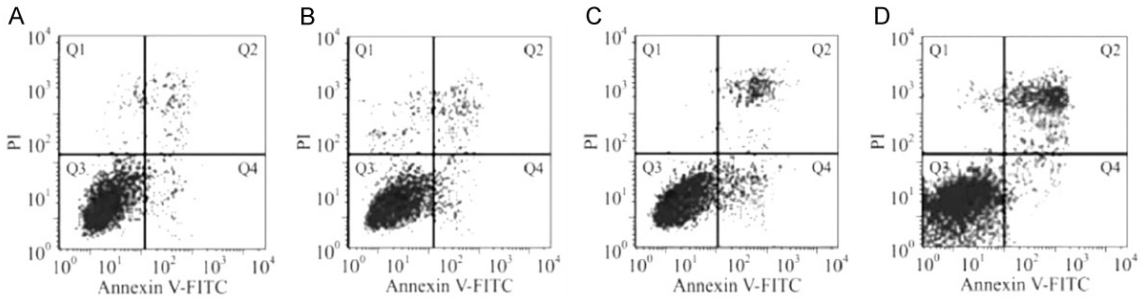

Figure 3. The effects of metformin on apoptosis of 253J non-invasive bladder cancer cells. A: Control; B: 5 mmoL/L; C: 10 mmoL/L; D: 20 mmoL/L.

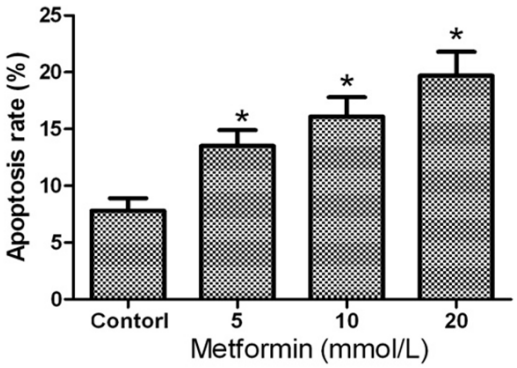

Figure 4. Apoptosis ratio of 253J non-invasive bladder cancer cells after treatment of different concentration of metformin. *Compared with the control group, P<0.05.

of G0/G1 phase in metformin treatment group was (67.4±3.2)%, (73.5±2.9)% and (79.3±3.4)%, respectively; compared with the control group, its cell ratio of G0/G1 phase increased significantly (P=0.002), and with the increasing concentration of metformin, the ratio of 253J cells in G0/G1 phase increased gradually. It indicated that metformin could block 253J non-invasive bladder cancer cells in the G0/G1 phase (**Figure 2**).

The effects of metformin on non-invasive bladder cancer cell apoptosis

The concentrations of metformin (5 mmoL/L, 10 mmoL/L and 20 mmoL/L) were respectively applied to 253J non-invasive bladder cancer cells for 48 h, then Annexin V-FITC/PI was used to stain necrotic and apoptotic cells. The results showed that the apoptotic ratio were (13.5±1.4)%, (16.1±1.7)% and (19.7±2.1)%, respectively. Compared with the control group, the difference was statistically significant (P=0.003). And with the increasing concentration of metformin, its apoptotic effects on 253J cells increased gradually. It indicated that metformin could significantly promote the apoptosis of 253J (as shown in **Figures 3** and **4**).

Regulation mechanisms of metformin on 253J non-invasive bladder cancer cells proliferation

Western Blot was performed to detect the protein expression levels of p-AMPK, AMPK, p38, p-p38 and Cyclin D1 in the 253J cells after the treatment of metformin for 48 h. The results showed that, compared with the control group, the protein expression levels of p-AMPK, AMPK, p38, p-p38 were strengthened significantly after the treatment of metformin at the concentration of 20 mmoL/L. However, the protein expression level of Cyclin D1 was decreased significantly (P<0.05, **Figure 5A** and **5B**).

Discussion

Because metformin has such characteristics as less side-effect, hypoglycemic stability, lactic acidosis and hypoglycemia, it has been widely used in the treatment of Type 2 mellitus. Recent studies showed that metformin could significantly inhibit the proliferation of esophageal carcinoma, breast cancer, lung cancer and other types of tumor cells [8, 9]. Consistent with the previous studies, this study found that metformin had significant inhibitory effects on the proliferation of low grade 253J non-invasive bladder cancer cells, which also showed time and concentration dependence (**Figure 1A** and **1B**). As for cell cycle and cell apoptosis, metformin could significantly promote the apoptosis of 253J cells and made the cell cycle block in

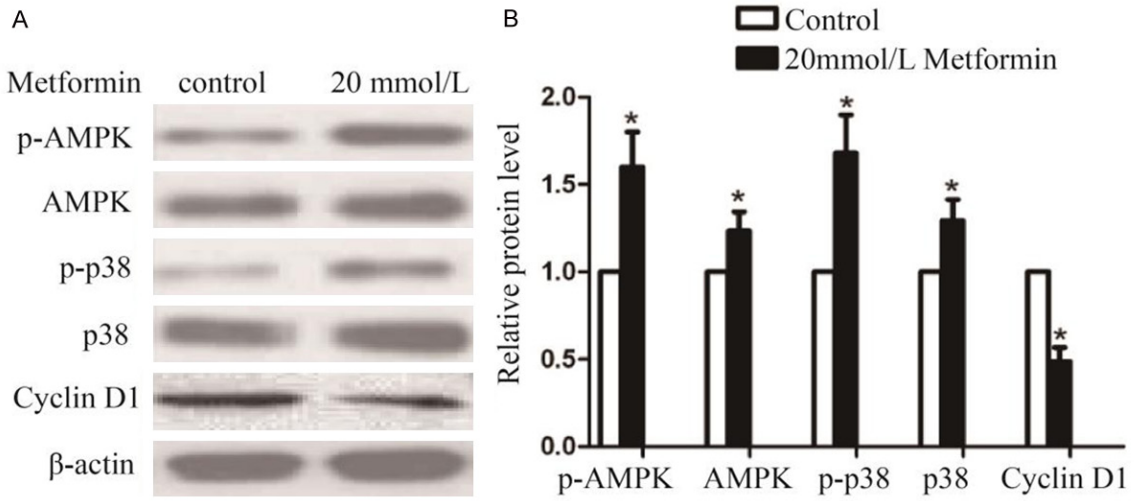

Figure 5. The effects of metformin on AMPK, P-38 MAPK phosphorylated proteins and Cyclin D1 protein expression in 253J cells. *Compare with control group, P<0.05.

the G0/G1 phase and these effects would be strengthened gradually with the increased concentration of metformin. Obviously, besides the effects on decreasing blood sugar, metformin also could prevent from non-invasive bladder cancer.

Other studies showed that metformin did not only have antitumor activity, but also could significantly reduce tumor incidence rate and relevant mortality. It promoted the apoptosis of tumor cells mainly by stagnating cell cycle and activating signal transduction pathway of cell apoptosis [10, 11]. The relationship between cell cycle and tumor development has always been a hot spot. In a series of cytokines regulating cell cycle, regulators for G1 phase had mutations or changes which could cause uncontrolled cell proliferation or even tumors [12]. Cyclin D1, one of the most important protein in cell cycle control, was regulated by protein kinase with cell cycle dependence. Some studies suggested that when the expression of Cyclin D1 was out of control, it could lead to the abnormal proliferation of the cells and the overexpression which was connected with the occurrence and the development of the tumors [13]. For example, in the cells of pancreatic cancer, metformin could block the cell cycle of the tumor in G0/G1 phase by lowering the expression of Cyclin D1 and thus inhibit the proliferation of the cells [14]. And other studies confirmed that Cyclin D1 proteins in low-grade and well-differentiated bladder cancer cells were usually overexpression and were related to the recurrence of the superficial bladder cancer [15]. In our research, the results showed that metformin could block 253J cells in G0/G1 phase to inhibit the proliferation of the cells by lowering the expression of Cyclin D1, suggesting that Cyclin D1 proteins might play an important role in the occurrence and the development of low-grade bladder tumors (**Figures 2** and **5**).

The mutation and abnormal expression of MAPK signaling pathway and its downstream target gene, Cyclin D1, are related with the occurrence and the development of various kinds of tumors. MAPK family includes many subfamilies such as p38, ERK and c-jun. These families form many signaling pathways, among which the p38 and AMPK are two major ways. As the energy receptor of the cells, MAPK signaling pathway plays an important role in regulating the energy metabolism, apoptosis and proliferation of the cells [16]. The deactivation of AMPK signaling pathway could lead to the abnormal proliferation of bladder cancer cells and could also inhibit the proliferation and the biosynthesis of the cells by two ways. One is inhibiting the expression of Cyclin D1 proteins through P53/p21 signal pathway to block the cells in G1 phase. Some studies confirmed that metformin could realize the antiproliferative effects of breast cancer by activating AMPK [17, 18]. Moreover, in the T24 cell lines of bladder tumors, metformin could inhibit the prolif-

eration of the T24 cells by activating AMPK [19]. Other studies also suggested that metformin could facilitate the apoptosis through AMPK signaling pathway [20]. For example, in the non-small cells of lung cancer, metformin could activate AMPK and then inhibit mTOR signaling pathway, which could thus facilitate the apoptosis of cells [21]. In addition, the activation of p38 AMPK signaling pathway could strengthen the antitumor effects of metformin. Some studies suggested that p38 AMPK blocker could inhibit the proliferation of SKOV3 ovarian cancer cell line. However, metformin could enhance the effects of the inhibition [22]. P38-AMPK signaling pathway played an important role in the apoptosis of cells and applying specific p38 MAPK blocker could lower the induction effects of metformin on the apoptosis of cell lines of lung adenocarcinoma [23]. In this study, we found that metformin could also activate AMPK and p-38 in 253J non-invasive bladder cancer cell, and these facts suggested that metformin could inhibit the cell proliferation and facilitate the cell apoptosis (**Figure 5**).

However, the occurrence and the development of the bladder tumors are multistep and complex processes. Besides AMPK signaling pathway and Cyclin D1 proteins, there may be other signaling pathways affecting metformin's inhibition effects on the proliferation and induction function of apoptosis of non-invasive bladder cancer cell. In addition, the anti-tumor effects of metformin still needs further confirmation from the multi-center randomized controls and clinical studies with a larger sample size. As a kind of hypoglycemic agent, the blood concentration of the maximum dosage of metformin in human body is 10 mmoL/L, which is lower than that of this study. Whether the human body owns good tolerance of high concentration of metformin also needs to be confirmed by a larger number of studies.

In conclusion, metformin could inhibit the proliferation of low-grade non-invasive bladder cancer cell and facilitate its apoptosis and make its cell cycle block in the G0/G1 phase. The mechanisms of action might be related with the decrease of Cyclin D1 expression and the activation of AMPK signaling pathway, suggesting that metformin might become the new aided drug to patients with non-invasive bladder cancer, which might provide a new method for the clinical treatment.

Disclosure of conflict of interest

None.

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