

Original Paper

Metformin Inhibits Esophagus Cancer Proliferation through Upregulation of USP7

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Key Words

Metformin • Esophagus cancer • USP7 • mTOR/p70S6K/pS6 signaling • AMPK signaling

Abstract

Background: Recent population studies suggest that the use of metformin is associated with reduced incidence and improved prognosis of certain cancers. **Methods:** In the current study, we assessed the effect of metformin on esophagus cancer cells using two cell lines (Eca-109 and TE-1 cells). **Results:** We found that metformin inhibited growth and decreased expression of cell-cycle regulators in these cells. Treatment with metformin was also associated with activation of AMP kinase and inhibition of mTOR/p70S6K/pS6 signaling in both cells. However, inhibition of AMPK signaling has little impact on the anti-proliferative roles of metformin. In addition, we found USP7, a positive regulator of tumor suppressor p53, as a new molecular target of metformin. Esophagus cancer cells can be protected against metformin-induced growth inhibition by small interfering RNA against USP7. **Conclusion:** These results provide evidence for a mechanism that may contribute to the antineoplastic effects of metformin suggested by recent population studies and justify further work to explore potential roles for it in esophagus cancer prevention and treatment.

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Introduction

Esophagus cancer, which has a particularly high incidence in East Asian countries, commonly has poor prognosis [1]. It is the eighth most common incident cancer and sixth in cancer mortality in the world [2]. Although the current treatment includes radiation therapy, chemotherapy, and esophagogastric resection, many patients with esophageal cancer

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experience progression of disease, indicating that these tumors are resistant to standard therapies [2].

Metformin (1,1-dimethylbiguanide hydrochloride), an oral anti-diabetic drug, is a biguanide usually used in the treatment of type 2 diabetes mellitus with obesity, insulin resistance, and hyperinsulinemia [3-5]. A growing body of evidence from clinical studies and animal models suggests that the primary function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis [6]. Moreover, recent studies carried out using population registries raise the notion that metformin could reduce cancer risk and/or improve cancer prognosis [7-10]. Besides, an anti-mitogenic effect of metformin has been suggested by *in vitro* and epidemiological studies [9, 10]. Metformin was suggested to inhibit cancer cell proliferation and tumor growth in animal models [11, 12]. For example, in prostate cancer cells, metformin provokes cell-cycle arrest in G0-G1 phase but does not induce apoptosis or autophagy [13]. Moreover, metformin inhibits growth of breast cancer cell lines, blocks cellular transformation in an inducible model system, and has anti-tumor effects in mouse xenografts [11, 14]. However, the functions of metformin on the esophagus cancer cells remain unexplored.

At the molecular level, metformin regulates the AMP-activated protein kinase signaling, consequently inhibits the mTOR pathway to suppress energy-consuming pathways and protein synthesis [15, 16]. In agreement, AMPK inhibition abolishes the anti-proliferative effects of metformin in ovarian and breast cancer cells [17, 18]. However, it was recently reported that metformin can act independent of AMPK signaling. Metformin could inhibit hepatic glucose production in both wild-type and AMPK α 1 α 2-deficient hepatocytes through the inhibition of ATP production [19]. In addition, metformin can act independently of AMPK to downregulate mTOR and, instead, signals through Rag GTPase [20]. Taken together, these results suggest a rather complicated mechanism for the metformin action.

Here, we describe *in vitro* experiments carried out to investigate the hypothesis that metformin exhibits direct antiproliferative actions on esophagus cancer cells. We also found USP7, a positive regulator of tumor suppressor p53, as a new molecular target of metformin.

Materials and Methods

Cell cultures

The esophagus cancer cell line Eca-109 and TE-1 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai), and cultured in Dulbecco modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, USA). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.

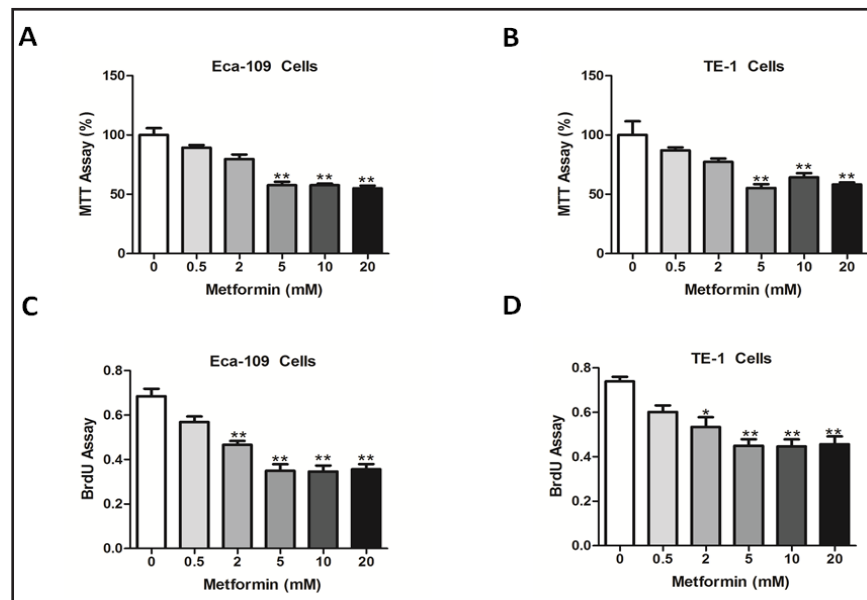
Cell viability and BrdU incorporation assays

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO). After preculture, cells were treated with medium containing different doses of metformin (Sigma) and/or different agents as described in Results and figure legends. MTT assay was performed by incubating the cells with 0.5 mg/ml MTT for 6 hours at 37°C in 5% CO₂. The formazan product was dissolved in dimethyl sulfoxide, and absorbance was read at 490 nm. A cell proliferation enzyme-linked immunosorbent assay kit (Beyotime, Shanghai) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were repeated at least three times in quadruplicate.

RNA isolation and Real-time PCR

Total RNAs were isolated from cells by TRIzol reagent (Invitrogen, USA), and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalian, China), following the manufacturer's instructions. In order to determine the transcripts of the interest genes, Real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Dalian, China) on an ABI 7500 machine.

Fig. 1. Metformin inhibits cell proliferation in esophagus cancer cells. (A-B) Cell viability was measured by MTT assays in Eca-109 (A) and TE-1 cells (B). Cells were treated with various concentrations of metformin as indicated. (C-D) Cell proliferation activity was measured by BrdU incorporation assays in Eca-109 (C) and TE-1 cells (D).



Western blot analysis

Cells after different treatments were lysed with RIPA buffer. An equal amount of protein was subjected to 8% SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. The membranes were blocked in 10% skim milk for 2 hour at room temperature. The immunoblots were incubated overnight at 4 °C with antibodies. Next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 2 hour at room temperature. The immunoreactive bands were detected with chemiluminescence substrate kit (ProteinSimple, Santa Clara, CA) under the Fluor Chem FC2 system. Antibodies were purchased from Abcam (anti- β actin, anti-AMPK, anti-ACC, anti-USP7, anti-YY1, anti-Mdm2 and anti-p53) or Cell signaling company (anti-p21, anti-p27 and anti-Cyclin D1).

Small interfering RNA (siRNA)

Cells were transfected with siRNA targeting the AMPK α 1 and α 2 subunit, the USP7 gene or a negative control (all siRNA oligos from QIAGEN, Valencia, CA) using Lipofectamine 2000 (Invitrogen, USA) as described by the manufacturer's instructions. Cell cultures were incubated for 18 hours with 100nM siRNA before metformin treatment.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 software. Numerical data are expressed as mean \pm SEM. Statistical significance is shown as * ($P < .05$), ** ($P < .01$), or *** ($P < .001$).

Results

Metformin treatment inhibited cell growth in a dose-dependent manner

To our knowledge, the effect of metformin on esophagus cancer cells remains unexplored. Thus, we selected two cell lines (Eca-109 and TE-1 cells) to investigate whether metformin has potential anti-proliferation roles. Both cell lines were treated with metformin at several concentrations. After 24 hours of treatment, growth was inhibited in a dose-dependent manner in both cell lines as determined by MTT and BrdU incorporation assays (Fig. 1A-1D). Moreover, these results suggested that the concentration of metformin at 5 mM was appropriate in both cell lines. Therefore, 5 mM of metformin was selected for the further analysis of genes expression in both cell lines.

Fig. 2. Metformin regulates expression of cell-cycle regulators. (A-B) The cell cycle phase of Eca-109 (A) and TE-1 (B) cells treated with vehicle control (Ctrl) or metformin (5mM) was analyzed by flow cytometry. Cells were labeled for 15 min with PI and immediately analyzed by flow cytometry. Histograms represent the percentage of cells in each phase of the cell cycle (G0/G1, S and G2/M). (C-D) mRNA (C) and protein (D) levels of p21, p27 and Cyclin D1 were determined by real-time PCR and western blot in Eca-109 cells treated with vehicle control (Ctrl) or metformin (5mM). (E-F) mRNA (E) and protein (F) levels of p21, p27 and Cyclin D1 were determined by real-time PCR and western blot in TE-1 cells treated with vehicle control (Ctrl) or metformin (5mM).

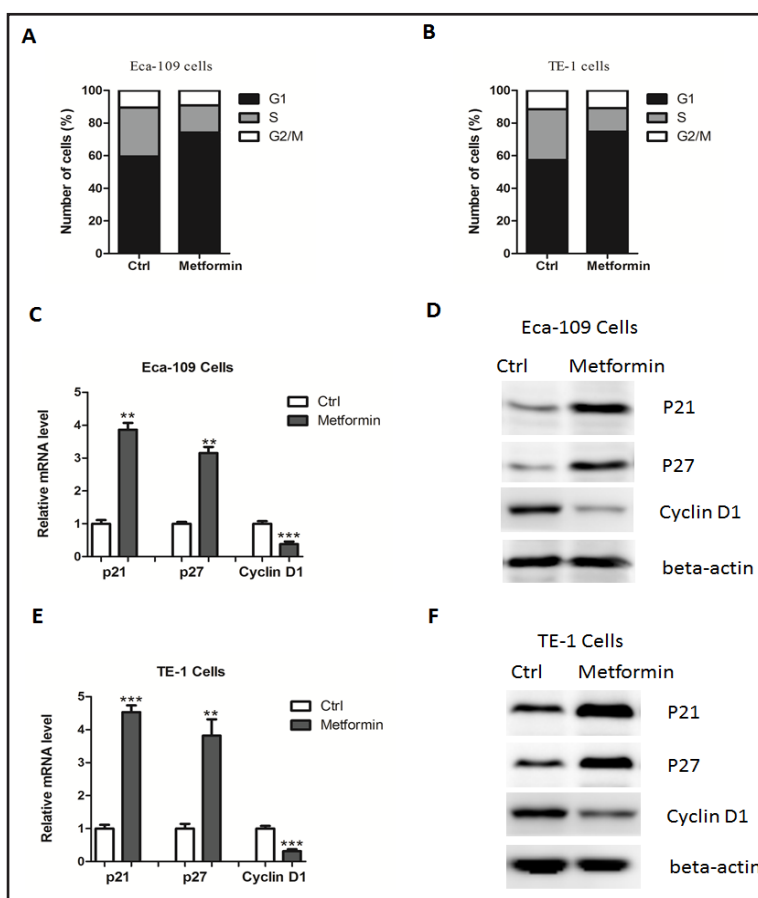
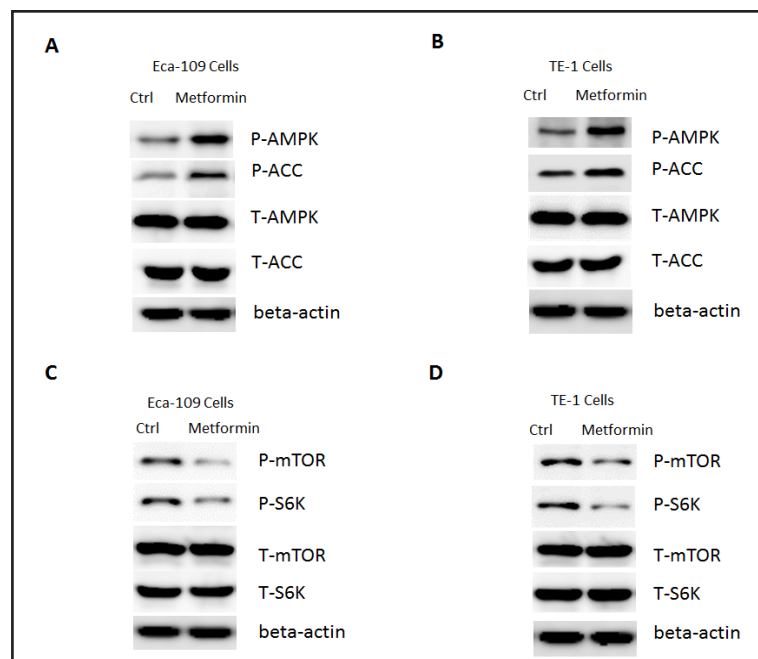


Fig. 3. Metformin induces AMPK activation in esophagus cancer cells. (A-B) Western blot analysis of phosphorylated AMPK and ACC in Eca-109 (A) and TE-1 cells (B). Contents of total AMPK, ACC and β -actin were used as loading controls. (C-D) Western blot analysis of phosphorylated mTOR and S6K in Eca-109 (C) and TE-1 cells (D). Contents of total mTOR, S6K and β -actin were used as loading controls.



Expression of p27, p21 and Cyclin D1 protein in metformin-treated cells

We speculate that growth inhibition in esophagus cancer cells might be caused by cell-cycle arrest following metformin treatment. Indeed, Eca-109 and TE-1 cells had a significantly increased percentage of cells in the G1/G0 phase and lower percentage of cells in the S phase,

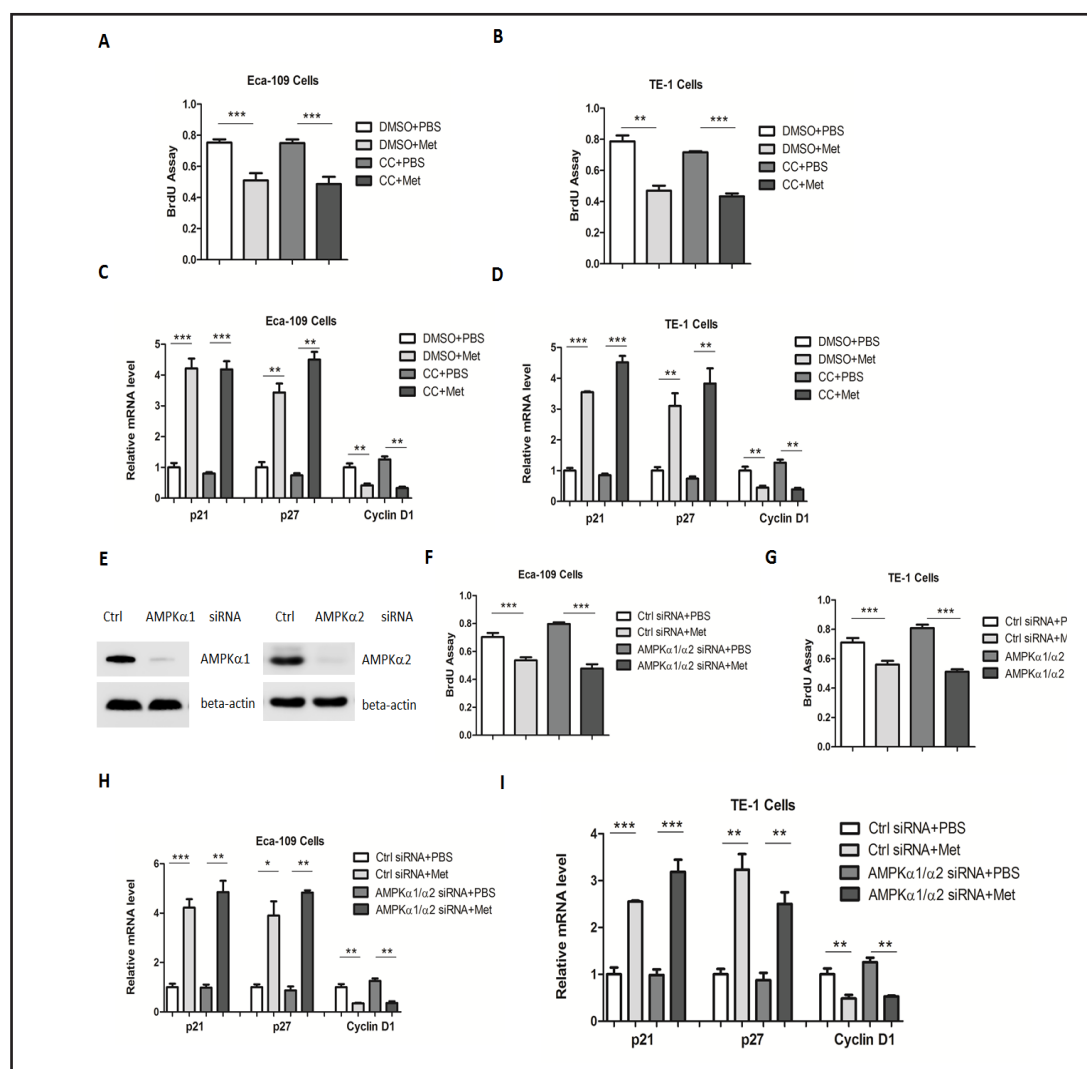


Fig. 4. The anti-proliferative action of metformin is independent of AMPK signaling activation. (A-B) Cell proliferation activity was measured by BrdU incorporation assays in Eca-109 (A) and TE-1 cells (B). Cells were pretreated with vehicle control (DMSO) or Compound C (CC) for 12 hours. (C-D) mRNA levels of p21, p27 and Cyclin D1 were determined by real-time PCR in Eca-109 (C) and TE-1 cells (D). (E) Western blot analysis of AMPK α 1 and AMPK α 2 in Eca-109 cells transfected with siRNA oligos against AMPK α 1/ α 2 or scramble siRNA (Ctrl). (F-G) Cell proliferation activity was measured by BrdU incorporation assays in Eca-109 (F) and TE-1 cells (G). Cells were pre-transfected with siRNA oligos against AMPK α 1 or scramble siRNA (Ctrl). (H-I) mRNA levels of p21, p27 and Cyclin D1 were determined by real-time PCR in Eca-109 (H) and TE-1 cells (I).

by 5mM metformin treatment (Fig. 2A-2B). Besides, we analyzed the expression contents of p21, p27 and Cyclin D1, which are known as key molecules involved in cell-cycle arrest. As shown in Fig. 2C-2F, expression levels of p21 and p27 were significantly increased in both cell lines. Besides, the contents of Cyclin D1 were markedly down-regulated in metformin-treated cells (Fig. 2C-2F).

Metformin up-regulates AMP kinase activity in esophagus cancer cells

Several studies have indicated that the antiproliferative effects of metformin involved the AMP kinase pathway [11]. Indeed, our western blot analysis indicated that metformin stimulated AMPK phosphorylation in Eca-109 and TE-1 cells (Fig. 3A-3B). Phosphorylated

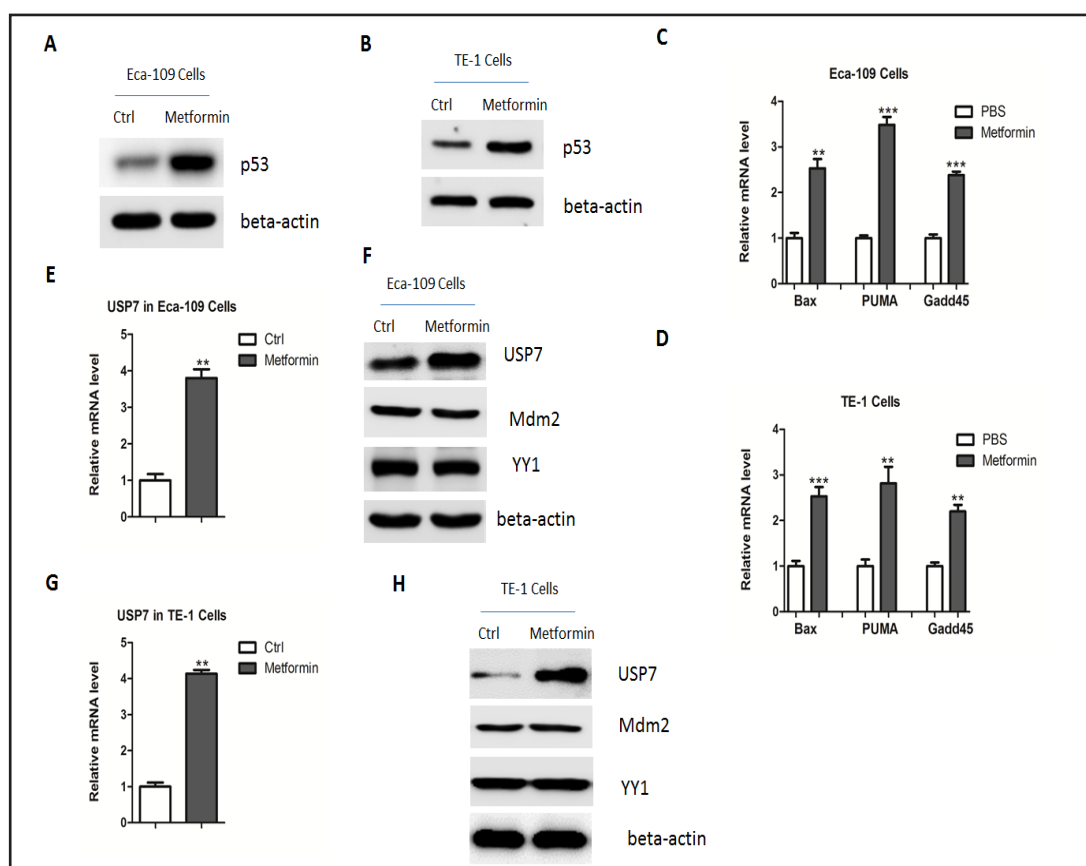


Fig. 5. Metformin increased USP7 expression in esophagus cancer cells. (A-B) Western blot analysis of USP7 expression in Eca-109 (A) and TE-1 (B) cells treated with vehicle control (PBS) or metformin. (C-D) Real-time PCR analysis of Bax, PUMA and Gadd45 expression in Eca-109 (C) and TE-1 (D) cells treated with vehicle control (Ctrl) or metformin. (E-F) mRNA (E) and protein (F) levels of USP7, YY1 and Mdm2 in Eca-109 cells treated with vehicle control (Ctrl) or metformin. (G-H) mRNA (G) and protein (H) levels of USP7, YY1 and Mdm2 in Eca-109 cells treated with vehicle control (Ctrl) or metformin.

ACC, a downstream target of AMPK, was also enhanced in cells treated with metformin (Fig. 3A-3B). Because AMPK activation inhibits energy-consuming pathways and protein synthesis, we observed that AMPK activation is associated with a decreased phosphorylation of mTOR and S6 kinase (Fig. 3C-3D).

Inhibition of AMPK pathway has little impact on the roles of metformin

We next test whether the inhibiting effect of metformin on cell proliferation is mediated by AMPK in esophagus cancer cells. As shown in Fig. 4A and 4B, pretreatment with the AMPK inhibitor (Compound C, CC) could not reverse the inhibitory effect of metformin on cell proliferation. Besides, expression levels of cell-cycle regulators were also inhibited by metformin in the presence of CC (Fig. 4C and 4D). To rule out possible nonspecific effects of CC, siRNA oligos-mediated knockdown of AMPK α 1 and α 2 subunit was performed (Fig. 4E). As a result, we also observed that metformin could regulate cell proliferation and expression levels of cell-cycle regulators in cells with AMPK α 1/ α 2 subunit depletion (Fig. 4F-4I). Therefore, our results suggest that the antiproliferative effects of metformin in esophagus is independent of AMPK signaling, although AMPK pathway was significantly activated by metformin treatment.

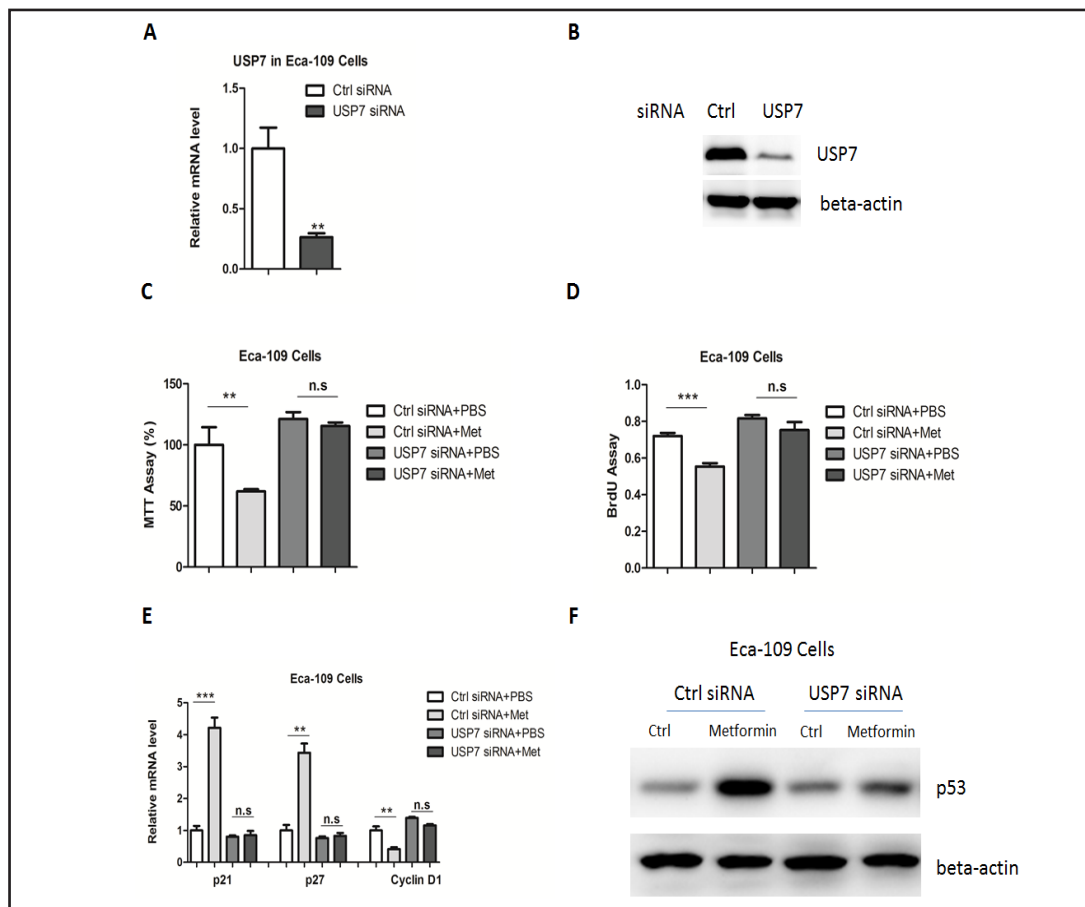


Fig. 6. USP7 deficiency reversed the anti-proliferative roles of metformin. (A-B) mRNA (A) and protein (B) levels of USP7 in Eca-109 cells transfected with siRNA oligos against USP7 or scramble siRNA (Ctrl). (C-D) Cell viability (C) and proliferation activity (D) were measured by MTT and BrdU assays in Eca-109 cells. (E) mRNA levels of p21, p27 and Cyclin D1 were determined by real-time PCR in Eca-109 cells. (F) Western blot analysis of p53 was determined in Eca-109 cells.

Metformin increases USP7 expression and p53 protein stability

Expression of several cell-cycle regulators including p21 and p27 is controlled by tumor suppressor p53. Given that these genes are regulated by metformin treatment in esophagus cancer cell, we analyzed p53 abundance in these cells. As shown in Fig. 5A and 5B, p53 protein contents were dramatically increased in cell treated with metformin, whereas its mRNA levels remain unchanged (data not shown). As a result, its down-stream target genes such as Bax, PUMA and Gadd45 were also up-regulated by metformin treatment (Fig. 5C and 5D).

Previous studies have demonstrated that p53 protein levels are regulated by several proteins such as USP7, Yin Yang 1 (YY1) and Mdm2 [21-23]. Here, we observed that USP7 expression was markedly increased after metformin treatment in Eca-109 and TE-1 cells (Fig. 5E-5H), while YY1 and Mdm2 expression remained unaffected by metformin (Fig. 5F and 5H).

siRNA against USP7 rescues cells from metformin-induced growth inhibition.

To determine if induction of USP7 by metformin is required for the anti-proliferative effect of the drug, we carried out experiments with USP7 knockdown using siRNA oligos (Fig. 6A and 6B). As a result, the siRNA rescued cells from the inhibitory effect of metformin in Eca-109 cells (Fig. 6C and 6D). Consistently, the inhibitory functions of metformin on the expression levels of cell-cycle regulators were also reversed by USP7 siRNA oligos (Fig. 6E).

Moreover, knockdown of USP7 reduced the protective effect of metformin on p53 protein accumulation (Fig. 6F). Similar results were also observed in TE-1 cells (data not shown). Therefore, our results highlight the USP7 as a new molecular target in anticancer therapy in response to metformin treatment.

Discussion

In the present study, we firstly explored the roles of metformin and its molecular mechanisms in esophagus cancer cells. Metformin was shown to inhibit cell proliferation in Eca-109 and TE-1 cells as evidenced by MTT and BrdU incorporation assays. Besides, metformin treatment led to a cell-cycle arrest, accompanied with up-regulation of p21 and p27 while repression of Cyclin D1 expression. Moreover, our results demonstrated that metformin activated AMP kinase activation as well as inhibition of mTOR signaling. Interestingly, inhibition of AMPK pathway using its antagonist or siRNA oligos did not affect the anti-proliferative roles of metformin. Indeed, Ben et al. revealed that antiproliferative action of metformin in prostate cancer cell lines is not mediated by AMPK, either [24]. They identified REDD1 (also known as DDIT4 and RTP801), a negative regulator of mTOR, as a new molecular target of metformin [24]. Therefore, together with their results, our data suggest that novel molecular targets in anticancer therapy in response to metformin treatment should be investigated in the future.

Here, we propose USP7 as a new molecular target of metformin in esophagus cancer cells. This is suggested by two lines of evidence. First, metformin treatment specifically up-regulates USP7 mRNA and protein levels in Eca-109 and TE-1 cells. Second, USP7 invalidation, using siRNA oligos, abrogated metformin inhibition of cell proliferation. Therefore, our observations show that USP7 might be a mediator of metformin-induced cell-cycle arrest in esophagus cancer cells and is one of the components of an important pathway for tumor suppression, although the mechanisms of USP7 up-regulation by metformin remain to be explored.

USP7, known as the herpes simplex virus associated ubiquitin-specific protease (HAUSP), is an evolutionarily conserved protein which was originally isolated as a binding partner of the herpes simplex virus protein Vmw110/ICP0 [25]. USP7 has critical roles in the p53 tumor suppressor pathway, whereby it stabilizes p53 protein [21]. Besides, USP7 was shown to be involved in tumorigenesis [26, 27]. For instance, mRNA and protein levels of USP7 are markedly decreased in several types of cancer [28, 29]. Moreover, dys-regulation of its expression and (or) activity contributes to oncogenic transformation and is crucial for cancer development [22, 29]. However, the roles of USP7 in esophagus cancer cells remain poorly understood, although we found that USP7 also positively regulated p53 abundance upon metformin treatment.

In conclusion, our results provide new evidence for a mechanism that may contribute to the antineoplastic effects of metformin suggested by recent population studies and justify further work to explore potential roles for it in esophagus cancer prevention and treatment.

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