Metformin inhibits mTOR and c-Myc by decreasing YAP protein expression in OSCC cells

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Abstract. Metformin is an antidiabetic drug that has been reported to have an inhibitory effect on different types of cancers, including oral squamous cell carcinoma (OSCC). However, few studies have explored the role of Yes-associated protein (YAP), a vital factor contributing to OSCC biology, in metformin-induced anticancer activity in OSCC cells. Thus, the purpose of the present study was to investigate the molecular relationship between metformin and YAP in OSCC cells. CAL27 and SCC25 cell lines were treated with various concentrations of metformin for 24 h. Cell proliferation was detected by Cell Counting Kit-8 (CCK-8) and flow cytometric assays. Cell apoptosis was analyzed using flow cytometry. The intracellular protein levels of target genes were determined by western blotting. It was demonstrated that metformin affected the cell cycle and apoptosis of CAL27 and SCC25 cells. Alteration of YAP protein expression affected metformin-mediated changes in the cell cycle and apoptosis of CAL27 and SCC25 cells. In addition, compared to the control treatment, metformin treatment decreased the protein levels of YAP, mTOR, p-mTOR and c-Myc. The overexpression of YAP alleviated the inhibitory effect of metformin on the protein expression of mTOR, p-mTOR and c-Myc. The combination of metformin and verteporfin markedly enhanced the effects of metformin on the protein expression of mTOR, p-mTOR and c-Myc. Therefore, the results of the present study suggest that metformin suppresses OSCC by inhibiting YAP protein expression and by suppressing the YAP-mediated effects of metformin on the protein expression of mTOR and c-Myc.

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Introduction

Oral squamous cell carcinoma (OSCC) is a major burden to human health according to a 2019 epidemiological investigation of cancer (1). The major carcinogenic factors of OSCC are tobacco, alcohol and ultraviolet rays. The typical clinical features of OSCC are obscure boundaries, bleeding and ulcers that affect the chewing, speech and appearance of patients (2). The biological behavior of OSCC cells, including rapid proliferation, resistance to apoptosis, rapid invasion and epithelial-mesenchymal transition, is related to the OSCC clinical phenotype (3). With the rapid development of molecular biology, it has been revealed that the expression of some intracellular molecules is correlated with the biological behavior of OSCC (4,5), and it has been demonstrated that high expression of Yes-associated protein (YAP) results in a rapid cell proliferation rate and poor differentiation of OSCC (6,7). Our previous study also revealed that YAP could be transported into the nucleus, driving the transcription of the *c-Myc* and *Bcl-2* genes, which impairs apoptosis and facilitates the proliferation of OSCC cells (8). In addition, it has also been reported that overexpression of YAP is related to the drug resistance and epithelial to mesenchymal properties of OSCC (9,10). Another study also indicated that cell growth and invasion could be inhibited by the suppression of YAP in OSCC (11,12). Therefore, YAP may be a tumor promoter and could be used as a potential anticancer drug target for OSCC (13).

Metformin, which is extracted from French lilac and easily produced, is an effective hypoglycemic drug with low toxicity (14). It has been revealed that metformin reduces the cancer risk and mortality of diabetic patients compared with that of nonusers via meta-analysis and epidemiology studies (15,16). Therefore, as metformin is widely used, researchers have revealed that metformin can effectively reduce the risk of cancer in people with diabetes, and a dose-response

relationship was proposed (16). In addition, previous studies have also revealed that metformin improves the survival of patients who suffer from head and neck squamous cell carcinoma (including oral cancer) (17,18). Recently, it has been demonstrated that metformin suppresses the growth of OSCC cells by promoting cell apoptosis and decreasing the malignant potential of OSCC (19,20). Metformin can also increase the sensitivity of OSCC cells to cisplatin and disulfiram (21,22). Thus, metformin may become a promising anticancer drug for OSCC. However, it has been confirmed that metformin inhibits OSCC by inhibiting the AMPK/mTOR (19), NF-κB/HIF-1α (21) and LSF/Aurora-A (23) pathways in cells. The relationship between metformin and YAP in OSCC, according to our knowledge, remains uncertain.

Metformin exerts its anticancer effect by reducing mTOR and c-Myc protein expression in cancer cells (24-27), and YAP expression is related to mTOR and c-Myc expression. YAP translocates from the cytoplasm to the nucleus once the Hippo signaling pathway is inactivated, which leads to increased expression of mTOR in tuberous sclerosis complex cells and colorectal cancer cells (28,29). Hansen *et al* also explained that YAP promotes the expression of mTOR protein via the high-affinity leucine transporter LAT1 (30). Moreover, it has been verified that the protein expression of c-Myc in gastric cancer cells and liver cancer cells is upregulated by the overexpression of YAP (31-34). Our previous research also indicated that YAP translocates to the nucleus, driving *c-Myc* gene transcription in OSCC cells (8). Thus, we hypothesized that metformin can downregulate the protein expression of c-Myc and mTOR via a YAP-dependent mechanism in OSCC cells.

In the present study, the relationship between metformin and YAP in OSCC and the regulatory mechanism by which YAP regulates the protein expression of c-Myc and mTOR in metformin-treated cells were investigated. The present study thus revealed a novel molecular mechanism of metformin in OSCC.

Materials and methods

Cell lines. Human tongue squamous cell carcinoma CAL27 and SCC25 cell lines were purchased from ATCC. CAL27 cells were cultured in high-glucose culture medium (DMEM; HyClone; Cytiva) containing 10% fetal bovine serum (FBS; Biological Industries) and 1% penicillin-streptomycin (HyClone; Cytiva) . SCC25 cells were cultured in high-glucose culture medium (DMEM F12; HyClone; Cytiva) containing 10% FBS and 1% penicillin-streptomycin.

Reagents and antibodies. Metformin (item no. D9351) was purchased from Solarbio Life Sciences, and Verteporfin (CL 318952) was purchased from Sigma-Aldrich; Merck KGaA. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Both cell cycle assay kit and Annexin V-PI cell apoptosis assay kit were purchased from Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd. APC-7AAD cell apoptosis assay kit was purchased from Sungene Biotech Co., Ltd. Antibodies against CDK4 (D9G3E; product no. 12790), CDK6 (D4S8S; product no. 13331), p21 (12D1; product no. 2947), Bax (D2E11; product no. 5023), Bcl-2 (D55G8; product no. 4223), mammalian STE20-like kinase 1 (MST1) (D8B9Q; product no. 14946), phosphorylated (p)-MST (Thr183; E7UD1; product no. 49332), large tumor suppressor 1 (LATS1) (C66B5; product no. 3477), p-LATS1 (Thr1097; D57D3; product no. 8654), YAP (D8H1X; product no. 14074), p-YAP (Ser127; D9W2I; product no. 13008), mTOR (7C10; product no. 2983), p-mTOR (Ser2448; D9C2; product no. 5536), c-Myc (D84C12; product no. 5605), SAV1 (D6M6X; product no. 13301) and MOB1 (E1N9D; product no. 13730) were obtained from Cell Signaling Technologies, Inc.

Cell proliferation assay. A Cell Counting Kit-8 (CCK-8) assay was used to detect changes in cell proliferation. First, the cells were seeded at 2,500 cells/well in 96-well plates and cultured in a 37˚C carbon dioxide incubator for 24 h. Next, 0, 5, 15 and 30 mmol/l metformin was added to 96-well plates, and the cells were cultured for 24, 48 and 72 h. Then, 10 μl of CCK-8 reagent was added to each well and incubated for 2.5 h. Subsequently, a microplate reader was used to detect the absorbance at 450 nm.

Cells treated without metformin or YAP overexpression lentiviral vector served as the control group. The experimental groups were as follows: Cells treated with metformin; cells treated with YAP overexpression lentiviral vector; and cells treated with metformin and YAP overexpression lentiviral vector. A 15-mmol/ml concentration of metformin was used in the relevant experimental groups. The cell viability of each group was detected by CCK-8 assay after 24 h of drug incubation, and a microplate reader was used to detect the absorbance at 450 nm.

Cell cycle assay. Firstly, cells ($2.5x10^6$) were treated with 0, 5, 15 and 30 mmol/l metformin for 24 h. Furthermore, cells treated without metformin and overexpression YAP gene lentiviral vector served as the control group; cells treated with metformin, overexpression YAP gene lentiviral vector as well as metformin and overexpression YAP gene lentiviral vector served as the experimental groups. In addition, cells treated without metformin and verteporfin served as the control group; cells treated with verteporfin, as well as metformin and verteporfin served as the experimental groups. A concentration of 15 mmol/l metformin or 1 μM verteporfin was used in the experimental groups. After 24-h drug incubation, 70% ethanol was used to fix cells for 12 h at 4˚C, after washing the cells with PBS. The cells were treated with cell cycle detection kit (item no. CA1510; Solarbio Life Sciences) according to the manufacturer's instructions and the proportion of each cell cycle was analyzed using a flow cytometer (FACSCanto II; BD Biosciences).

Cell apoptosis assay. Cells treated with 0 mmol/l metformin served as the control group and cells respectively treated with 5, 15 and 30 mmol/l metformin served as the experimental groups. In addition, cells treated without metformin and overexpression YAP gene lentiviral vector served as the control group while cells treated with metformin, overexpression YAP gene lentiviral or cells treated with metformin combined with overexpression YAP gene lentiviral vector served as the experimental groups. Similarly, cells treated without metformin or verteporfin served as the control group while cells treated with verteporfin, metformin or metformin combined with verteporfin served as the experimental groups. The concentration of metformin was 15 mmol/l and the concentration of verteporfin was 1 μl in the experimental groups. After 24 h of drug

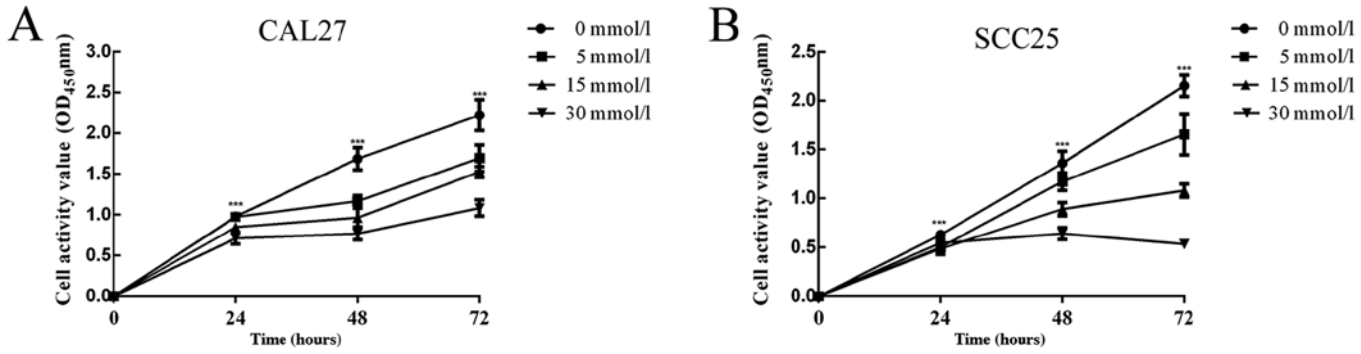

Figure 1. Metformin inhibits OSCC cell proliferation. (A) CAL27 and (B) SCC25 cells ($2.5x10^3$ cells/well) were plated into a 96-well cell culture plate and treated with 0, 5, 15 and 30 mmol/l metformin for 24, 48 and 72 h. Each set had 5 replicates. The proliferative activity of cells was detected by CCK-8 assay. Data are presented as the mean SEM. ***P<0.001 denotes significance for comparisons between the untreated control group and the treatment group. OSCC, oral squamous cell carcinoma; CCK-8, Cell Counting Kit-8.

incubation, cells were stained using a green fluorescent-labeled Annexin V-APC staining kit (Sungene Biotech Co., Ltd.) according to the manufacturer's instructions. Cells ($2x10^5$) were collected from each group of samples. Secondly, 500 μl of 1X binding buffer and 8 μl of Annexin V-fluorescein APC were used to suspend cells for 10 min. Finally, the cells were incubated with 5 μl of 7-AAD solution for 5 min. Similarly, Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) (Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.) was used to detect cells with no staining. Finally, the proportion of apoptotic cells was calculated by flow cytometry (FACSCanto II; BD Biosciences).

Western blotting. Cells ($8x10^6$) were cultured in a culture plate and were collected, and the total protein in cells was extracted with an ice-cold lysate containing a phosphatase inhibitor and a protease inhibitor (Solarbio Life Sciences). The protein concentration was determined by a bicinchoninic acid assay (Boster Biological Technology, Co., Ltd.). The proteins (20 μg total protein/lane) were separated using 10% SDS-PAGE. Electrophoresis and transfer were performed according to the standard protocol of western blotting. After blocking the PVDF membrane for 1 h at 4˚C with 5% non-fat milk in Tris-buffered saline (TBST) containing 0.1% Tween-20, the membrane was probed with a primary antibody (1:1,000) overnight at 4˚C. Then the membrane was incubated with HRP-conjugated secondary antibody (1:20,000; product no. 7074; Cell Signaling Technology, Inc.) for 40 min at 25˚C. Finally, protein bands were detected using the Immobilon Western Chemiluminescent HRP Substrate Kit (Vazyme Biotech Co., Ltd.). Densitometric analysis was performed using ImageJ v1.52 (National Institutes of Health).

Construction of YAP-overexpressing cell lines. A YAP overexpression lentiviral vector ($1x10^8$ TU/ml) was constructed, and an empty lentiviral vector was used as a control. Both vectors were prepared by the Shanghai Genechem Co., Ltd. and transfected into the CAL27 and SCC25 cell lines for 72 h at 27˚C. Subsequently, 24 h later the transfection efficiency was detected by western blotting and RT-qPCR.

Reverse transcription quantitative (RT-q)PCR analysis. Cells treated with lentiviral blank vector served as the control group while cells treated with overexpressed YAP vector served as the experimental group. Then, 72 h later, total RNA in cells was extracted by TRIzol total RNA isolation (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The purity of the obtained total RNA samples was detected by NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed into cDNA using PrimeScript™ RT II reagent kit (Takara Bio, Inc.) on Biometra PCR thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). Then, 2 μl RNase-free water (Takara Bio, Inc.), 2 μl primers (Sangon Biotech Co., Ltd.), 1 μl obtained cDNA samples and 5 μl SYBR-Green fluorescent dye (Takara Bio, Inc.) were mixed into a 10-μl reaction system. The forward primer of YAP was 5'-TCTTACACCGTGCTGCCATT-3' and the reverse primer was 5'-AGCACCTGTCCAGGTATCAC-3'. The forward primer of GAPDH was 5'-GCACCGTCAAGGCTGAGAAC-3' and the reverse primer was 5'-TGGTGAAGACGCCAGTGGA-3'. Finally, the gene expression was detected by Light Cycler Roche 480 RT-qPCR instrument (Roche Diagnostics). The results of fold-changes in mRNA levels were calculated by using a $2^{-\Delta\Delta Cq}$ method (35).

Statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used to analyze the differences between several research groups, and the difference between two groups was analyzed by two-tailed t-test (P<0.05 and P<0.01 were considered to indicate statistically significant differences). All the statistical results were analyzed by SPSS software 20.0 (IBM Corp.).

Results

Metformin inhibits cell proliferation and promotes cell apoptosis in OSCC. To illustrate the effect of metformin on the growth of CAL27 and SCC25 cells, cells were first treated with 0, 5, 15 and 30 mmol/l metformin, and the changes in cell proliferation were assessed at 24, 48 and 72 h by CCK-8 assay. It was evident that cell proliferation decreased with increasing metformin concentration and increasing dosing time (Fig. 1). The results of flow cytometric assays revealed that the proportion of cells arrested in the G0/G1 phase was increased, while the proportion of cells in the S phase and G2 phase were reduced in a dose-dependent

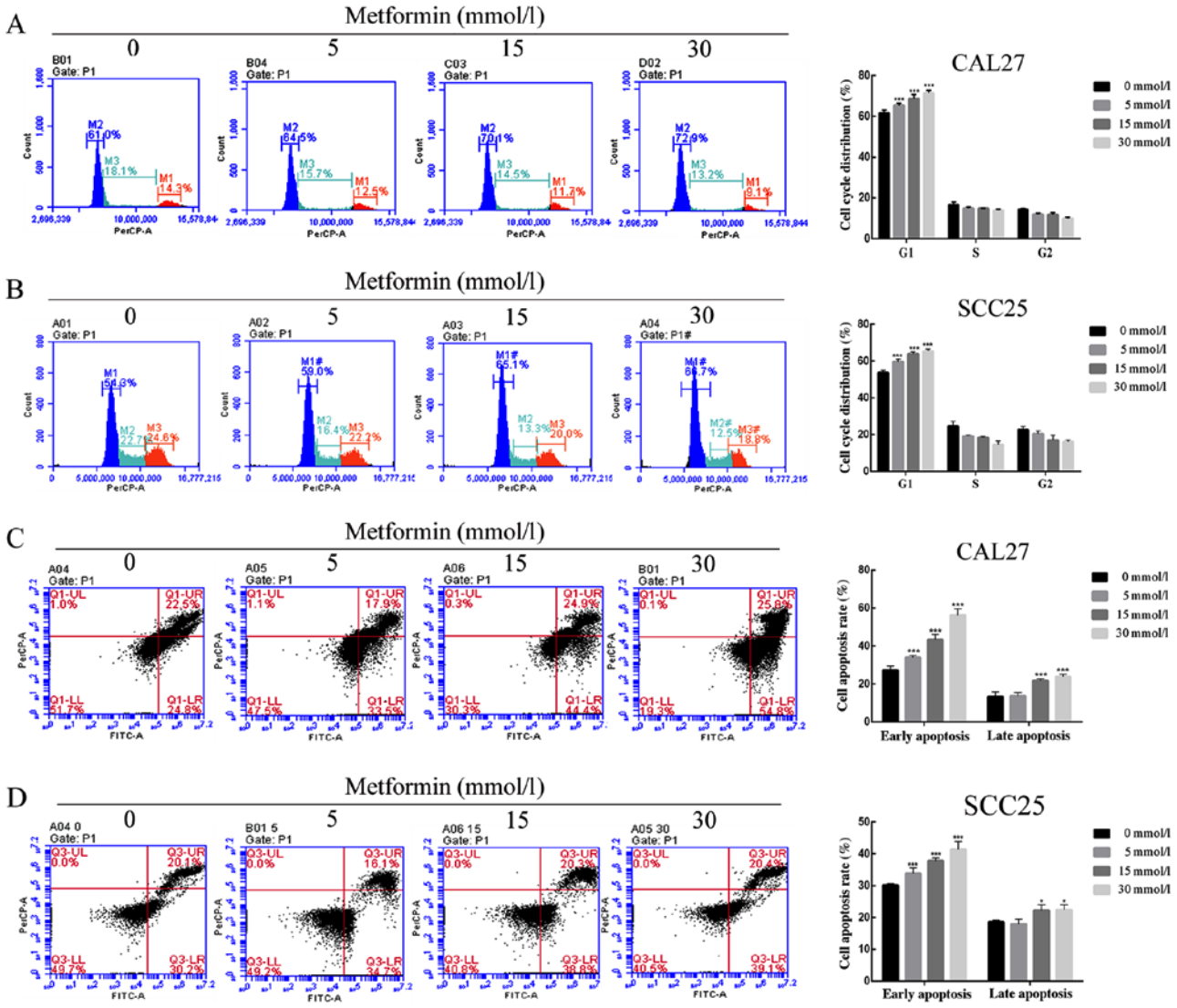

Figure 2. Effect of metformin on OSCC cell cycle and apoptosis. CAL27 and SCC25 cells ($2.5x10^6$ cells/well) were plated into a 6-well cell culture plate and treated with 0, 5, 15 and 30 mmol/l metformin for 24 h. Each set had 3 replicates. The proportions of cells in each phase of the cell cycle and the proportion of cells undergoing apoptosis were detected by flow cytometry. (A and B) The proportion of CAL27 and SCC25 cells in each phase of the cell cycle under different concentrations of metformin. (C and D) The percentage of apoptotic CAL27 and SCC25 cells under different concentrations of metformin. Data are presented as the mean SEM. $^*P<0.05$ and $^{***}P<0.001$ denote significance for comparisons between the untreated control group and the treatment group. OSCC, oral squamous cell carcinoma.

manner (Fig. 2A and B). Then, FITC/PI staining and flow cytometry were used to detect the cell apoptosis induced by metformin and it was revealed that the early apoptosis rate and late apoptosis rate were significantly increased with metformin treatment compared with the control treatment (Fig. 2C and D). In addition, biomarkers of proliferation and apoptosis were detected by western blotting. The protein expression levels of CDK4 and CDK6, which are involved in the G0/G1 phase (36), were markedly decreased in metformin-treated cells compared to control cells. p21 has the ability to inhibit the transition from the G1 to S phase in the cell cycle (37), and its expression was increased accordingly (Fig. 3A). The decreased protein expression of Bcl-2 and the increased expression of the proapoptotic protein Bax in the metformin group vs. the control group confirmed that metformin inhibited cell growth by promoting apoptosis in the CAL27 and SCC25 cell lines (Fig. 3A). The results revealed that metformin arrested the cell cycle in the G1/S phase and promoted cell apoptosis *in vitro*.

Metformin stimulates the Hippo signaling pathway in OSCC cells. YAP is one of the transcription coactivators inhibited by the Hippo pathway and is related to OSCC cell growth (6). The signaling molecules related to the Hippo pathway rely on a phosphorylation cascade. MST1 can be phosphorylated at Thr183 as a result of activation of the Hippo pathway. Then, phosphorylated MST1 can bind with SAV1 to phosphorylate LATS1 at T1097, which can bind MOB1. Next, upstream activation of the Hippo pathway can result in the phosphorylation of Yap at S127. Subsequently, YAP is susceptible to proteasomal degradation (38). In the present results, metformin increased the phosphorylation of MST1 (Thr183) and LATS1 (T1097), and the expression of total protein was not markedly altered as the dose of metformin increased. The protein expression of SAV1 and MOB1 also increased with metformin treatment (Fig. 3B).

Metformin inhibits OSCC cell growth by decreasing YAP. The YAP expression level was enhanced in CAL27 and SCC25

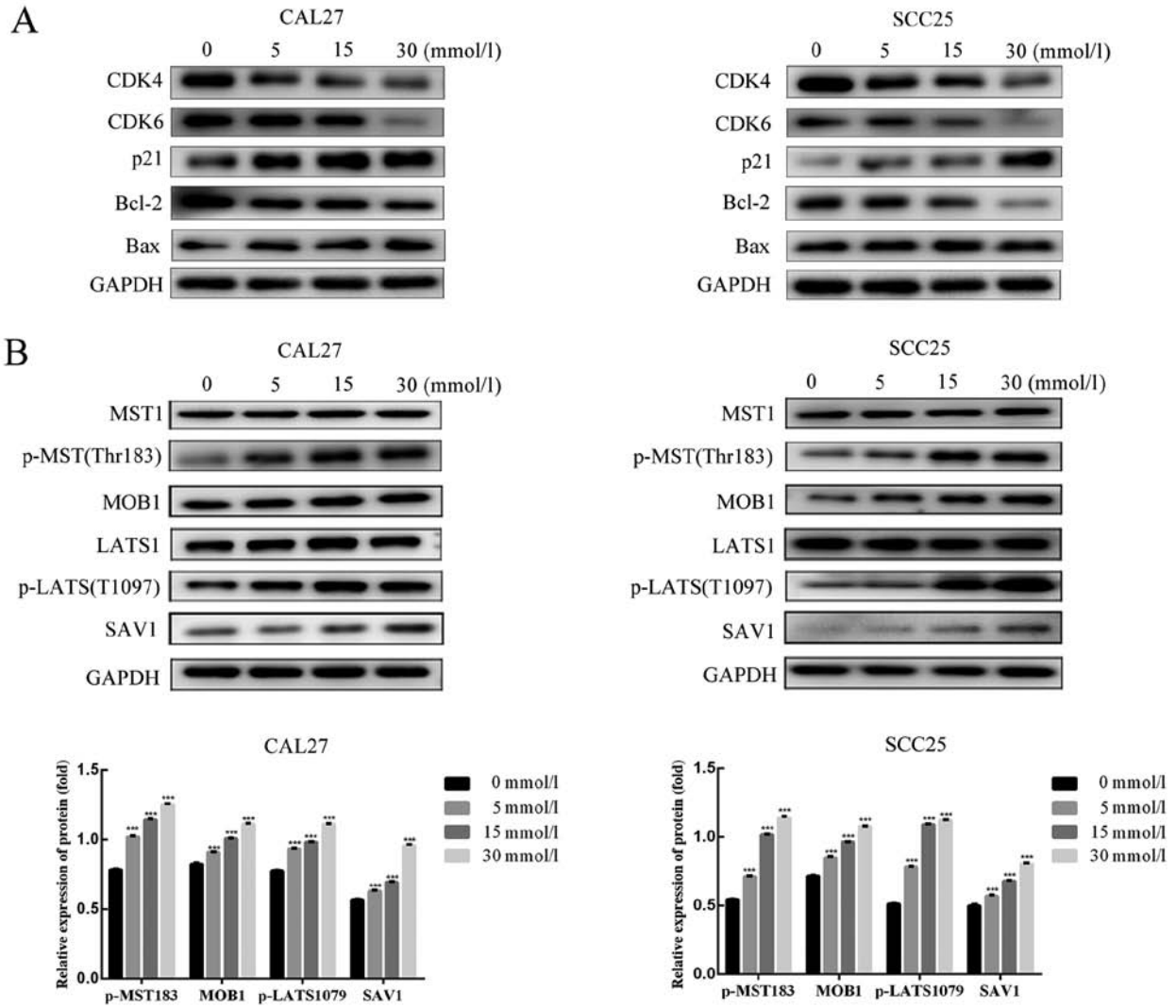

Figure 3. Effect of metformin on protein expression in OSCC cells. CAL27 and SCC25 cells were treated with 0, 5, 15 and 30 mmol/l metformin for 24 h. Total protein was extracted from cells. Then, proteins related to the cell cycle and cell apoptosis were detected by western blotting. The experiment was repeated three times. (A) CDK4, CDK6, p21, Bcl-2 and Bax protein expression in CAL27 and SCC25 cells following 24 h of metformin treatment. (B) MST1, p-MST1, MOB1, LATS1, p-LATS1SAV1protein expression in CAL27 and SCC25 cells following 24 h of metformin treatment. GAPDH served as a loading control. Data are presented as the mean SEM. ***P<0.001 denote significance for comparisons between the untreated control group and the treatment group. OSCC, oral squamous cell carcinoma; MST1, mammalian STE20-like kinase 1; p-, phosphorylated; LATS1, large tumor suppressor 1; YAP, Yes-associated protein.

cells by using YAP overexpression lentiviral particles. YAP protein expression was confirmed to be higher in the overexpression group than in the control group (Fig. 4A). The mRNA expression of YAP in the YAP overexpression group was also higher than that in the control group (Fig. 4B). In contrast to transfection with empty lentiviral vector, transfection with YAP overexpression vector significantly weakened the effects of metformin on OSCC cells. The optical density (OD) value at 450 nm in the CCK-8 assay of YAP-overexpressing cells was higher than that of empty lentivirus-transfected cells after metformin treatment for 24 h (Fig. 4C). The proportion of cells arrested in G1 phase of the cell cycle in the YAP overexpression group was lower than that in the empty lentivirus group after metformin treatment for 24 h (Fig. 5A and B). The cell apoptosis rate in the YAP overexpression group was lower than that in the empty lentivirus group after metformin treatment for 24 h (Fig. 5C and D). To further identify the role of YAP in metformin treatment, metformin was combined with the YAP inhibitor verteporfin. The proportion of cells at the G1 phase (Fig. 6A and B) and the proportion of apoptotic cells in the metformin and verteporfin treated group were higher than those in the metformin-treated group (Fig. 6C and D). The YAP overexpression group exhibited higher expression of CDK4, CDK6 and Bcl-2 and lower expression of Bax than the empty lentivirus group after metformin treatment for 24 h (Fig. 7A). However, the CDK4, CDK6, and Bcl-2 protein expression was lower and the Bax expression was higher in the verteporfin and metformin combination treatment group than in the metformin treatment group (Fig. 7B). These results clearly indicated that metformin had an inhibitory effect on the proliferation of OSCC cells by decreasing YAP protein.

Metformin decreases mTOR and c-Myc through the downregulation of YAP. Other studies have revealed that YAP promotes the expression of mTOR in hepatocellular cells and colorectal cancer cells (39,40). Our previous study also revealed that YAP facilitated the protein expression of c-Myc (8). However, few studies have reported that metformin inhibits the expression

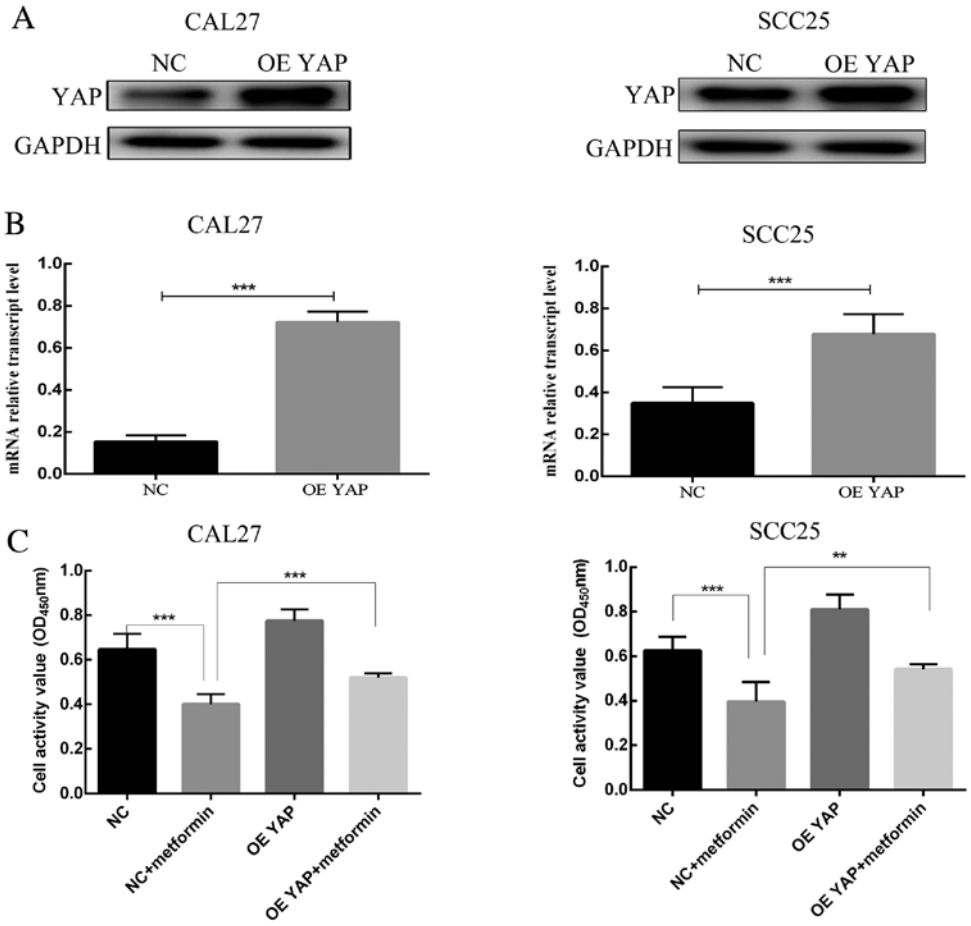

Figure 4. YAP overexpression reverses the inhibitory effect of metformin on OSCC cells. YAP protein expression was enhanced by lentiviral transfection of CAL27 and SCC25 cells for 72 h. Total mRNA and protein were extracted from cells. The transfection efficiency was detected by RT-qPCR and western blotting. The effect of YAP overexpression on the proliferative activity of cells upon metformin treatment was detected by CCK-8 assay. (A) YAP protein expression in the control group and the YAP overexpression group of CAL27 and SCC25 cells. The experiment was repeated three times. (B) YAP mRNA expression in the control group and the YAP overexpression group of CAL27 and SCC25 cells. The experiment was repeated three times. (C) The proliferative activity of CAL27 and SCC25 cells in the control group, metformin group, YAP overexpression group and the combined treatment group. Data are presented as the mean SEM. **P<0.01 and ***P<0.001 denote significance for comparisons between the metformin group and the combined treatment group. Each set had 5 replicates. OSCC, oral squamous cell carcinoma; YAP, Yes-associated protein; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; OE, overexpression; NC, negative control.

of mTOR and c-Myc through a YAP-dependent mechanism in OSCC cells (24-27). To further illustrate the molecular relationship between metformin and YAP in OSCC cells, western blotting was used to detect changes in mTOR and c-Myc expression in OSCC cells. As anticipated, a gradual reduction in mTOR expression, mTOR phosphorylation at Ser2448 and c-Myc expression was observed as the metformin concentration increased (Fig. 8A). Overexpression of YAP facilitated mTOR and c-Myc protein expression in CAL27 and SCC25 cells treated with metformin (Fig. 8B). The metformin and verteporfin combination group exhibited a decreased expression level of mTOR and c-Myc protein than the metformin treatment groups (Fig. 8C). These results indicated that YAP inhibition was associated with the metformin-induced decrease in mTOR and c-Myc expression in OSCC cells.

Discussion

Metformin, discovered from French lilac, has been widely studied for its role in cancer treatment since it has an anti-tumor ability with characteristics of low toxicity and low cost production (41). It has been revealed that metformin-treated head and neck squamous cell carcinoma patients had a higher survival rate than those not treated with metformin, according to a meta-analysis (17,18). Previous studies have indicated that metformin promotes the toxic effects of disulfiram, 5-fluorouracil and cisplatin on OSCC cells since metformin enhances the inhibitory effects of these traditional chemotherapeutics, even at low concentrations (21,22,42,43). In addition, it has also been demonstrated that metformin suppresses cell proliferation and increases cell apoptosis in OSCC *in vivo* and *in vitro* (19). Although some studies have elucidated the effects of metformin on OSCC, the molecular mechanism of metformin on OSCC cells remains undefined.

Prolonged proliferation and insensitivity to apoptosis are two main types of biological characteristics typically featured in human tumors (44). Therefore, controlling cancer cell proliferation and apoptosis has a certain significance for the clinical treatment of cancer. YAP, as a vital carcinogenic factor, is important in OSCC growth, and high expression of YAP protein may accelerate the progression of OSCC (6). YAP was demonstrated to enhance the proliferation rate of cells

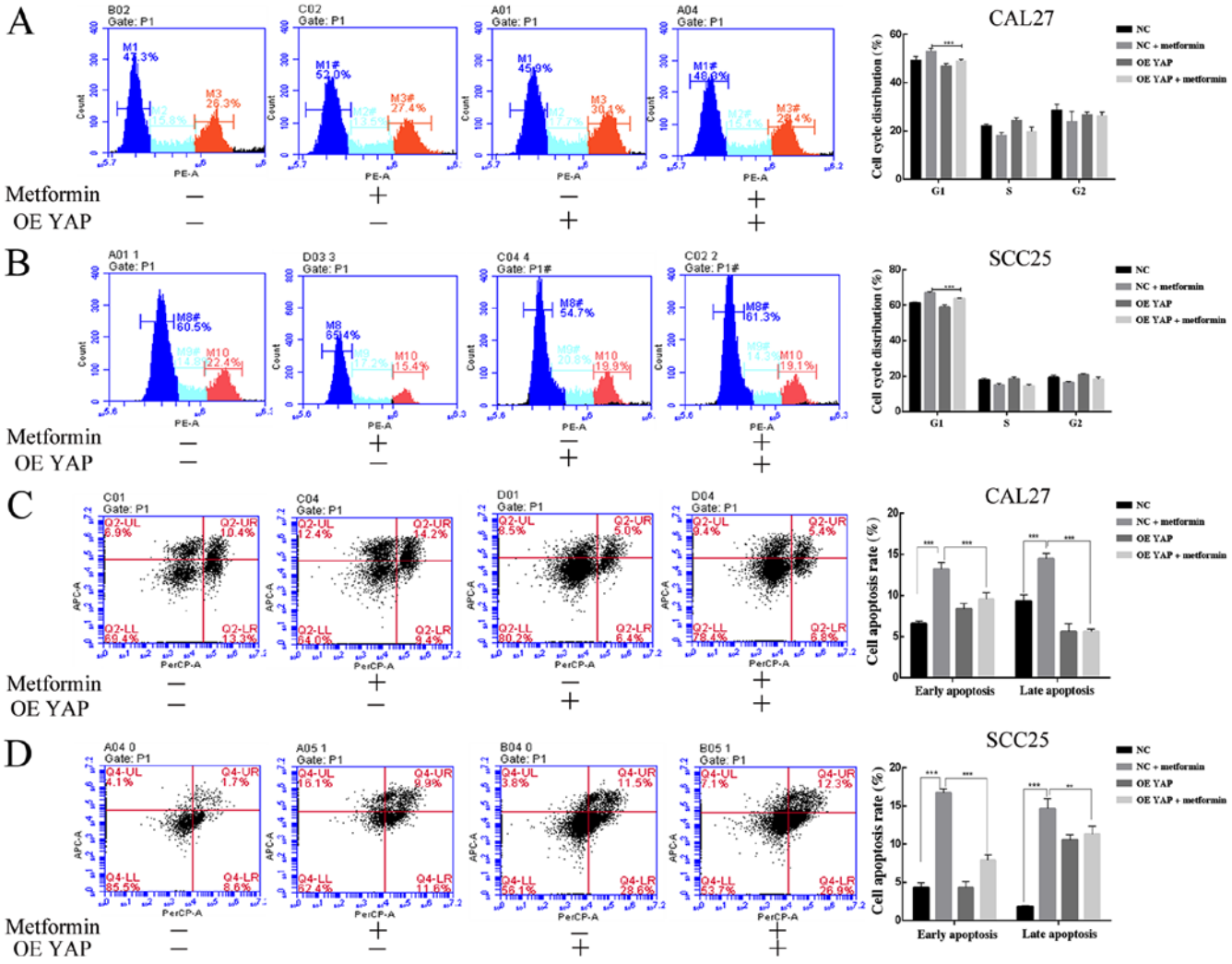

Figure 5. YAP overexpression weakens the function of metformin in OSCC cells. CAL27 and SCC25 cells ($2.5x10^6$ cells/well) were plated into 6-well cell culture plates. The proportions of cells in each phase of the cell cycle and the proportion of apoptotic cells in each group were detected by flow cytometry. Metformin (15 mmol/l) was used in the experimental group. The experiment was repeated three times. (A and B) The proportion of CAL27 and SCC25 cells in each phase of the cycle in the control group, metformin group, YAP overexpression group and combined treatment group. (C and D) The percentage of apoptotic CAL27 and SCC25 cells in the control group, metformin group, YAP overexpression group and combined treatment group. The data are presented as the mean SEM. **P<0.01 and ***P<0.001 denote significance for comparisons between the metformin group and the combined treatment group. OSCC, oral squamous cell carcinoma; YAP, Yes-associated protein; OE, overexpression; NC, negative control.

and reduce apoptosis by promoting c-Myc and Bcl-2 protein expression in OSCC (8). Moreover, cell growth and invasion were inhibited by the suppression of YAP in OSCC (11). Inhibition of YAP protein also decreased the drug resistance of cisplatin in OSCC cells (9). Thus, to determine whether metformin has an inhibitory effect on OSCC, the YAP protein was inhibited.

The protein binding between YAP and TEAD4 has been demonstrated to accelerate the cell proliferation rate by markedly promoting the transition of the cell cycle from the G1 to S phase and reducing cell apoptosis (8). In addition, the protein expression of CDK4/6 and p21 is involved in the transition of the cell cycle from the G1 to S phase. CDK4, CDK6 and other cyclin-dependent kinases can accelerate the transition of the cell cycle from the G1 to the S phase (36). p21 is an inhibitor of cyclin-dependent kinases, and it has a negative regulatory effect on the cell cycle transition from the G1 to the S phase (37). The Bcl-2 protein family can control the apoptosis process of cells by influencing the outer mitochondrial membrane potential. Bcl-2 protein and Bax protein belong to the Bcl-2 protein family. The former blocks the process of cell apoptosis, while the latter stimulates the processes of cell apoptosis (45). As anticipated, in the present study, metformin inhibited the transition of the cell cycle from the G1 to S phase and increased cell apoptosis in CAL27 and SCC25 cells. The protein expression of YAP in CAL27 and SCC25 cells was also decreased with metformin treatment compared to control treatment. The protein expression of CDK4, CDK6, p21, Bcl-2 and Bax also paralleled the phenotypic changes upon metformin exposure.

Next, YAP overexpression lentiviral particles or empty lentiviral vectors were transfected into CAL27 and SCC25 cells. The cells were divided into a YAP overexpression group and a negative control group. The protein and mRNA expression levels of YAP in the YAP overexpression group were higher than those of the empty vector group. In fact, metformin inhibited the transition of the cell cycle from the G1 to S phase and increased cell apoptosis in the control group. However,

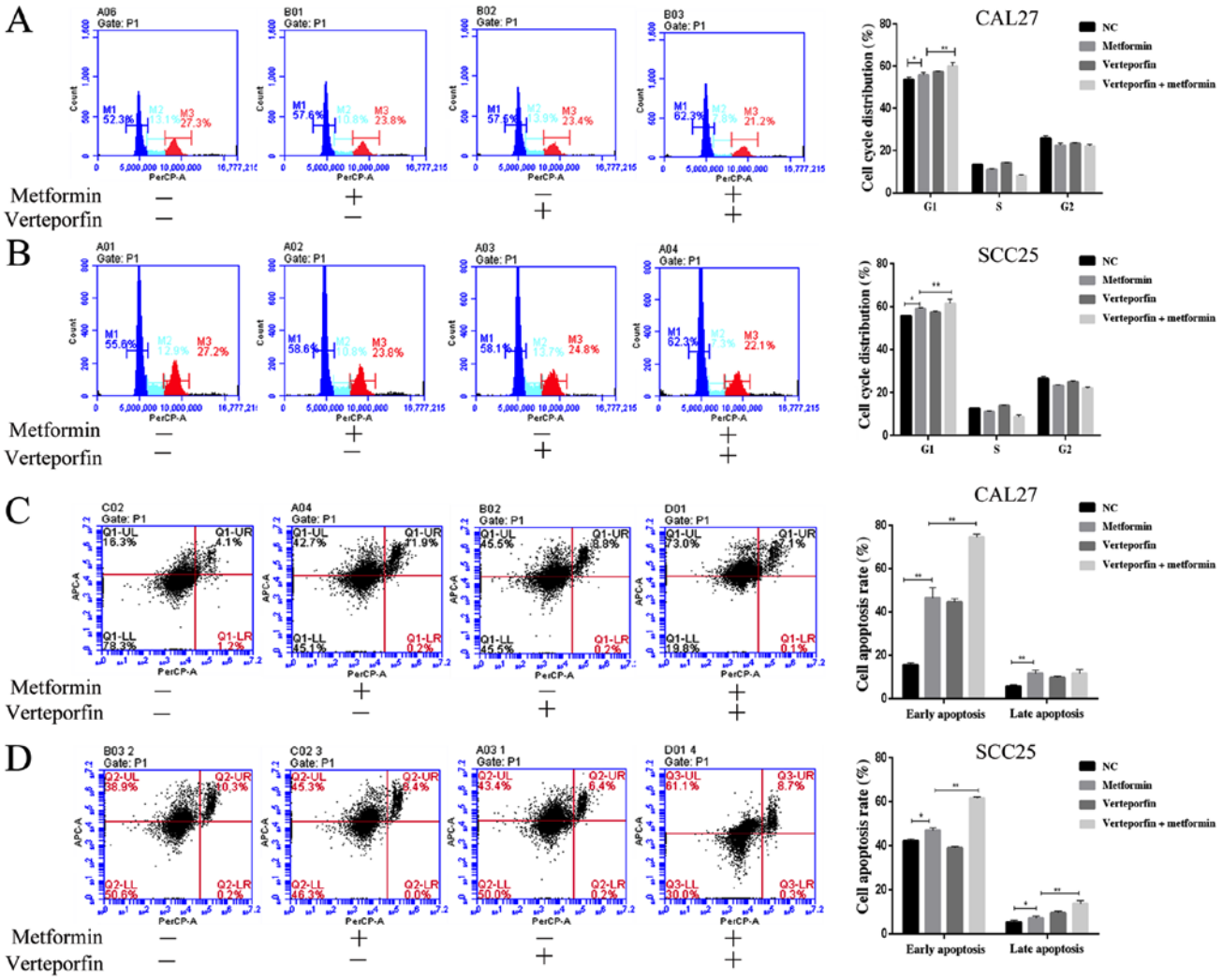

Figure 6. Combination of the YAP inhibitor and metformin blocks the cell cycle and facilitates cell apoptosis in CAL27 and SCC25 cells. CAL27 and SCC25 cells ($2.5x10^6$ cells/well) were plated into a 6-well cell culture plate and treated with 15 mmol/l metformin, 1 μM verteporfin or a combination for 24 h. The proportion of cells in each phase of the cell cycle and the proportion of apoptotic cells in each group were detected by flow cytometry. The experiment was repeated three times. (A and B) The proportion of CAL27 and SCC25 cells in each phase of the cell cycle in the control group, metformin group, verteporfin group and the combined treatment. (C and D) The percentage of apoptotic CAL27 and SCC25 cells in the control group, metformin group, verteporfin group and combined treatment group. The data are presented as the mean SEM. *P<0.05 and **P<0.01 denote significance for comparisons between the metformin group and the combined treatment group. YAP, Yes-associated protein; NC, negative control.

overexpression of YAP reversed the inhibitory effects of metformin on the cell cycle and attenuated metformin-induced cell apoptosis. The protein expression of CDK4, CDK6, p21, Bcl-2 and Bax also paralleled the phenotypic changes in the YAP overexpression group.

To further confirm that metformin affected the cell cycle and apoptosis by decreasing YAP protein, cells were treated with both metformin and verteporfin, which is an inhibitor of YAP protein. Verteporfin is a photosensitizer that can be used for photodynamic therapy to treat macular degeneration with limited toxicity (46). Recent studies have illustrated that verteporfin effectively inhibits the development of cancer by decreasing the expression of YAP protein in cancer cells (47,48). For example, verteporfin inhibits the translocation of YAP protein into the nucleus by inhibiting the binding of YAP and TEAD (49) or promotes the expression of 14-3-3 protein so that YAP remains in the cytoplasm (50). Our previous experiments also verified that the expression of YAP protein in OSCC cells decreased with increasing verteporfin drug concentration (8). Therefore, verteporfin was combined with metformin. As anticipated, the effects of the combined use of metformin and verteporfin on the cell cycle and apoptosis were greater than those of a single medication. The protein expression of CDK4, CDK6, Bcl-2 and Bax also paralleled the phenotypic changes in the metformin and verteporfin combination group. These data clarified that metformin has an inhibitory effect on OSCC by inhibiting the YAP protein.

YAP is a main downstream factor of the Hippo signaling pathway and can be inhibited by the Hippo signaling pathway (6). MST1, LATS1, SAV1 and MOB1 constitute the upstream regulatory signal transduction of the Hippo signaling pathway. It is known that MST1 can be phosphorylated at Thr183 as a result of activation of the Hippo pathway. Then, phosphorylated MST1 can bind with SAV1 to phosphorylate LATS1 at T1097, which can bind MOB1. Subsequently, the upstream Hippo pathway is activated, which could result in the phosphorylation of YAP at S127. Phosphorylated YAP is susceptible to proteasomal degradation (51). Phosphorylation

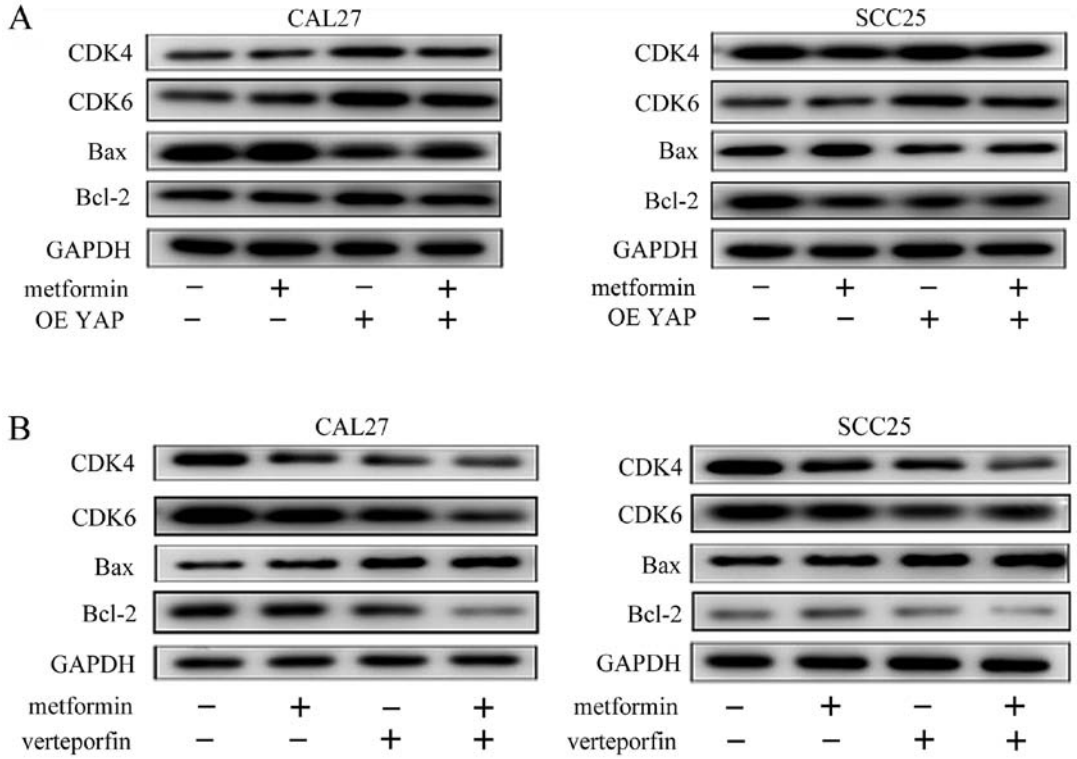

Figure 7. Expression of proteins involved in the cell cycle and cell apoptosis. (A) The expression of proteins involved in the cell cycle and cell apoptosis of the control group, metformin group, YAP overexpression group and the combined treatment. (B) The expression of proteins involved in the cell cycle and cell apoptosis of the control group, metformin group, verteporfin group and the combined treatment group. YAP, Yes-associated protein; OE, overexpression.

of S127 results in YAP sequestration in the cytoplasm via interaction with 14-3-3 (11) or SCFβ-TrCP E3 ubiquitin ligase (52). The present results demonstrated that metformin increased the phosphorylation of MST1 (Thr183) and LATS1 (T1097), with virtually no change in the expression level of total protein as metformin concentration increased. The protein expression of SAV1 and MOB1 also increased with metformin treatment. A change in YAP phosphorylation (S127) and a progressive decrease in YAP expression followed the activation of the upstream signaling factor in the Hippo pathway. These results indicated that metformin may reduce the protein expression of YAP by activating the Hippo pathway.

The mTOR signaling pathway transmits information concerning synthesis or catabolism in cells and affects the metabolism of proteins, lipids and nucleotides in cells, thereby providing sufficient substances for cancer growth (53). Observation of patients with tongue squamous cell carcinoma revealed high expression levels of mTOR and p-mTOR proteins in patient tissue sections (54). It has been demonstrated that circular RNA hsa-circ-0007059 or activated C kinase 1 receptor promotes the proliferation of OSCC cells by increasing the expression of mTOR protein (55,56). YAP serves as a vital association between the Hippo pathway and the PI(3)K-mTOR pathway. It has been confirmed that SAV1 inhibits the protein expression of mTOR by inactivating the YAP protein in colorectal cancer (29). It has also been illustrated that mTOR blocks the inhibitory effect of AMOTL2 on YAP to promote cell growth and invasion in glioblastoma (57). In addition, YAP has been revealed to increase the expression level of miR-29, which indirectly increases the protein expression of mTOR in cells (40). In the present study, metformin decreased the protein expression of mTOR in a dose-dependent manner. YAP overexpression inhibited the ability of metformin to decrease the protein expression of mTOR. In contrast, the combination of metformin with verteporfin enhanced the ability of metformin to decrease the protein expression of mTOR.

The *Myc* genes are a family of proto-oncogenes. MYC family proteins are involved in regulating the proliferation, differentiation and apoptosis of cancer cells and are closely related to cell division activities (58). A study of OSCC patients revealed that the level of c-Myc protein expression was closely related to disease severity and survival rate (59). Our previous study revealed that YAP is transported into the nucleus, where it drives the transcription of the *c-Myc* gene to accelerate OSCC cell growth (8). Other studies also demonstrated by RNA sequencing that c-Myc serves as a definitive target of YAP and mediates the ability of YAP to promote cancer growth (31,33). Thus, it was investigated whether metformin has an inhibitory effect on c-Myc in OSCC cells by inhibiting the YAP protein. In the present study, metformin decreased the protein expression of c-Myc in a dose-dependent manner. YAP overexpression inhibited the ability of metformin to decrease the protein expression of c-Myc. Conversely, the combination of metformin with verteporfin enhanced the ability of metformin to decrease the protein expression of c-Myc.

In conclusion, the present experiments demonstrated that metformin prevented OSCC by reducing the expression of YAP protein. Furthermore, it was revealed that YAP-mediated metformin also had regulatory effects on c-Myc and mTOR proteins. This provides a new reference for the anticancer mechanism of metformin. However, this study still has some

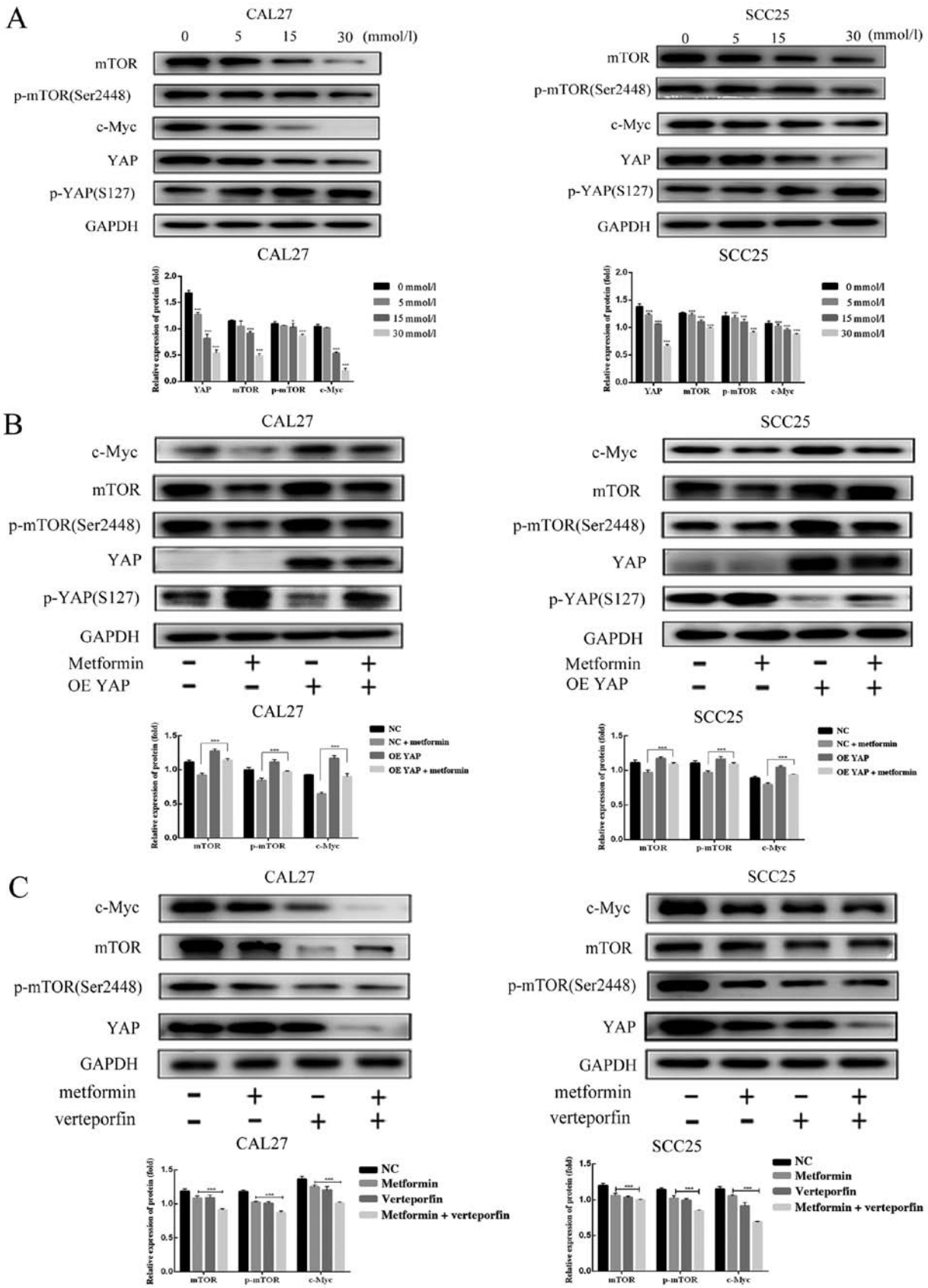

Figure 8. Effect of metformin on mTOR, p-mTOR and c-Myc protein expression in OSCC cells. CAL27 and SCC25 cells were treated with 0, 5, 15 and 30 mmol/l metformin for 24 h. To verify the effect of YAP protein on the protein expression of mTOR, p-mTOR and c-Myc, the protein expression of YAP was changed by lentivirus infection or verteporfin treatment. (A) The protein expression of mTOR, p-mTOR and c-Myc in CAL27 and SCC25 cells upon treatment with different concentrations of metformin. (B) The protein expression of mTOR, p-mTOR and c-Myc in the control group, metformin group, YAP overexpression group and the combined treatment group. (C) The protein expression of mTOR, p-mTOR and c-Myc in the control group, metformin group, verteporfin group and the combined treatment group. GAPDH served as the loading control. The data are presented as the mean SEM. *P<0.05 and ***P<0.001 denote significance for comparisons between the metformin group and the combined treatment group. YAP, Yes-associated protein; mTOR, mammalian target of rapamycin; p-, phosphorylated; OSCC, oral squamous cell carcinoma; OE, overexpression; NC, negative control

limitations. For example, this molecular mechanism has not been verified by *in vivo* experiments in the present study. If conditions permit, further experiments will be designed to explore and verify this related hypothesis in the future. The present study thus revealed that YAP may be recognized as a potential target for metformin treatment in OSCC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YWa, YWe, WG and YZ designed and executed the study. YZ, XF, HT and XF analyzed the data. WY wrote the study. All authors read and approved the 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

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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