Metformin inhibits epithelial-mesenchymal transition of oral squamous cell carcinoma via the mTOR/HIF-1α/PKM2/STAT3 pathway

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Abstract. Epithelial-mesenchymal transition (EMT) serves an important role in the formation and development of various types of cancer, including oral squamous cell carcinoma (OSCC). Metformin, used for treating type 2 diabetes, has been revealed to exert an anticancer effect in various types of cancer, including liver, breast and colorectal cancer. However, its role in the EMT of OSCC has been rarely reported. Therefore, the present study aimed to investigate the effects of metformin on EMT and to identify its underlying mechanism in OSCC. Firstly, EMT was induced in CAL-27 cells using CoCl2. Subsequently, the effects of metformin on cell viability, migration and xenograft growth were evaluated in vitro and in vivo. Reverse transcription-quantitative PCR was performed to detect the expression levels of E-cadherin, vimentin, snail family transcriptional repressor 1, mTOR, hypoxia inducible factor 1α, pyruvate kinase M2 and STAT3. The results demonstrated that metformin abolished CoCl2-induced cell proliferation, migration, invasion and EMT. Moreover, metformin reversed EMT in OSCC by inhibiting the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway. Overall, the present findings characterized a novel mechanism via which metformin modulated EMT in OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common type of cancer in the world (1), and is the most common primary oral cancer type in the oral maxillofacial region, with a 5-year survival rate of 50–60% (2). Smoking and alcohol are major risk factors for oral cancer, both exerting synergistic effects (3). Epithelial-mesenchymal transition (EMT) serves an important role in tumorigenesis and tumor development. EMT is a process that involves loss of cell polarity and cell-cell adhesion conferring tumor cells the ability to migrate and metastasize (4).

mTORs, functioning as mechanistic targets, are regulators of cell proliferation and metabolism (5). mTOR principally controls cell metabolism by regulating the translation and transcription of metabolic genes (6). It has been revealed that mTOR can be activated in kidney cancer and accelerates cancer progression (7). Previous studies have reported that suppressing the mTOR-associated signaling pathway can inhibit EMT (8-10).

Hypoxia inducible factor 1α (HIF-1α) leads to insufficient blood supply and hypoxia in the tumor microenvironment and affects tumor metabolism (11). Moreover, the upregulation of HIF-1α serves a crucial role in tumorigenesis, tumor angiogenesis, glycolysis and chemoresistance (12). The downstream factor of HIF-1α, pyruvate kinase M2 (PKM2), can interact with HIF-1α to regulate cancer metabolism (13). In addition, STAT3 can promote EMT progression (14), and HIF-1α, PKM2 and STAT3 are all modulated by mTOR (15,16).

Metformin, used for treatment of type 2 diabetes, has been associated with decreasing cancer incidence and mortality (17). A previous study revealed that metformin decreased the risk of liver, breast and colorectal cancer (2). Other studies have indicated that metformin inhibits EMT in prostate cancer, cervical cancer and rectal cancer (18-20). However, to the best of our knowledge, no reports have studied EMT in OSCC. Moreover, the potential mechanism via which metformin inhibits tumor growth is yet to be fully elucidated. Therefore, the aims of the present study were to investigate the role of metformin on inhibiting CoCl2-induced EMT in OSCC cells and to examine whether EMT could be suppressed via the mTOR/HIF-1α/PKM2/STAT3 signaling pathway.

Materials and methods

Cell lines and culture. The human OSCC CAL27 cell line was acquired from the Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Jilin University, Changchun, Jilin 130021, P.R. China.
Surgery, Tooth Development and Maxillary Reconstruction and Regeneration Laboratory at Jilin University (Changchun, China). Cells were cultured in Dulbecco’s modified Eagle’s (DMEM) medium supplemented with 10% FBS and 100 mg/ml penicillin/streptomycin (all purchased from Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in a humid incubator with 5% CO₂.

Metformin (MedChemExpress) and CoCl₂ (Sigma-Aldrich; Merck KGaA) were dissolved in PBS (Invitrogen; Thermo Fisher Scientific, Inc.) at a stock concentration of 160 mM and 500 μM, respectively. Both were stored at -80°C.

**Cell proliferation assay.** Cells were seeded at a density of 1x10⁴ cells/well in 96-well plates and cultured overnight at 37°C. After treatment by indicated concentrations of CoCl₂ (0, 50, 100, 200, 300, 400 and 500 μM) and metformin (0, 2.5, 5, 10, 20, 40, 80 and 160 mM) for 24 h at 37°C, 10 μl of the Cell Counting Kit-8 (CCK-8; Invigentech) was added to each well according to the manufacturer’s protocol. After culturing for 2 h, the absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Inc.). After the screening process, the medium of optimal concentrations (10 mM metformin with or without 300 μM CoCl₂) was used to culture cells for 24 h at 37°C. Then, the above process was repeated. All results were measured three times.

**Cell migration assay.** A wound-healing assay was performed to assess cell migration, and 10⁴ cells/well were seeded onto 6-well plates for 24 h at 37°C. A wound was scraped using a 1,000-μl pipette tip, and plates were washed three times with PBS. The cells were cultured in fresh serum-free medium containing 300 μM CoCl₂, with or without 10 mM metformin, for 24 h at 37°C. Images were captured at the time points of 0 and 24 h after wounding. The migration rate was quantified using the following equation: (0-h scratching distance - 24-h scratching distance)/0-h scratching distance. Representative images were obtained at x40 magnification using an Olympus light microscope (Olympus Corporation). All experiments were repeated at least three times.

**Cell invasion assay.** Transwell assay was performed using 24-well Transwell units (Corning, Inc.) with an 8-μm pore size polycarbonate membrane which has been precoated with Matrigel (Becton Dickinson) for 1 h at 37°C. Cells (1x10⁵), 300 μM CoCl₂, with or without 10 mM metformin, were suspended in 100 μl DMEM without FBS and seeded onto the upper unit, while 600 μl DMEM with 10% FBS, was added to the lower units. After incubation for 24 h at 37°C, cells on the upper side of the membrane were removed using PBS-soaked cotton swabs. The membrane was fixed in paraformaldehyde for 30 min at 37°C and then stained with 0.1% crystal violet for 30 min at room temperature. Cell numbers under the membrane were counted using an Olympus light microscope (magnification, x400; Olympus Corporation).

**RNA isolation and reverse-transcription-quantitative (RT-q) PCR.** Cells were cultured in DMEM with 10% FBS containing 300 μM CoCl₂, with or without 10 mM metformin (metformin and CoCl₂ were added at the same time), for 48 h at 37°C. Total RNA was extracted using TRIzol® (TRIeasyTM Total RNA Extraction reagent; Shanghai Yeasen Biotechnology Co., Ltd.) from the specified treated cells and maintained at -20°C for 12 h. Total RNA was reverse transcribed using Hifair™ II 1st Strand cDNA Synthesis SuperMix (TRIeasy™ Total RNA Extraction reagent; Shanghai Yeasen Biotechnology Co., Ltd.) for qPCR under the recommended conditions: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and holding at 4°C (GeneAmp PCR System 9700; Thermo Fisher Scientific, Inc.). cDNA corresponding to 25 ng RNA was used for qPCR using a HeiFtM qPCR SYBR® (Role of Metformin in the EMT of OSCC). The following thermocycling conditions were used: Initial denaturation at 95°C for 5 min and 40 cycles at 95°C for 10 sec and 60°C for 30 sec (ProFlex PCR System; Thermo Fisher Scientific, Inc.). The expression levels of human β-actin, E-cadherin, vimentin, snail family transcriptional repressor 1 (Snail1), mTOR, HIF-1α, PKM2 and STAT3 were detected. Gene expression normalized to β-actin was calculated using the 2⁻ΔΔCq method (21). The RT-qPCR primers were as follows: β-actin forward, 5'-CTCCATCCTGGCCTCTCGTGT-3' and reverse, 5'-GCTGTCACCTTCACCAGTCC-3'; E-cadherin forward, 5'-GCCTCTCTGAAAGAGAGTTGAGAAG-3' and reverse, 5'-TGCGAGCTGTCTCCTAAAATCCG-3'; vimentin forward, 5'-AGGCCAACGGAGAGTCCACTGA-3' and reverse, 5'-ATCTGGGCGGAGCGTTTCCA-3' and reverse, 5'-GAGGCCTGAGCTGTTGACCAGCTCAT-3'; Snail1 forward, 5'-ATCTGGCGCGAGGCTGG-3' and reverse, 5'-CAGGACGATCTTGGGACCC-3'; mTOR forward, 5'-AGCATCGGATGTCTTAGGATGGG-3' and reverse, 5'-CAGGACGATCTTGGGACCC-3'; HIF-1α forward, 5'-TAGCCCGAGGAAGACTATGAAC-3' and reverse, 5'-CTGAGGTGTTGTTACTGTTGGTA-3'; PKM2 forward, 5'-ATGGCTGACATCTCCTCAGTGACC-3' and reverse, 5'-CCTCTACAGTCTCCACTGATCG-3'; and STAT3 forward, 5'-CTTTGAGAACCAGGTTATCACC-3' and reverse, 5'-GGTCAGCATGGTTGGACCGAG-3'.

**Xenograft mouse studies.** To investigate whether metformin inhibited CoCl₂-induced EMT in vivo, the subcutaneous xenografted growth of OSCC cells was monitored. For the experiment, cells were cultured in DMEM with 20% FBS containing 300 μM CoCl₂, with or without 10 mM metformin, for 48 h at 37°C. There were 28 male BALB/C nude mice (Shanghai Vital River Laboratory Animal Technology Co., Ltd.; age, 4-6 weeks; weight, 15-20 g) were housed under specific pathogen-free conditions, with food and water provided ad libitum. After 1 week of acclimation, the mice were randomly divided into four groups (seven mice per group) and injected with 5x10⁵ indicated OSCC cells subcutaneously which was resuspended by PBS into the right flank. Xenograft tumor volume and weight were measured every other day. After 24 days, nude mice were euthanized by cervical dislocation and tumors were collected. Tumor volume (mm³) was calculated as follows: 1/2 x long diameter (mm) x short diameter (mm)². The present study was approved by the Animal Research Ethics Committee of Jilin University. All animal treatments were performed in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals.

**Statistical analysis.** Statistical analysis was performed using SPSS v21 (IBM Corp.) and GraphPad Prism 8 (GraphPad Software).
Results

CoCl₂ promotes proliferation and induces EMT of OSCC cells. Cells were treated with 0, 50, 100, 200, 300, 400 and 500 µM CoCl₂ to evaluate the potential effect of CoCl₂ in OSCC cells. CoCl₂ at concentrations ≥100 µM could induce cell proliferation (Fig. 1A). The concentration of 300 µM CoCl₂ with the lowest error bar was chosen for subsequent experiments. Moreover, the expression levels of E-cadherin, vimentin and Snail1. *P<0.05; **P<0.01; ***P<0.001. (C) Metformin inhibited the proliferation of OSCC cells, evaluated via CCK-8 assay. *P<0.05; **P<0.01; ***P<0.001 vs. 0 mM. (D) CAL-27 cells were treated with metformin (10 mM) with or without CoCl₂ (300 µM), CCK-8 assay was used to compare the four groups, revealing that metformin inhibited CoCl₂-induced proliferation. *P<0.05; #P<0.05 vs. control. CCK-8, Cell Counting Kit-8; OSCC oral squamous cell carcinoma; EMT, epithelial-mesenchymal transition; Snail1, snail family transcriptional repressor 1.

Metformin prevents proliferation, migration, invasion and EMT of OSCC cells induced by CoCl₂. Cells were treated with 0, 2.5, 5, 10, 20, 40, 80 and 160 mM metformin to investigate the underlying anti-proliferative effect of metformin in OSCC. Proliferation of cells treated with metformin decreased significantly in a dose-dependent manner compared with that of untreated cells (Fig. 1C). A concentration of 10 mM metformin was used for CCK-8, wound-healing, Transwell, RT-qPCR and nude mice xenograft assays. Moreover, compared with the group treated with CoCl₂, cell proliferation in the CoCl₂ + metformin group was significantly attenuated by the addition of metformin (Fig. 1D). Additionally, cells were treated with 300 µM CoCl₂, with or without 10 mM metformin and the result revealed that CoCl₂ significantly increased the migration of cells compared with the control group. This phenomenon could be abolished by the addition of metformin (Fig. 2A and B).

The markers of EMT were detected using RT-qPCR. In the CoCl₂ group, E-cadherin expression was decreased, while vimentin and Snail1 expression was increased. In vivo compared with the control group, the volume and weight of xenografts in the metformin group were reduced. Using CoCl₂ alone promoted tumor growth, which could be inhibited by the addition of metformin (Fig. 3A and B). These findings suggested that metformin inhibited the cell proliferative, migratory and invasive abilities, as well as reversed CoCl₂-induced EMT.
Metformin prevents EMT of OSCC induced by CoCl₂ via the mTOR/HIF-1α/PKM2/STAT3 signaling pathway. The expression levels of mTOR, HIF-1α, PKM2 and STAT3 in the EMT process were detected via RT-qPCR. High expression levels of mTOR and PKM2 in CoCl₂-induced EMT were identified, which were inhibited by metformin (Fig. 4A and C). Moreover, HIF-1α was upregulated in the CoCl₂ group compared with the control group, but its expression was highest in the metformin group. In the CoCl₂ + metformin group, HIF-1α’s expression was decreased compared with the CoCl₂ group (Fig. 4B). Thus, it was suggested that metformin suppressed CoCl₂-induced EMT, but using metformin independently did not exert this effect. STAT3 expression was significantly increased in the CoCl₂ group, but was significantly decreased by the addition of metformin (Fig. 4D).

Discussion

EMT is a major process in tumor metastasis, as well as a vital factor involved in mortality in patients with OSCC (22). Previous studies have reported that CoCl₂ at an appropriate concentration promotes cell proliferation and induces EMT in liver and mammary gland cancer (23,24). The present study used CoCl₂ to induce EMT and then investigated the underlying mechanism of metformin inhibition on CoCl₂-induced EMT in OSCC.

Metformin, as an antidiabetic drug, has been revealed to exert effects to decrease cancer incidence and mortality rates in various types of human cancer (17). The use of metformin in diabetic patients is associated with a decreased incidence in cancer types, including pancreatic, liver and colon cancer, and can decrease cancer-associated mortality (25). Moreover, several studies have reported that metformin inhibits tumor growth via multiple mechanisms, including by suppressing tumor cell proliferation (26) and EMT, affecting tumor autophagy and metabolism (12,27), and inducing apoptosis of cancer stem cells (28). Although no studies on the effects of metformin on EMT in OSCC, previous studies have demonstrated that metformin suppresses EMT in other types of cancer, including cervical and breast carcinoma (4,18).
The present study demonstrated that metformin inhibited CoCl₂-induced proliferation, migration, invasion and EMT in OSCC.

The inhibitory effect of metformin on EMT in cervical carcinoma via the mTOR-p70s6k relative signaling pathway has been observed (19). mTOR can accelerate cell proliferation and adjust cellular energy homeostasis (29). In EMT, overactivation of mTOR is closely associated with tumor progression (30). Thus, targeting the mTOR-associated pathway is the key to research.

mTOR regulates HIF-1α, and overexpression of HIF-1α facilitates tumor metastasis and EMT in colorectal cancer (15). Moreover, PKM2 is an important downstream executor of HIF-1α; the expression and function of PKM2 is associated with HIF-1α in feedforward loops, and HIF-1 is also a target gene of the PKM2/STAT3 signaling pathway (16). Metformin inhibits the expression levels of HIF-1α and PKM2 in gastric cancer (12), as well as preventing the activation of STAT3 in breast cancer (31) and colorectal cancer (20). The aforementioned studies suggest that the mechanism of metformin suppressing CoCl₂-induced EMT in OSCC may also occur by inhibiting the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway. The present results confirmed that metformin significantly inhibited CoCl₂-induced EMT via the mTOR/HIF-1α/PKM2 signaling pathway in OSCC cells. However, there are certain limitations to the current study, and additional cell lines are required to further validate the present results. In addition, the expression levels of markers

Figure 3. Metformin inhibits CoCl₂-induced EMT in vivo. (A) Tumor volume and (B) tumor weight were measured. **P < 0.01; ***P < 0.001; ###P < 0.001 vs. control.

Figure 4. Metformin inhibits CoCl₂-induced EMT via the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway. CAL-27 cells were treated with metformin (10 mM) with or without CoCl₂ (300 µM) and the expression levels of (A) mTOR, (B) HIF-1α, (C) PKM2 and (D) STAT3 were detected via reverse transcription-quantitative PCR. *P < 0.05; **P < 0.01; ***P < 0.001. EMT, epithelial-mesenchymal transition; HIF-1α, hypoxia inducible factor 1α; PKM2, pyruvate kinase M2.
including mTOR/HIF-1α/PKM2/STAT3 should be further analyzed using western blotting or immunohistochemical staining. Additionally, rapamycin, an mTOR inhibitor, should be used to confirm that metformin inhibits EMT via the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway.

In conclusion, the present study demonstrated that EMT served an important role in the proliferation, migration and invasion of OSCC cells. Metformin was capable of reversing CoCl2-induced EMT. Furthermore, it was identified that metformin exerted its effects by suppressing mTOR, HIF-1α, PKM2 and STAT3 activation. The current results were obtained using OSCC cell models in vitro and xenograft nude-mice models in vivo, and indicated that metformin may offer a novel strategy for the treatment of patients with OSCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY and ZY conceived the experiments. YL and BS designed the experiments. WY, YL, XL, XM, BS and ZY performed the experiments. WY and YL analyzed the data and wrote the manuscript. ZY and BS reviewed and edited the manuscript, and acquired the funds. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Animal Research Ethics Committee of Jilin University (Changchun, China). All animal treatments were performed in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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