Original Article

Study on the inhibiting effect of metformin on the growth of osteosarcoma

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Abstract: Objective: To investigate the effects of metformin on the proliferation, migration and invasion of the MG63 osteosarcoma cell line. Methods: MG63 cells treated with 10 mmol/L metformin were selected as the metformin group and the cells treated without metformin were selected as the control group. The effects of metformin on proliferation, colony formation, migration and invasion were detected by CCK-8 assay, colony formation test, and transwell assay, respectively. The levels of MMP-2 and MMP-9 were detected by Real-time PCR and Western blot methods. Results: Compared with control group, the proliferation, colony formation, migration and invasion of MG63 cells in the metformin group were significantly inhibited and statistical differences were found between the two groups (all P<0.05). Real-time PCR and Western blot results showed that the expression levels of MMP-2 and MMP-9 in the metformin group were significantly lower than those in the control group and there were significant statistical differences (all P<0.001). Conclusion: Metformin can significantly inhibit the proliferation, migration and invasion of MG63 osteosarcoma cells, and the mechanism may be associated with the regulation of MMP-2 and MMP-9 expression levels.

Keywords: Metformin, osteosarcoma, matrix metalloproteinase, proliferation, migration and invasion

Introduction

Osteosarcoma is one of the most common malignant tumors of bone and the incidence of osteosarcoma increases every year [1, 2]. With the development of medical technology, the treatment of osteosarcoma has made great progress, however, the 5-year survival rate is still unsatisfactory due to drug resistance and high rate of recurrence and metastasis [3]. Some studies found that the degradation of the extracellular matrix is necessary for the growth and metastasis of osteosarcoma, and MMP-2 and MMP-9 which can degrade the extracellular matrix play key roles in the growth and migration of tumor cells [4, 5]. Other studies have reported that the efficacy and side effects of new targeting drug agents for osteosarcoma are needed to be confirmed through a lot of clinical research [6, 7]. Therefore, the search for a safe and effective treatment regime against osteosarcoma as well as understanding its mechanism of action has become the focus of orthopaedic scholars.

Metformin, as the first-line pharmacotherapy for glucose control, has been a widely applied therapy for diabetics due to its safety, efficacy and tolerability [8]. Besides inhibiting the levels of blood glucose, metformin can also obviously enhance the antitumor effect of chemotherapy drugs and decrease the risk of tumors in patients with diabetes mellitus [9, 10]. Some studies reported that metformin was correlated with a reduced risk of many cancers [11]. Another study reported that metformin use in lung cancer patients can improve the overall survival and progression of survival [12]. However, little is still known about the role of metformin in osteosarcoma. In this context, the effect of metformin on osteosarcoma cell line MG63 was explored by cell experiments in vitro and the molecular mechanism of metformin was preliminarily investigated. The results of
this study provide an experimental foundation for metformin as a new anti-tumor drug in osteosarcoma.

**Materials and methods**

**Cell culture**

Human osteosarcoma cell line MG63 was obtained from the American type culture collection (ATCC). MG63 cells were cultured using RPMI-1640 medium with 10% fetal bovine serum, and 100 μg/mL streptomycin and 100 U/mL penicillin. MG63 cells were kept in an incubator under the conditions of 5% CO$_2$ and 37°C. These cells were used for subsequent experiments when they were in the logarithmic phase of growth.

MG63 cells were divided into the control group and experimental group. Cells in the experimental group were stimulated with 10 mmol/L metformin, while those in control group were treated without metformin.

**Reagents and materials**

Fetal bovine serum and RPMI-1640 culture medium were purchased from the American company Gibico; CCK-8 kit was obtained from DOJINDO Laboratories, Japan; Transwell chambers were from Corning Corp., American; crystal violet, metformin and ECL chemiluminescence reagent was purchased from the American company Sigma; GAPDH antibody, rabbit-anti-human matrix metalloproteinase-2 (MMP-2) and MMP-9 antibody were obtained from Santa Cruz, American; Trizol Regent and PCR kits were purchased from the American company Invitrogen.

**CCK-8 assay**

MG63 cells at a density of 3×10$^3$ cells/well were cultured in 96-well plates. Ten μL of CCK-8 was added to each well after stimulating with metformin at different time points of 24 h, 48 h and 72 h. The cells continued to incubate for 1 h. Then, an enzyme linked immuno-sorbent assay instrument was used to detect optical density value of each well at 450 nm. The blank well containing medium and CCK-8 reagent without MG63 cells was used to zero. The OD value indicates the proliferation ability of cells.

**Colony formation assay**

MG63 cells in a logarithmic phase of growth were cultured in 6-well plates at a density of 2×10$^3$ cells/well, overnight in the incubator. Then, fresh culture medium containing 10 mmol/L metformin was added. The cells were continually cultured for 14 d. MG63 cells were fixed with 4% formaldehyde for 10 min and 0.1% crystal violet was used for staining for 3 min after cells were washed three times. Then, MG63 cells were rinsed until colorless with double distilled water. Under the light microscope, ten fields of view were randomly selected and the number of clone formation was analyzed quantitively.

**Cell migration assay**

MG63 cells in two groups were incubated in Transwell chambers with 5 repeated well in each group. One hundred μL of serum-free culture medium was added into the upper chambers and culture medium containing 20% fetal bovine serum was added into the lower chamber. The cells were cultured continually for 12 h under the conditions of 5% CO$_2$ and 37°C in an incubator. The upper unmigrated cells were wiped off by a cotton swap and the migrated cells were stained with 0.1% crystal violet for 8 min. The migrated cells were observed under a light microscope. The crystal violet was completely eluted by 33% acetic acid dehydrating. Then, the OD value of the eluting solutions was detected by Microplate Reader at 570 nm. The OD value indicated the migration ability of MG63 cells.

**Cell invasion assay**

Matrigel was diluted by pre-cooling serum-free culture medium and it was then spread evenly on the membranella (8 μm) of Transwell chambers. The following operations were conducted according to the methods of the cell migration experiment. Finally, the OD value was detected after staining. The OD value suggested the invasion ability of MG63 cells.

**Real-time PCR assay**

Total RNA of MG63 cells in two groups were isolated and reversely transcribed into cDNA following the instructions of RT-PCR Kits. MMP-2 forward primer, 5'-TCCATGACGGAGGAGCTGAC-
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**Table 1.** Comparison of OD values between control group and metformin group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Culture time</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.52±0.07</td>
<td>0.86±0.09</td>
<td>1.27±0.12</td>
<td></td>
</tr>
<tr>
<td>Metformin group</td>
<td>0.41±0.05</td>
<td>0.61±0.04</td>
<td>0.94±0.08</td>
<td></td>
</tr>
<tr>
<td>t value</td>
<td>2.859</td>
<td>5.676</td>
<td>5.116</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.021</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

ATC-3' and reverse primer, 5'-TCCATACCTCACA-CGCACACTTG-3'; MMP-9 forward primer, 5'-AGTGGGCTACGTGACCTATGA-3' and reverse primer, 5'-CGGCAAATGGCTCTTT-3'. The reaction system was as follows: forward/reverse primer was 0.4 μL, SYBR Green Real-time PCR Master Mix 10 μL, cDNA 1.6 μL, ddH₂O 8.0 μL. PCR amplification was conducted according to the following reaction conditions: 95°C for 2 min, denaturation at 95°C for 35 s, annealing at 65°C for 20 s, extension at 72°C for 30 s and a total of 40 cycles. The Ct values of MMP-2 or MMP-9 gene were calculated with ABI 7300 System software. The relative expression levels of MMP-2 or MMP-9 mRNA were calculated using 2^ΔΔCt method.

**Western blot assay**

The cell lysates were used to collect the total protein and BCA protein assay was applied to examine the concentrations of proteins. The total proteins were separated by SDS-PAGE gels electrophoresis. Then, the proteins from each group were transferred onto a PVDF membrane. Next, the membranes were blocked with TBS-T solution with 5% non-fat milk powder at the room temperature for 1 h. The rabbit-anti-human matrix metalloproteinase-2 (MMP-2) and MMP-9 primary antibody were added and incubated with the membranes at 4°C for overnight. The secondary antibody was added and incubated with membranes at room temperature for 1 h. After washing with TBS-T solutions three times, the membranes were developed by ECL chemiluminescence reagent and scanned by Gel imaging system. GAPDH was selected as an internal reference.

**Statistical analysis**

All the data in this study were statistically analyzed by SPSS 22.0 software. The measurement data were presented by Mean ± Standard deviation (SD). The comparison between two groups was conducted by independent sample t test. Counting data were presented by percentage. Comparison between two groups was performed by chi-square test. P<0.05 suggested that the statistical differences were significant.

**Results**

**Comparison of cell proliferation and number of colony formations between the two groups**

As shown in Table 1, the OD values in the metformin group were significantly lower than those in control group at the different time points of 24 h, 48 h and 72 h, respectively. There were significant statistical differences (all P<0.05). As seen in Figure 1, compared with that in control group, the number of colony formations in the metformin group was significantly reduced, and there was a significant statistical difference (t = 2.027, P = 0.004).

**Comparison of cell migration and invasion between the two groups**

As shown in Figure 2, compared with that in control group, the OD value in the metformin group was remarkably decreased (0.78±0.15 vs 0.62±0.10), and there was an obviously statistical difference (t = 2.807, P = 0.012). As seen in Figure 3, the OD value in the metformin group was obviously lower than that in control group (0.67±0.13 vs 0.55±0.09), and there was a significant statistical difference (t = 2.400, P = 0.027).
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As shown in Table 2, the levels of MMP-2 and MMP-9 mRNA in the metformin group were significantly lower than those in control group, and significant differences were found between two groups (all $P<0.001$). As shown in Figure 2, compared with control group, the levels of MMP-2 and MMP-9 proteins in the metformin group were significantly reduced, and a statistical differences was found between the two groups.

Discussion

In recent years, the disability and morbidity rate from osteosarcoma has been increasing both at home and abroad. The main reason is growth and metastasis in patients with osteosarcoma. Some researchers reported that growth and metastasis in tumors is a complex biological process. Many factors interact with each other and the extracellular matrix, basement membrane decomposition, proliferation, migration and invasion of cancer cells reduced the adhe-
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Metformin has been widely applied as anti-diabetic medication in the world for over half a century. Some studies reported that metformin is not only available at a low cost, but also its toxicity profile has been obtained from clinical trials [15, 16]. At present, metformin has been reported to have antitumor activity both in vitro and in vivo. Some studies indicated that metformin can obviously decrease the proliferation of lung cancer, breast tumors, esophageal carcinoma and other kinds of cancers [17, 18]. Another study reported that low-dose metformin use in patients without diabetes mellitus was confirmed to be effective in chemoprevention of metachronous colorectal adenomas in contrast to a control group [19]. It was also reported that the beneficial effects of metformin use in prostate cancer was limited to those patients who underwent radical radiotherapy [20]. Other studies indicated that metformin has the function of prevention in non-invasive bladder cancer [21]. However, the effect of metformin in osteosarcoma is still unknown. Thus, this study aimed to investigate the potential role of metformin in inhibiting the proliferation, migration and invasion of osteosarcoma cells.

In this study, the effects of metformin on proliferation, migration and invasion of osteosarcoma cells line MG63 were observed by CCK-8 and Transwell assays. The results suggested that 10 mmol/L metformin could significantly inhibit the proliferation, migration and invasion of MG63 cells and decrease the number of colonies. These results were accordance with the role of metformin in other kinds of tumors reported by previous studies [22, 23]. As we can see, metformin can be considered as an anti-tumor drug and may be used as an adjuvant therapy in osteosarcoma.

Previous studies reported that metformin exerts anti-tumor effects through the regulation of the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway [24]. In this study, the exploration of a mechanism for metformin in osteosarcoma focused on matrix metalloproteinase expression and MMP-2 and MMP-9, which were selected as targeted proteins. The extracellular matrix is a barrier for the metastasis of tumor cells. Matrix metalloproteinase belong to a class of proteolytic enzymes which degrade the extracellular matrix and play an important role in proliferation and metastasis of tumor cells [25]. It was reported that MMP-2 was able to degrade Collagen IV which could enhance the infiltration of cancer cells to surrounding environment and increase the spread of tumors via nascent capillaries [26]. MMP-9 can degrade the extracellular matrix and break down the basement membrane, which leads to the proliferation and metastasis of tumors cells [27]. Some studies reported that MMP-2 and MMP-9 were expressed at high levels and involved in the migration and invasion of osteosarcoma cells [28]. In this study, it was found that metformin could significantly down-regulate the expression levels of MMP-2 and MMP-9 in osteosarcoma MG63 cells by real-time PCR and Western blot methods. This indicated that metformin may influence the proliferation, migration and invasion of cancer cells via deceasing the expression of MMP-2 and MMP-9 in osteosarcoma, which impacts the clinical outcomes of patients. The above results in this study are similar with those reported by Liang et al [29].

In conclusions, this study demonstrated that metformin can significantly inhibit proliferation, migration and invasion of osteosarcoma MG63 cell line, which may be associated with the
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down-regulation of MMP-2 and MMP-9 proteins. However, further studies are required to make deeper explorations over time with a dose dependence of metformin and the involved targeted signaling pathways. The results of this study provide the experimental foundation for new ideas in treatment of osteosarcoma in clinical practice.

Disclosure of conflict of interest

None.

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