Metformin Induces G1 Cell Cycle Arrest and Inhibits Cell Proliferation in Nasopharyngeal Carcinoma Cells

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ABSTRACT

It has been reported that metformin, a biguanide derivative widely used in type II diabetic patients, has antitumor activities in some cancers by activation of AMP-activated protein kinase (AMPK). But its role in nasopharyngeal carcinoma (NPC) is not known. Here, we reported for the first time that 1–50 mM of metformin in a dose- and time-dependent manner suppressed cell proliferation and colony formation in NPC cell line, C666-1. Further studies revealed that the protein level of cyclin D1 decreased and the percentage of the cells in G0/G1 phase increased by 5 mM metformin treatment. Metformin also induced the phosphorylation of AMPK (T172) in a time-dependent manner. Mammalian target of rapamycin complex 1 (mTORC1), which is negatively regulated by AMPK and plays a central role in cell growth and proliferation, was inhibited by metformin, as manifested by dephosphorylation of its downstream targets 40S ribosomal S6 kinase 1 (S6K1) (T389), the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (T37/46) and S6 (S235/236) in C666-1 cells. In a summary, metformin prevents proliferation of C666-1 cells by down-regulating cyclin D1 level and inducing G1 cell cycle arrest. AMPK-mediated inhibition of mTORC1 signaling may be involved in this process. Anat Rec, 294:1337–1343, 2011. © 2011 Wiley-Liss, Inc.

Key words: nasopharyngeal carcinoma; metformin; mammalian target of rapamycin complex 1; cell cycle; AMP-activated protein kinase; proliferation

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma that occurs in the epithelial lining of the nasopharynx. It is endemic in southern parts of China, Southeast Asia, the Mediterranean basin, and Alaska (McDermott et al., 2001). Epidemiologic investigations suggest that the etiology of NPC is associated with a complex interaction of genetic, viral, environmental, and dietary factors (Yip et al., 2008). Because of the anatomic location of nasopharyngeal tumors, they are traditionally treated by the combination of radiotherapy and adjuvant platinum-based chemotherapy, rather than surgery (Al-Sarraf et al., 1998), but the 5-year survival rate after this treatment is only about 50%–60%, and

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the rates of 5-year cumulative local relapse and distant metastasis are 20%–30% and 20%–25%, respectively (Dickson and Flores, 1985; Fandi et al., 1994). Because side effects such as hematologic, gastrointestinal, renal toxicity, and hearing impairment, infection, dry mouth, and taste alteration would suffer the patients (Al-Sarraf et al., 1998) therefore, newer therapies are needed.

Metformin, a biguanide derivative which is widely given to patients with type II diabetes, is inexpensive, well tolerated and causes decrement in insulin levels by 25%. Two population-based observational studies have demonstrated that cancer incidence and mortality were reduced in diabetics treated with metformin (Bowker et al., 2006; Evans et al., 2005). Metformin may have direct effects on cancer cells through the activation of AMP-activated protein-kinase (AMPK). Furthermore, it is able to inhibit mammalian target of rapamycin complex 1 (mTORC1), a down-stream effector of AMPK and is expected to decrease oncogenic proliferation in some cancers (Dowling et al., 2007; Isakovic et al., 2007; Jiang et al., 2008; Zakikhani et al., 2006).

The cell line of C666-1, which has been established from undifferentiated NPC, consistently carries the Epstein-Barr virus (EBV) in long-term cultures and expresses EBV-encoded RNAs. It is positively stained for cytokeratin, an epithelial marker. In addition, it expresses EBV nuclear antigen (EBNA), latent membrane proteins 1(LMP1) and LMP2 transcripts and thus resembles the EBV latency II pattern (Cheung et al., 1999). The LPM 1 and LMP 2A activate the phosphatidylinositol 3′-OH kinase (PI3-K)/protein kinase B (Akt) pathway, which is deregulated in various human malignancies. Recently, metformin has been shown to inhibit cell growth in breast cancer cells through activation of AMPK and inhibition of translation initiation (Dowling et al., 2007; Zakikhani et al., 2006). It may also prevent cell proliferation in prostate and colon cancer cells (Zakikhani et al., 2006) and reduce the risk of human pancreatic cancer in diabetic patients (Li et al., 2009). However, the effects of metfomin on the NPC have not been reported. Considering that Akt is frequently activated in NPC cell lines and in NPC tissues (Morrison et al., 2004; Yip et al., 2008), and that the potential involvement of Akt/mTORC1 signaling in NPC carcinogenesis (Ma et al., 2009), in current study we examined the effect of metformin on mTORC1 signaling, cell cycle progression, and cell proliferation in NPC cell line C666-1.

MATERIAL AND METHODS

Reagents

Metformin (1,1-dimethylbiguanide hydrochloride) was purchased from Sigma-Aldrich (Sigma-Aldrich). Antibodies against AMPK, phospho-AMPK (T172), phospho-S6K (T389), phospho-S6 (S235/236), phospho-Akt (S473), 4E-BP1, phospho-4EBP1(T37/46), and cyclin D1 were purchased from Cell Signaling (Cell Signaling Technology); anti-S6, S6K1, and β-actin antibody were from Santa Cruz Biotech (Santa Cruz Biotechnology). All other reagents were purchased from Sigma-Aldrich, unless otherwise specified.

Cells and Cell Culture

The undifferentiated human NPC cell line, C666-1 was a gift from Professor Yao Kaitai (Cancer Research Institute of Southern Medical University, Guangzhou, China). The cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and incubated in a 37°C humidified atmosphere containing 95% air and 5% CO_2. Culture was trypsinized upon confluence and propagated to passage 2 before being subcultured into 12, 6, or 96 well plates for further experiments.

Cell Proliferation Assay

Cell proliferation was determined using WST-8 cell proliferation assay kit (Dojindo Molecular Technologies, Japan) according to manufacture's instructions. Briefly, 2.5×10^3 cells/well were seeded in a 96-well flat-bottomed plate, grown at 37°C for 24 hr. Cells were subsequently treated with metformin at increasing concentrations (0, 1, 2, 5, 10, and 20 mM) for 48 hr or at concentration of IC50 (5 mM) for 24, 48, or 72 hr in the presence of 10% FBS. Then 10 μL WST-8 dye was added to each well and incubated at 37°C for 4 hr. Finally, the absorbance of each well was determined at 450 nm using a microplate reader (Bio-Tek instruments) (Hou et al., 2007). The percentages of surviving cells from each group relative to control were defined as proliferation rate, using the following formula: % proliferation rate = A450 of treated cells/A450 of control cells × 100%. All experiments were performed in triplicate.

Flow Cytometric Analysis

Flow cytometric analysis was performed as described previously to define the cell cycle distribution for metformin-treated and untreated cells (Liu et al., 2001). Briefly, cells grown in six well plates (2×10^5 cells/well), exponentially growing C666-1 cells were synchronized at the G1/S boundary after starvation with basal medium for 24 hr, followed by incubation in the presence or absence of 5 mM metformin for 24, 48, and 72 hr. At the indicated intervals, cells were harvested by trypsinization, fixed with 70% ethanol and measured following the instruction of the cell cycle detection kit (KEY GEN, Nanjing, China). The cell cycle distribution was analyzed by flow cytometry (FACSCalibur TM BD). DNA histograms were measured using FACS Express software and the percentage of G0/G1, S, and G2/M cells were calculated.

Colony Formation Assay

Colony formation assays were performed as described previously (Liu et al., 2007). Briefly, cells were seeded into six-well plates in triplicates at a density of 500 cells/well in 2 mL medium containing 10% FBS. After 24 hr, cultures were replaced with fresh medium containing 10% FBS (control) or same medium containing 1, 2, or 5 mM metformin in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and grown for 2 weeks. The formed colonies were stained with a solution containing 0.5% crystal violet and 25% methanol for 15 min, followed by three rinses with water to remove excess dye. The colony numbers were counted by 1-D gel analysis software QUANTITY ONE (Bio-Rad, Bio-Rad Laboratories, CA). The percentage of colony formation of C666-1 cells from each group relative to control was defined as colony formation rate, using the following formula: % colony formation rate = number of colony of treated cells/number colony of control cells × 100%. All experiments were performed in triplicate.

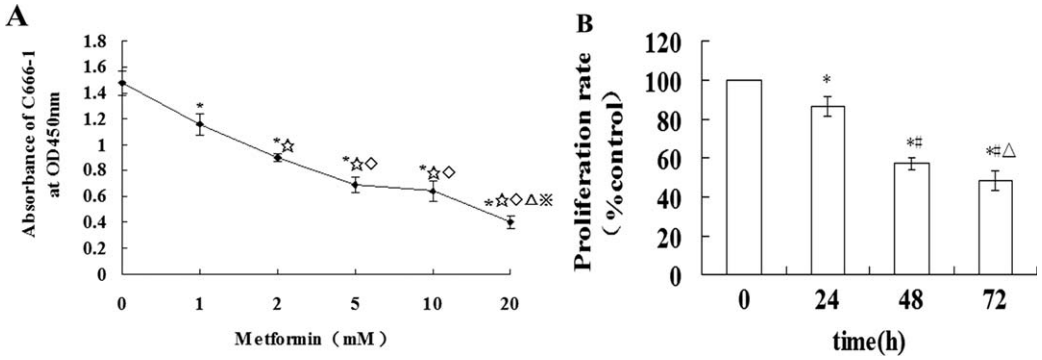

Fig. 1. Metformin dose- and time-dependently suppresses C666-1 proliferation. **A:** Human nasopharyngeal cancer (NPC) C666-1 cells were treated with Metformin at increasing concentrations (0, 1, 2, 5, 10, 20 mM) for 48 hr in the presence of 10% FBS. Cell proliferation was detected by WST-8 assay. $^{*}P < 0.05$ vs. 0 mM; $^{\star}P < 0.05$ vs. 1 mM; $^{\diamond}P < 0.05$ vs. 2 mM; $^{\triangle}P < 0.05$ vs. 5 mM; $^{\ast}P < 0.05$ vs. 10 mM. **B:** C666-1 cells were treated with fresh medium containing 10% FBS as control, or same medium containing 5 mM Metformin for 24, 48, or 72 hr, and cell proliferation was detected by WST-8 assay. $^{*}P < 0.05$ vs. 0 hr; $^{\#}P < 0.05$ vs. 24 hr; $^{\triangle}P < 0.05$ vs. 48 hr. Proliferation rates were calculated as described in "Material and Methods" section. Data show the representative of four independent experiments. Bars, SD.

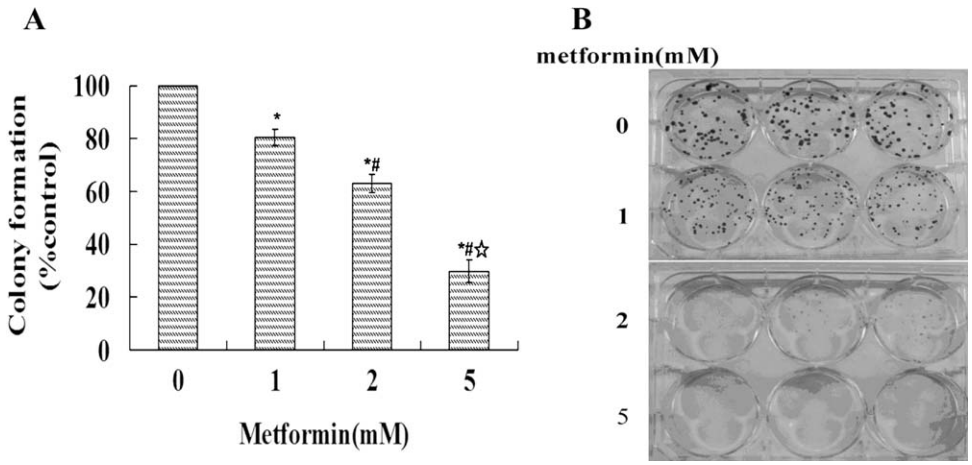

Fig. 2. Metformin reduces C666-1 cell colony formation *in vitro*. C666-1 cells were seeded into six-well plates (1,000 cells/well) in triplicates. After 24 hr, the culture medium was replaced with fresh medium containing 10% FBS as control, or same medium containing 1, 2, or 5 mM metformin, and the culture medium was changed once every 3 days for 2 weeks. **A:** the bar graph was obtained by calculating the percentages of colony numbers relative to controls, defined as 100%, measured by 1-D gel quantity software QUANTITY ONE. $^{*}P < 0.05$ vs. 0 mM; $^{\#}P < 0.05$ vs. 1 mM; $^{\star}P < 0.05$ vs. 2 mM. **B:** The pictures of six-well plates with colonies were taken by a digital camera on day 14. Data show the representative of three independent experiments. Bars, SD.

Western Blotting Analysis

Protein expression levels were determined by western blot analysis as previously described (Li et al., 2009). Briefly, after treatment, cells were lysed immediately in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenol blue) for 5 min at 95°C. Cell lysates were analyzed by SDS/PAGE and transferred electrophoretically to nitro cellulose membrane (Bio-Rad Corp Hercules, CA). Blots were probed with specific antibodies and immunoreactive proteins were revealed by the enhanced chemiluminescence kit (Santa Cruz Biotechnology, CA).

Statistical Analysis

All experimental results reported here were from at least three separate experiments. The data were performed by One-way ANOVA using SPSS version 13.0 (SPSS, Chicago, USA). Summary statistics were expressed as mean ± standard deviations, unless otherwise stated. In all statistical analyses, a P value <0.05 was considered statistically significant, and all P values were two-sided.

RESULTS

Metformin Inhibits C666-1 Cell Proliferation and Reduces Colony Formation *In Vitro*

Firstly, we evaluated the effect of metformin on cell proliferation of C666-1 cells. It was revealed that metformin inhibited proliferation of C666-1 cells in a dose- and time-dependent manner (Fig. 1A). After 48 hr of incubation, the IC50 value of C666-1 cells was 5 mM. Compared with the untreated cells, the proliferation rate of C666-1 cells was 86.47%, 57.06%, and 48.69% respectively after 5 mM metformin treatment for 24, 48, and 72 hr (Fig. 1B).

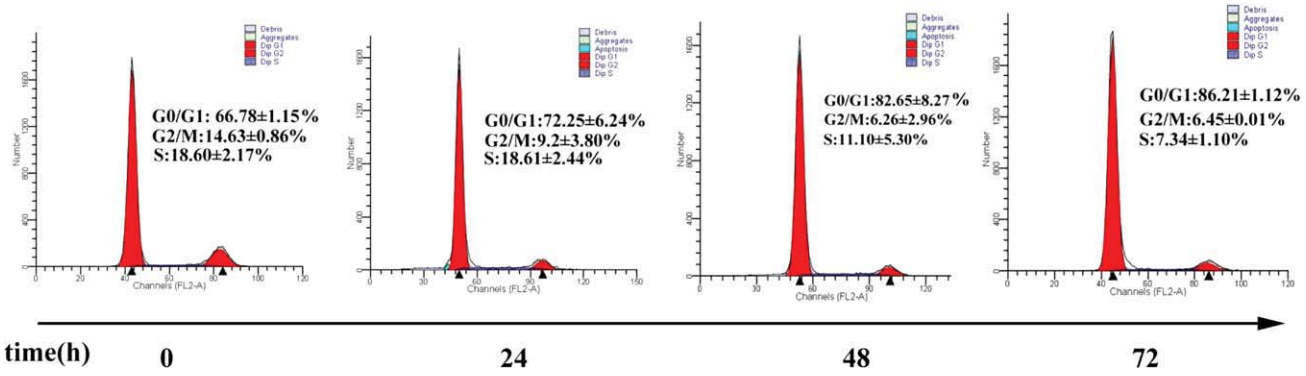

Fig. 3. Effect of metformin on cell cycle progression in C666-1 cells. C666-1 cells were exposed to 5 mM metformin for the indicated time. Both adherent and nonadherent cells were harvested. Cell cycle distributions were analyzed by flow cytometry as described in the "Material and Methods" section.

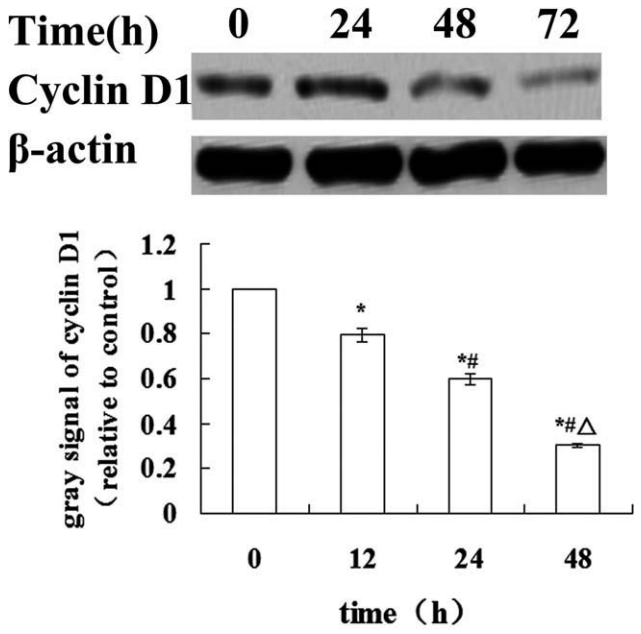

Fig. 4. Metformin down-regulates level of cyclin D1 in C666-1 cells. C666-1 cells were incubated with 5 mM metformin for 24, 48, and 72 hr, cell lysates were subjected to Western blot analysis with antibodies against cyclin D1 and β-actin. $^{*}P < 0.05$ vs. 0 hr; $^{\#}P < 0.05$ vs. 12 hr; $^{\triangle}P < 0.05$ vs. 24 hr. Data show the representative of three independent experiments.

We then examined the ability of C666-1 cells to form colonies on 6-well cell culture plates in the presence or absence of metformin for 2 weeks. Metformin reduced colony formation at concentrations as low as 1 mM (Fig. 2A). The number of colonies formed was reduced in a dose-dependent manner. At the concentration of 5 mM, colony number was reduced to 30% of untreated cells (Fig. 2B).

Metformin Induces G1 Cell Cycle Arrest and Down-Regulates Level of Cyclin D1 in C666-1 Cells

To further determine if metformin inhibits cell proliferation by induction of cell cycle arrest, C666-1 cells were exposed to 5 mM metfromin for 24–72 hr and cell cycle distribution was evaluated by flow cytometric analysis. It was revealed that metformin treatment resulted in partial G1 cell cycle arrest, and the number cells in G1 phase increased in a time-dependent manner (Fig. 3).

We next examined the effect of metformin on protein level of cyclin D1, a critical factor for G1 checkpoint. As expected, metformin treatment time-dependently reduced the level of cyclin D1 in C666-1 cells (Fig. 4).

Metformin Activates AMPK and Inhibits mTORC1 and Akt Signaling in C666-1 Cells

It has been reported that metformin inhibits proliferation by activation of AMPK in some cancer cells. We examined the effects of metformin on AMPK in C666-1 cells. Activity of AMPK was evaluated by monitoring the phosphorylation of AMPK on Thr172, which is required for activation of AMPK (Kahn et al., 2005). We found that 5 mM metformin activated AMPK time-dependently, when compared with controls (Fig. 5A). We next examined the effects of metformin on mTORC1 signaling, which is negatively regulated by AMPK and a major regulator of translation initiation. We assessed the phosphorylation status of its two direct downstream targets as mTORC1 readout, 40S ribosomal S6 kinase 1 (S6K1) (T389) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (T37/46). Metformin time-dependently decreased the phosphorylation of S6K1 (T389) and consequently reduced the phosphorylation of ribosomal S6 protein (S235/236), which is a component of the 40S ribosomal subunit (Fig. 5B). Three isoforms of 4E-BP1 have been detected. Metformin treatment induced dephosphorylation of 4E-BP1, as manifested by shift of 4E-BP1 isoforms detected by anti-4E-BP1 antibody, and decrease of 4E-BP1 phosphorylation detected anti phospho-4E-BP1 (T37/46) antibody (Fig. 5C). Moreover, the phosphorylation of Akt (S473), a critical factor for cell survival, was also reduced by metformin treatment (Fig. 5D).

DISCUSSION

NPC is a highly invasive and metastatic head and neck cancer prevalent in Southeast Asia with a high incidence

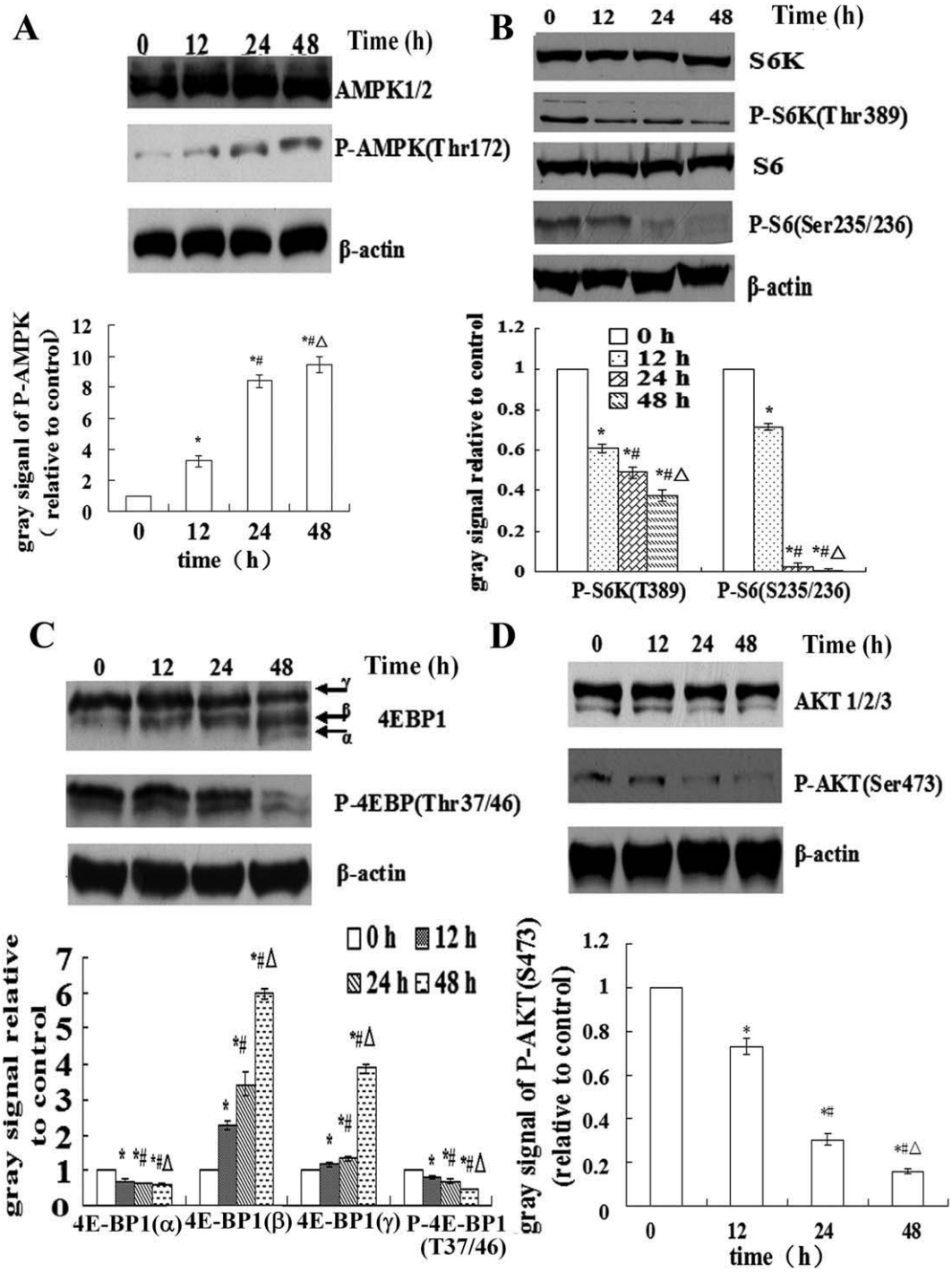

Fig. 5. Metformin induces the phosphorylation of AMPK and reduces phosphorylation of S6K, S6, 4E-BP1, and Akt in C666-1 cells. C666-1 cells were treated with 5 mM metformin for 12, 24, and 48 hr in RPMI1640 containing 10% FBS. Cell lysates were resolved by 15% (4E-BP1) or 10% (all other proteins) SDS-PAGE. Immunoblot analysis was carried out using antibodies against. **A:** AMPK, phospho-AMPK1(T172), $^{*}P < 0.05$ vs. 0 hr; $^{\#}P < 0.05$ vs. 12 hr; $^{\triangle}P < 0.05$ vs. 24 h. **B:** S6K1, phospho-S6K1 (T389), S6, phospho-S6 (S235/236), $^{*}P < 0.05$ vs. 0 hr; $^{\#}P < 0.05$ vs. 12 hr; $^{\triangle}P < 0.05$ vs. 24 hr. **C:** 4E-BP1, phospho-4E-BP1(T 37/46) , $^{*}P < 0.05$ vs. 0 hr; $^{\#}P < 0.05$ vs. 12 hr; $^{\triangle}P < 0.05$ vs. 24 hr. **D:** Akt1/2/3, phospho-AKT(S473), and β-actin. $^{*}P < 0.05$ vs. 0 hr; $^{\#}P < 0.05$ vs. 12 hr; $^{\triangle}P < 0.05$ vs. 24 hr. Data show the representative of three independent experiments.

rate of 15–50/100,000 persons/year (comparable with that of pancreatic cancer in the US). Over 50%–70% of NPC patients present with advanced disease (stages II b–IV) with lymph node invasion or metastasis at the time of diagnosis. The rate of distant metastasis in recurrent NPC patients is up to 37% (Chan et al., 2002; Hong et al., 2010; Isobe et al., 1998; Skinner et al., 1991; Spano et al., 2003). Because of the special location of NPC, radiotherapy

combined with chemotherapy is the traditional treatment, but side effects will suffer the asthenic patients.

As a drug used for the treatment of type II diabetes, the majority of studies on the effects of metformin that have been carried out involved insulin signaling and metabolism, such as muscle, adipose tissue, and liver (Kolak et al., 2007; Musi et al., 2002; Shaw et al., 2005). More and more data suggest that metformin has inhibitory effect on cell proliferation of human cancer cells such as breast cancer, prostate, and colon cancer cells. Metformin attenuates the stimulatory effect of a high-energy diet on *in vivo* Lewis lung LLC1 carcinoma growth in C57BL/6J mice (Algire et al., 2008). We found no reports about the effects of metformin on NPC.

In this study, we demonstrated that cell proliferation and colony formation of C666-1 was inhibited by metformin in a dose- and time-dependent manner (Fig. 1A). Cell cycle analysis revealed that metformin induced G1 cell cycle arrest in C666-1 cells (Fig. 3). Further studies showed that protein level of cyclin D1, a protein which is frequent amplifiable and altered expression in a variety of tumors including NPC (Lamb and Ewen, 2003; Lin et al., 2006), decreased markedly by metformin treatment (Fig. 4). Decreased level of cyclin D1 might be responsible for G1 cell cycle arrest and growth inhibition induced by metformin (Diehl, 2002).

The LPM 1 and LMP 2A of EBV activate the PI3K/Akt pathway, one of the most common molecular alterations in various human malignancies. Consequently, the mTORC1 signaling, a downstream of Akt, which plays a central role in cell growth and proliferation, may contribute to NPC carcinogenesis. To determine whether mTORC1 signaling is involved in metformin-mediated inhibition of cell proliferation in C666-1 cells, the effects of metformin on mTORC1 signaling in C666-1 cells were examined. We demonstrated that metformin inhibited mTORC1 signaling, as manifested by dephosphorylation of S6K1 (T389), 4E-BP1 (T37/46), and S6 (S235/236) in C666-1 (Fig. 5B–C). Metformin caused a shift of the phosphorylation state of 4E-BP1 from the hyperphosphorylated form to the hypophosphorylated form of the protein. Hypophosphorylated 4E-BP1 inhibits translation initiation by binding to eIF4E with high affinity. The binding of 4E-BP1 to eIF4E prevents the formation of the eIF4F complex, which is the rate-limiting step in translation initiation (Beretta et al., 1996). Metformin also induced phosphorylation of AMPK (T172) (Fig. 5A) and dephosphorylation of Akt (S473) (Fig. 5D). Inhibition of mTOR signaling often induces a G1 cell cycle arrest that correlates with down-regulation of cyclin D1 levels in some cell types (Beretta et al., 1996). We found that metformin induced G1 cell cycle arrest and decreased levels of cyclin D1. It is suggested that inhibition of mTOR signaling may be involved in prevention of cell proliferation by metformin in C666-1 cells. The significance of metformin-induced inhibition of mTOR signaling in C666-1 and whether it is related to cellular apoptosis or necrosis should be further investigated.

In a summary, our findings demonstrate that metformin induces G1 cell cycle arrest and inhibits cell proliferation and colony formation in NPC C666-1 cell line. The activation of AMPK and subsequently inhibition of mTORC1 signaling, reduction of cyclin D1 level, and dephosphorylation of Akt (S473) may be involved in this process. Taken together, results from this study provide the basis for the potential cocktail therapy which will include radiotherapy, chemotherapy, together with long-term metformin use for EBV-positive NPC.

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