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Metformin inhibits glioma cells stemness and epithelial-mesenchymal transition via regulating YAP activity

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ABSTRACT

This work aims to study the roles and mechanisms of metformin in glioma cells stemness and epithelial-mesenchymal transition. Here, we found that metformin suppressed glioma cells spheroid formation and size, inhibited the expression of glioma stemness-related marker, CD133. Additionally, Metformin attenuated TGF-β-induced epithelial-mesenchymal transition in glioma cells. Mechanistically, metformin inhibited the nuclear abundance of YAP, a key effector of Hippo pathway, subsequently leading to its cytoplasmic retention, and thus reduced YAP transcriptional modulating activity. Importantly, overexpression of a mutant form of YAP (YAP-5SA) attenuated the inhibition of metformin on glioma cells stemness and epithelial-mesenchymal transition. Thus, metformin inhibits glioma cells stemness and epithelial-mesenchymal transition via regulating YAP activity.

1. Introduction

Metformin, which is a widely used medication for the treatment of type 2 diabetes mellitus, has been reported to hold diverse correlations with the mortality and morbidity of patients with glioma, and reduce the viability of cancer stem cells (CSCs) in glioma [1,2]. These data highlight the roles of metformin in regulating stem cells traits in glioma. However, the roles and related mechanisms of metformin in regulating CSCs-related traits in glioma are still confusing.

The transcription modulators YAP/TAZ act as the downstream effectors of Hippo signaling pathway [3]. Upon Hippo signaling activation, YAP/TAZ translocate to the nucleus where they act as transcriptional co-activators of other key regulators [4]. The transcription modulating activity of YAP/TAZ plays critical roles in organismal development, cell growth and is often dysregulated during cancer progression [5]. TAZ could confer cancer stem cell-related traits on breast cancer cells [6]. The increasing of YAP transcriptional modulating activity could also promote tumor epithelial-mesenchymal transition (EMT) and metastasis [7]. Therefore, it could be targeted that promoting the translocation of YAP/TAZ from the nucleus to cytoplasm for tumor treatment [8]. However, efforts in this direction are frustrated by the fact that the regulation on YAP/TAZ activity is complicated [3], and is still unclear that the correlation between metformin treatment and the YAP/TAZ transcriptional modulating activity in glioma. Thus, based on the efficient and various functions for treating diseases of metformin, it is necessary to investigate whether metformin could regulate YAP/TAZ activity and thus be used for glioma treatment.

In this context, we decided to perform a detailed investigation of the involvement of YAP/TAZ activity in the effects of metformin on glioma CSCs formation and EMT. Here, we indicated that YAP/TAZ activity, and in particular YAP activity, is a key contributor for metformin effects on glioma CSC-related traits and EMT.

2. Materials and methods

2.1. Cell culture and plasmid acquisition

Human glioma cell lines U87 and U251 were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). Both of the two cell lines were cultured in 1640 medium (Gibco, Grand Island, NY) with 10% FBS (fetal bovine serum, Gibco, Grand Island, NY) under humidified atmosphere with 5% CO_2 at 37 °C. Independent authentication of cells was performed before using. pQCXIH-Myc-YAP-5SA (Plasmid #3309), and 8xGTIIC-luciferase (Plasmid #34,615), a YAP-responsive synthetic promoter driving luciferase expression plasmid, were purchased from addgene.

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2.2. Cell sphere formation assay

Cells were plated on ultra-low attachment 24-well plates (Corning, Union City, CA) at 500 cells/well, and maintained in MammoCult™ Human Medium Kit (Cat#05620, Stemcell Technologies, Vancouver, BC, Canada). Cells were treated with various concentrations of metformin or solvent for 10 days, followed by detecting the number and size of mammospheres with the diameters greater than 50 μm using a microscope fitted with a ruler. All experiments were performed at least in triplicate.

2.3. Cell viability assay

Cells with different treatment were seeded into 96-well plates at 3×10^3 cells/well and incubated at 37 °C for 24 h, 48 h and 72 h, respectively. The cell viability was measured using a Cell Counting Kit-8 assay (Beyotime, Nanjing, China) following the manufacturer's protocol.

2.4. Flow cytometry analysis

Glioma cells with different treatment were resuspended and incubated with antibody against CD133 (BD Biosciences, Franklin Lakes, NJ) for 30 min in PBS on ice according to the manufacturer's recommendation, and then washed with 1 ml ice-cold PBS two times. The collected cells were resuspended in 500 ul PBS and sorted out by FACS Calibur flow cytometry (Becton Dickinson, USA).

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from glioma cells with different treatment with TransZol Up (Transgen Biotech, Beijing, China). cDNA was obtained with EasyScript First-Strand cDNA Synthesis SuperMix (Transgen Biotech). qRT-PCR was carried out on the StepOne Plus PCR system with TransStart Green qPCR SuperMix (Transgen Biotech). The primers for qRT-PCR were provided in Table 1. All samples including negative control without template were analyzed in triplicate. The relative expression genes was normalized to GAPDH. The quantification method of gene expression was performed using using $2^{-\triangle\triangle ct}$.

2.6. Western blot analysis

Cells with different treatment were harvested by ice-cold scraping and extracted with ProteinExt Mammalian Total Protein Extraction Kit (Transgen Biotech). The protein concentration was determined by Bradford protein concentration assay kit (Cat#P0009, Beyotime, China). An equal amount of protein with loading buffer was separated by SDS-PAGE, and transferred on PVDF membrane. The membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1.5 h at room temperature and then incubated with respective primary antibodies overnight at 4 °C, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were washed with TBST for 15 min for three times before incubating with antibodies. Enhanced chemiluminescence (ECL) kit (Beyotime) was used to develop image in Tanon 5200 machine (Tanon, Shanghai, China). The primary antibodies against YAP (ab52771), TAZ (ab224239), ALDH1 (ab23375), E-cadherin (ab1416), Vimentin (ab8978), CTGF (ab6992), β-actin (ab82227) and Histone H2A (ab18255) were purchased from Abcam. P-YAP (Cat # 13,008) and p-TAZ (Cat # 75,275) were purchased from CST.

Table 1
qRT-PCR Primer sequences.

Gene	Sequences (5' to 3')
E-cadherin forward	GCTTGCGGAAGTCAGTTCAGACTCC
E-cadherin reverse	TTCATCATAGTAATAAACG
Vimentin forward	AAGAAAAACCTTCCCGGTGCAATCG
Vimentin reverse	TTGCTGTGTGCGGTCGGGC
GAPDH forward	CGGAGTCAACGGATTTGGTCGTAT
GAPDH reverse	AGCCTTCTCCATGGTGGTGAAGAC

2.7. Transfection

Lipofectamine^{TM2000} (Invitrogen) was used for transfection. The concentration of plasmid was referred to the manufacturer's protocol.

2.8. Luciferase reporter assay

Luciferase reporter assay was used to detect the change of YAP transcriptional modulating activity within metformin treatment. Briefly, 8xGTIIC-luciferase was transfected into glioma cells following metformin treatment. After 72 h, the luciferase activity was examined, β-gal (Ambion, USA) was used as a normalization control for luciferase activity. Luciferase activity was measured as described previously [9].

2.9. Statistical analysis

All data were obtained from at least three independent experiments ($n \geq 3$), and presented as the mean ± SD (standard deviation). Datasets with only two groups were analyzed using a student's *t*-test. The differences between the groups were analyzed using One-way ANOVA with the Tukey – Kramer post-test, and $P < 0.05$ was considered significant.

3. Results

3.1. Metformin suppresses glioma cells stemness in a concentration-dependent manner

We firstly investigated whether metformin could affect glioma cells stemness. Cell sphere formation assay showed that metformin significantly decreased the sphere-forming ability in a concentration-dependent manner (Fig. 1A). Consistently, metformin attenuated the fraction of CD133 + cells (Fig. 1B), which has been regarded as glioma stemness marker [10]. Furthermore, qRT-PCR and western blot assays indicated that metformin inhibited the expression of ALDH1A1 in a concentration-dependent manner, which is another stemness marker [11] (Fig. 1C and D). Furthermore, metformin had no effects on cell viability (Fig. 2A) and cell cycle distribution (Fig. 2B–E). Thus, these results demonstrate that metformin could suppress glioma cells stemness.

3.2. Metformin suppresses EMT process in glioma cells

Since the interplay of CSCs and EMT has been elucidated in tumors [12], we sought to explore whether metformin could also inhibit EMT in glioma cells. As shown in Fig. 3A, metformin significantly decreased the mRNA level of mesenchymal marker—Vimentin, while upregulated the level of epithelial marker—E-cadherin. Identical results were acquired in protein level of Vimentin and E-cadherin (Fig. 3B). Therefore, our results indicate that metformin could suppress EMT in glioma cells.