Metformin suppresses hepatocellular carcinoma cell growth through induction of cell cycle G1/G0 phase arrest and p21[CIP] and p27[KIP] expression and downregulation of cyclin D1 *in vitro* and *in vivo*

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Abstract. Metformin is used as a first-line therapy for type 2 diabetes, with reports of its usefulness for the prevention and control of several types of cancers. This study investigated the effects of metformin on hepatocellular carcinoma (HCC). The human HCC cell lines HepG2 and PLC/PRF/5 were cultured and treated with metformin or 5-aminoimidazole-4-carbox-amide ribonucleoside (AICAR), an activator of adenosine monophosphate (AMP)-activated protein kinase. Changes in cell viability and cell cycle distribution were evaluated by MTT and flow cytometry, respectively. Apoptosis was assessed by fluorescent-dye staining. An HCC model was established in 6- to 8-week-old BALB/c-nu mice by subcutaneous injection of PLC/PRF/5 cells. After 1 week, mice were treated intra-gastrically with metformin or vehicle. Tumor xenograft tissues were examined using immunohistochemistry for evaluation of the the expression of cyclin D1, p21[CIP] and p27[KIP]. HCC cells and tissues from the *in vitro* and *in vivo* experiments, respectively, were subjected to protein extraction and western blotting. We found that metformin treatment reduced HCC cell viability in a dose-dependent manner similar to AICAR treatment. In addition, metformin treatment induced HCC cell cycle arrest at G1/G0 phase and apoptosis. Intragastric treatment of the mouse PLC/PRF/5 cell xenograft model with metformin showed that metformin not only blocked tumor progression, but also reduced tumor morbidity. Treatment with metformin upregulated the expression of p21[CIP] and p27[KIP], but downregulated cyclin D1 levels, both *in vitro* and *in vivo*. Metformin treatment also upregulated the expression of phos-phorylated AMPK protein in xenograft tissues. These findings indicate that metformin warrants further evaluation as a novel therapeutic and preventive strategy against HCC.

Introduction

Liver cancer is a significant health issue, particularly in developing countries where it is inevitably fatal (1,2), and worldwide it is the third most common cause of death from cancer. In 2008, an estimated 749,000 newly diagnosed liver cancer cases and 695,000 deaths were reported (1). The 5-year survival rate of these patients is between 6.5 and 8.3% (2).

The most common form of liver cancer is hepatocellular carcinoma (HCC). It is well accepted that the primary causes of HCC are nonspecific cirrhosis, steatohepatitis and viral hepatitis, and according to clinical data the incidence of HCC and the associated mortality continue to increase (3,4). However, due to the lack of specific early symptoms and symptoms that overlap with other liver diseases, HCC is often diagnosed at an advanced stage when curative surgical resection is no longer viable. At this advanced stage of disease, liver transplantation may be an option, but it is difficult to carry out due to limited donor livers, fierce immune rejection and huge expenditure. Chemotherapy or radiation treatment may be helpful for some patients, but are usually palliative. Many HCC patients have concomitant liver cirrhosis or other liver diseases, and the above therapies have only limited applications. Thus, novel approaches for HCC patients are urgently needed to provide both preventive and curative strategies.

Towards this end, the drug metformin, commonly used to suppress glucose production by the liver (5), has recently been targeted as a chemoprophylatic agent and for treatment of various human cancers. Belonging to the biguanide class of drugs, metformin is used as a first-line therapy for diabetes mellitus type 2 (adult-onset diabetes), but epidemiological studies have shown that when used against type 2 diabetes

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it also reduced the risk of cancer (6,7). Moreover, according to several clinical trials, type 2 diabetes is an independent risk factor for HCC development, and use of metformin appeared specifically to be associated with a lower risk of liver cancer (8,9). Features of diabetes such as insulin resistance, glycometabolic disorder, and hepatic lipid accumulation may also occur in HCC, precancerous nonalcoholic fatty liver disease and liver cirrhosis (10-12). Metformin can increase insulin sensitivity and reduce serum levels of glucose, lipotoxicity and inflammatory cytokines, which may in turn help to suppress oncogenesis (13,14).

Metformin inhibits glucose production in the liver and improves hyperglycemia via activation of AMP-activated protein kinase (AMPK), an enzyme with an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fat. AMPK is a serine/threonine kinase, which for its activation requires phosphorylation by upstream kinases at a specific threonine residue (Thr-172) (15-19). AMPK has been considered a target for cancer therapy or prophylaxis.

Previous studies have reported that metformin is able to inhibit the viability of ovarian cancer cell lines (8) and to suppress renal carcinoma *in vivo* (20). In experiments with hamsters, the drug also showed preventive activity for pancreatic cancer (21). The multiple effects of metformin in cancers such as breast, ovarian, prostate and colon cancers, appear to act through cell cycle arrest, apoptosis, prevention of angiogenesis and enhancement of chemosensitivity (22-24) through AMPK-dependent or -independent pathways (25,26).

In the present study, we investigated the effects of metformin on HCC *in vitro* and *in vivo*, and the underlying molecular events.

Materials and methods

Cell culture and cell viability assay. The human HCC cell lines HepG2 and PLC/PRF/5 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin and 100 U/ml streptomycin, in a humidified incubator at 37°C with 5% CO_2 and 95% air.

To assess changes in cell viability, we performed a methyl thiazolyl tetrazolium (MTT) assay. Briefly, HepG2 and PLC/PRF/5 cells were seeded at a density of $3x10^3$ cells/well in 96-well plates and cultured for 24 h. The medium was then replaced with RPMI-1640 medium supplemented with 2.5, 5.0, 10 or 20 mM metformin (American Biomol, Farmingdale, NY, USA) or 100, 250, 500 or 1,000 μM 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; Cell Signaling Technology, Inc., Danvers, MA, USA). Following the above treatments, MTT (Sigma Chemicals, St. Louis, MO, USA) at 5 mg/ml in phosphate-buffered saline (PBS) was added to each well, and the cells were incubated for an additional 4 h. The supernatant was discarded, and 150 μl of dimethyl sulfoxide was added to each well for 10 min to dissolve the formazan crystals. The optical density levels of the cell cultures were measured spectrophotometrically using a dual beam microplate reader at 490 nm.

Flow cytometric assessment of cell cycle distribution. Cells ($5x10^5$) were grown and treated with metformin (10 mM) or AICAR (500 μM) for 72 h. The cells were trypsinized and fixed in 70% ethanol overnight. The following day, the cells were stained with propidium iodide (50 μg/ml; Sigma) for 30 min at 4°C and then analyzed with a flow cytometer. The data were further analyzed using Windows Multiple Document Interface for Flow Cytometry (WinMDI) software.

DAPI staining to visualize apoptosis. To assess apoptosis after drug treatment, cells were stained fluorescently with 4',6-diamidino-2-phenylindole (DAPI) to detect nuclear condensation and fragmentation. Cells ($1x10^5$) were cultured in medium with or without metformin (10 mM) or AICAR (500 μM) for 72 h. The cell culture medium was then discarded, and 1 μg/ml of DAPI solution (Roche, Shanghai, China) was added to the cell culture and incubated for 15 min in the dark. After that, the cells were washed with methanol and examined under a fluorescence microscope.

Nude mouse tumor model. The Ethics Committee for Animal Experimentation of the Shantou University Medical College approved the animal experiments. BALB/c-nu mice were obtained from SLC (Guangzhou, China). Freshly cultured (human hepatoma) PLC/PRF/5 cells ($1x10^6$) were injected subcutaneously into 6- to 8-week-old male nu/nu mice at 4 sites in both flanks (x2). After 1 week, metformin dissolved in PBS was intragastrically administered at 250 mg/kg body weight/day. The control group received PBS only. Tumor formation and growth were measured every 2 days when xenografts were visible. Mouse body weight was measured the day before intragastric administration and was repeatedly measured at week 7. Three hours after intragastric drug administration, serum glucose was measured with a blood glucose monitor.

Immunohistochemistry. Immunohistochemistry was performed on tumor xenograft tissues for assessment of gene expression. After animals were euthanized, tumor xenograft tissues were resected for tissue processing (fixed in 10% buffered formalin, embedded in paraffin, and cut into 4-mm sections). For immunohistochemistry, the sections were deparaffinized, rehydrated, and then incubated in 3% (v/v) hydrogen peroxide for 10 min at room temperature. The sections were subjected to antigen retrieval by heating in 10 mM (pH 6.0) citrate buffer for 20 min in a microwave oven, incubation in 20% normal serum for 50 min at room temperature, and further incubation with a rabbit anti-cyclin D1, anti-cyclin E or anti-p27[KIP] antibody (Maixin Bio, Fuzhou, China) overnight at 4°C. The following day, the sections were washed with PBS thrice and then incubated with a secondary antibody, followed by incubation with streptavidin-peroxidase solution (both from Vector Laboratories, Burlingame, CA, USA). The color reaction was performed using 3,39-diaminobenzidine (DAB; Sigma) and counterstaining with hematoxylin. The negative control was carried out omitting the primary antibody in the tissue sections.

The stained sections were reviewed and scored under a light microscope independently by 2 investigators. The images of the immunostained tissue sections were scanned with an Olympus charged coupled device (CCD) camera
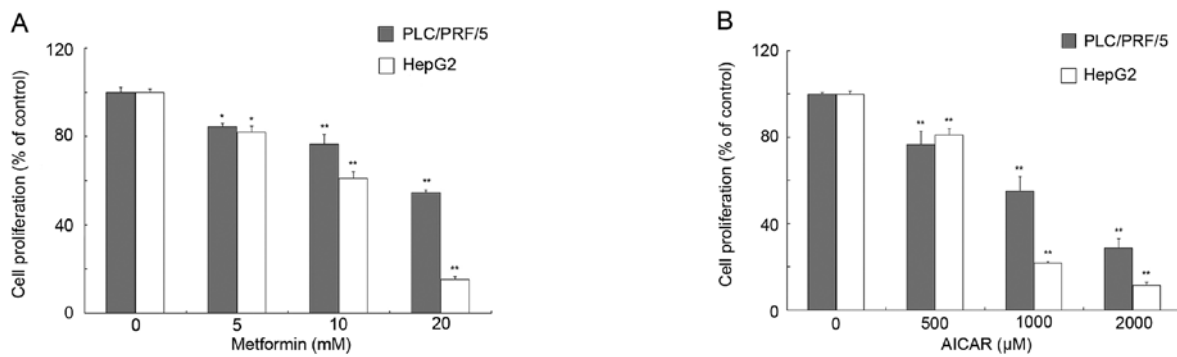
Figure 1. Effects of metformin and AICAR on induction of HCC cell viability. (A) PLC/PRF/5 and HepG2 cells were exposed to different concentrations of metformin (0, 5, 10 or 20 mM) for 72 h. The cells were then subjected to an MTT assay of cell viability. (B) PLC/PRF/5 and HepG2 cells were exposed to different concentrations of AICAR (0, 500, 1,000, 2,000 μM) for 72 h. The cells were then subjected to an MTT assay of cell viability. Data from 3 independent experiments are expressed as means ± SEM. *P<0.05 and **P<0.01 compared to the control. AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside. HCC, hepatocellular carcinoma.

and analyzed by ImageJ (National Institutes of Health). The positive expression rate was calculated as the positive cell number/total cell number in a field. The mean of 5 low-power fields was used.

Protein extraction and western blotting. Cultured tumor cells and HCC tissues were lysed in RIPA buffer (Bocai, Shanghai, China) supplemented with a protease and phosphatase inhibition mixture (10 μl), NaF (10 μl of 100 mM), sodium orthovanadate (10 μl of 100 mM) and phenylmethylsulfonyl fluoride (PMSF; 10 μl of 100 mM).

For western blotting, equal amounts of the protein samples were resolved via SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 10% non-fat dried milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C. The next day, the membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma) for 2 h at room temperature.

The peroxidase activity was detected using enhanced chemiluminescence (ECL; Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to X-ray film (Kodak, Guangzhou, China). The positive protein bands were quantitated using laser densitometry.

Statistical analyses. Data are expressed as means ± standard error and were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The statistical difference between two measurements was calculated using the Student's t-test. Results were considered statistically significant at P<0.05.

Results

Effects of metformin on HCC cell viability. To determine the effects of metformin on HCC cell viability, the HCC cell lines PLC/PRF/5 and HepG2 were treated with different concentrations of metformin or the AMPK activator AICAR for 72 h *in vitro*. The data showed that metformin significantly inhibited the viability of HCC cells in a dose-dependent

manner (Fig. 1A), and AICAR was also able to reduce HCC cell viability (Fig. 1B).

Metformin induces cell cycle arrest at the G0/G1 phase in HCC cells. Treatment of HCC cells with 10 mM metformin for 72 h induced HCC cell cycle arrest at the G1/G0 phase (58.99% compared with 69.28% in HepG2 cells and 50.14% compared with 65.96% in PLC/PRF/5 cells). Moreover, treatment with 500 μM AICAR for 72 h replicated the metformin-induced cell cycle arrest phenomenon in these 2 HCC cell lines (Fig. 2).

Molecularly, treatment of HepG2 cell lines with metformin or AICAR induced expression of p21[CIP] and p27[KIP], but downregulated cyclin D1 expression (Fig. 2). These data suggest that metformin and AICAR modulated expression of cyclin D1, p21[CIP] and p27[KIP] to arrest the cell cycle at the G0/G1 phase, and that AMPK activation may be responsible for this effect.

Metformin induces apoptosis of HCC cells in vitro. We next assessed the effect of metformin on induction of apoptosis of tumor cells. Treatment of HepG2 cell lines with 10 mM metformin for 24 h induced apoptosis. Apoptotic cells with condensed and fragmented nuclei were visualized by staining with DAPI (Fig. 3).

Association of metformin with AMPK activation in vitro. We further investigated expression of phosphorylated AMPK (p-AMPK), an active form of AMPK, in HCC cells. Metformin was able to activate AMPK, similar to AICAR (Fig. 4). This result indicates that AMPK activation may be involved in the antiproliferative effects of metformin on HCC cells.

Metformin inhibits HCC cell xenograft formation and growth in nude mice. To assess the *in vivo* effects of metformin, we produced an HCC cell xenograft model in nude mice and then treated the mice with metformin through intragastric administration (250 mg/kg body weight/day for 7 weeks) 1 week after subcutaneous injection of PLC/PRF/5 cells. Our data showed that metformin markedly suppressed the growth of tumor xenografts when compared to the isovolumic PBS-treated controls (Fig. 5A and B). In addition, the morbidity due to HCC in the mouse model was reduced from 41 to 16%, after
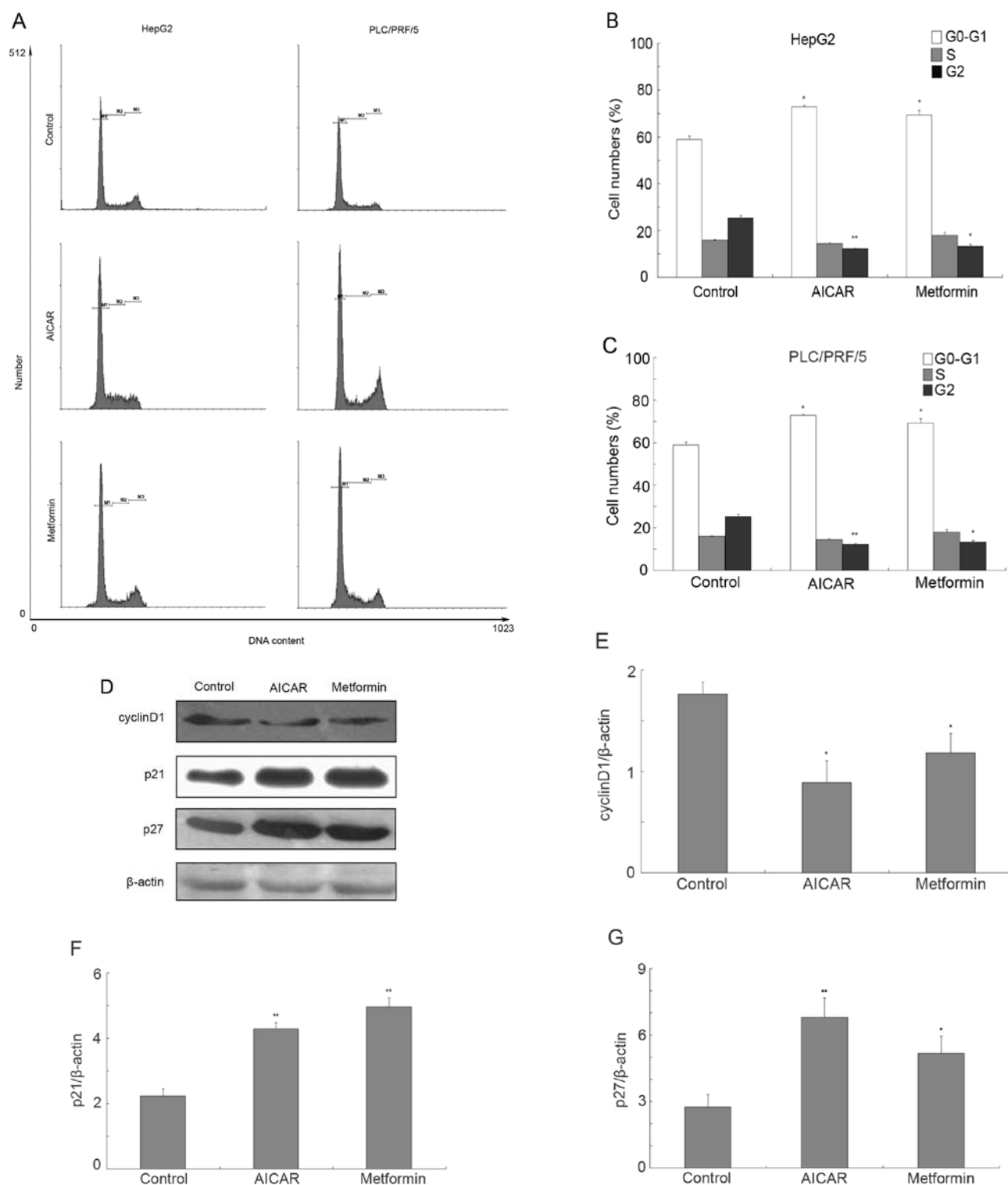
Figure 2. Effects of metformin and AICAR on induction of HCC cell cycle arrest. (A) Flow cytometric assay. (B) HepG2 and (C) PLC/PRF/5 cells were untreated or treated with (10 mM) metformin or AICAR (500 μM) for 72 h and then subjected to analysis of cell cycle distribution via flow cytometry. (D) The duplicated HepG2 cells were subjected to analysis of p21^{CIP}, p27^{KIP} and cyclin D1 expression using western blotting. β-actin is shown as a loading control. (E-G) Data were quantitatively analyzed using gray value histograms of the respective detected proteins. (B, C and E-G) Data from 3 independent experiments are expressed as means ± SEM. *P<0.05 and **P<0.01 compared with the control. AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside. HCC, hepatocellular carcinoma.

metformin treatment (compared with the control, n>10). However, metformin did not have any effect on mouse body weight and serum glucose level (Fig. 5C and D). These data may support the view that metformin is relatively safe and contributed to the prophylaxis and treatment of HCC.

Effects of metformin on expression of cell cycle regulators and p-AMPK in vivo. To explore the molecular mechanisms responsible for the metformin-induced antiproliferative effects *in vivo*, expression of different cell cycle regulator genes was analyzed in the PLC/PRF/5 cell tumor xenograft tissues.
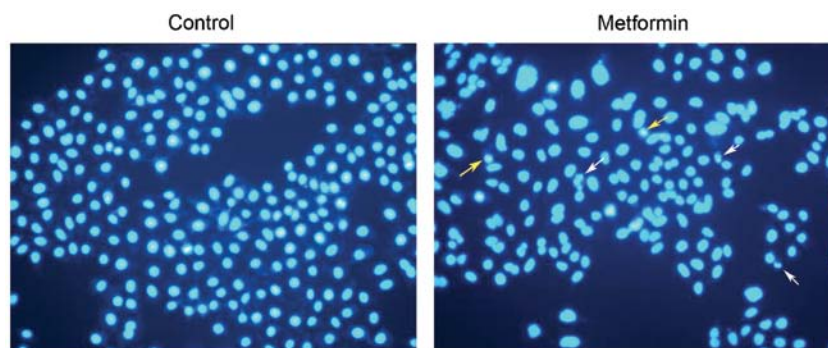
Figure 3. Effects of metformin on the induction of HCC cell apoptosis. HepG2 cells were treated with 10 mM metformin for 24 h and then stained with DAPI for observation under a fluorescence microscope. The control cells showed intact nuclei of uniform shape and size with smooth edges, whereas the metformin-treated cells showed nuclei and chromatin condensation (yellow arrow) and cracking (white arrow). HCC, hepatocellular carcinoma.
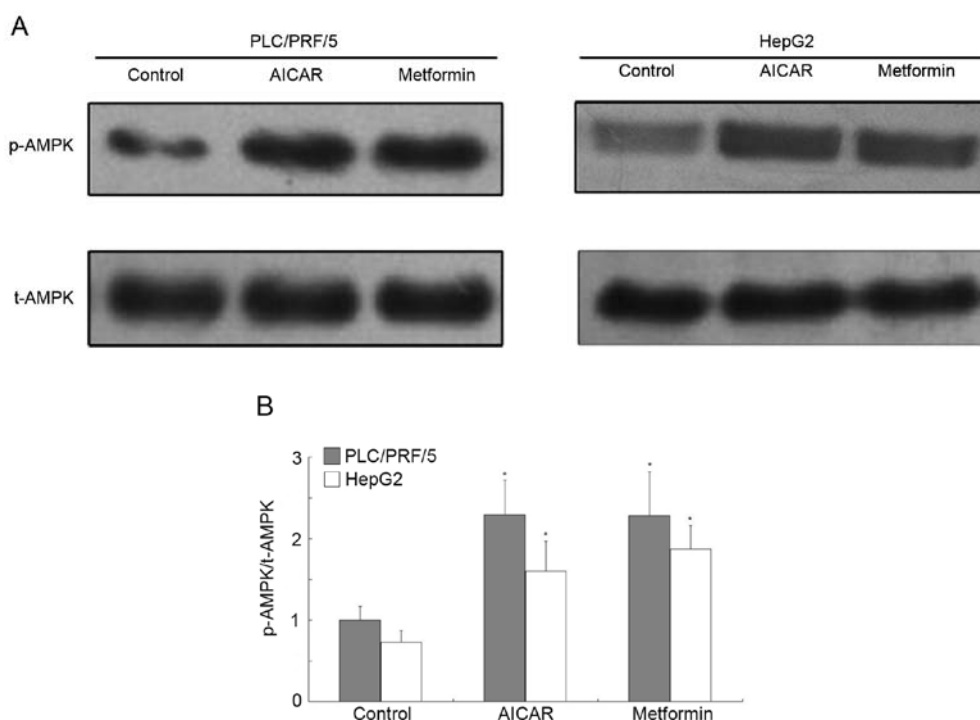

Figure 4. Effects of metformin on AMPK activation in HCC cells. (A) Western blotting. HCC cell lines were treated with 10 mM metformin or 1 mM AICAR for 24 h and then subjected to western blot analysis of total t-AMPK and p-AMPK expression. (B) Quantitative data of A. Graph represents the gray scale value ratio of p-AMPK to t-AMPK of 3 independent experiments expressed as means ± SEM. $^*P<0.05$ compared with the control. AMPK, AMP-activated protein kinase. HCC, hepatocellular carcinoma. AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside.

Western blotting data showed that the expression of p21[CIP] and p27[KIP] proteins was significantly increased by metformin treatment when compared with that of the controls (Fig. 6A). Immunohistochemical data showed that metformin inhibited expression of cyclin D1 and cyclin E proteins, but upregulated the level of p27[KIP] in the metformin-treated tumor xenografts (Fig. 6B-E). Furthermore, we found that metformin treatment upregulated expression of phosphorylated AMPK protein (Fig. 7).

Discussion

In the present study, we investigated the effects of metformin *in vitro* and *in vivo*, and the underlying molecular events. Our data showed that treatment with metformin *in vitro* reduced HCC cell viability in a dose-dependent manner. *In vitro*, metformin treatment induced HCC cell cycle arrest at the G1/G0 phase and apoptosis. In the mouse HCC cell xenograft model, metformin not only blocked tumor progression, but also reduced tumor morbidity. Molecularly, metformin upregulated p21[CIP] and p27[KIP] expression, but downregulated cyclin D1 levels both *in vitro* and *in vivo*. We also found evidence to suggest that these effects of metformin may be through the upregulation of p-AMPK protein. Thus, metformin warrants further evaluation as a novel strategy for the treatment and prevention of HCC.

The conventional view of HCC risk factors focused on viral hepatitis and cirrhosis. Yet recent epidemiological data have shown an association between diabetes, obesity, and insulin resistance and the increased incidence of HCC (13).
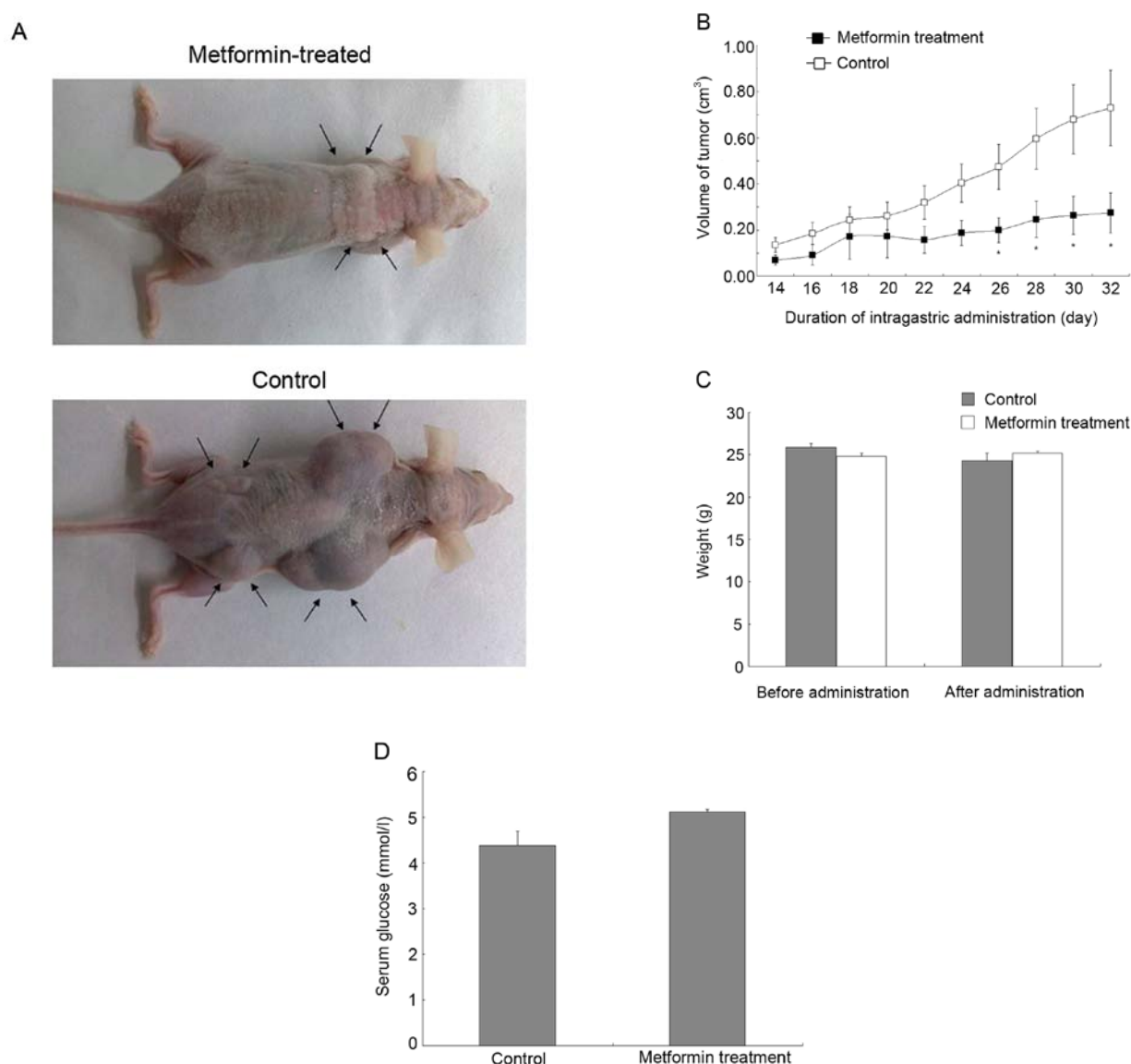
Figure 5. Effects of metformin treatment on the inhibition of HCC cell xenograft growth in nude mice. PLC/PRF/5 cells (1×10^6) were subcutaneously inoculated into 6- to 8-week-old male nu/nu mice in both flanks at 4 sites. One week later, metformin was intragastrically administered to the mice at a dose of 250 mg/kg body weight/day for 7 weeks, while the control mice received vehicle only. Tumor volumes were measured every 2 days. (A) Photographs showing the xenograft tumors on metformin-treated or vehicle-treated nude mice (arrow). (B) The average tumor volumes of PLC/PRF/5 cell xenografts. (C) The mouse body weight was measured before and after metformin administration. (D) Blood glucose was also measured 3 h after metformin administration. Data are shown as means ± SEM. n>10; *P<0.05 compared with the control. HCC, hepatocellular carcinoma.

The HCC risk in type 2 diabetics was found to be as high as 7.1-fold higher than in non-diabetic patients, depending on the duration of diabetes and the treatment protocol used (27). Thus, metformin could be useful for the prevention of HCC, particularly in type 2 diabetic patients. Our current data that metformin induces cell cycle arrest at the G1/G0 phase supports this notion.

Previous studies, including *in vitro* experiments, animal models, and epidemiological analyses have shown that metformin use is associated with a lower rate of cancer development and that metformin inhibits tumor cell proliferation (6,20). Previous studies have revealed that metformin is able to inhibit HCC growth by arresting the tumor cell cycle at G1 phase, through regulation of the AMPK-dependent pathway (28). However, Xiong *et al* (31) showed that the induction of HCC cell cycle arrest and apoptosis by metformin was

through an AMPK-independent pathway. In the present study, we confirmed that metformin treatment upregulated p-AMPK levels in HCC cells *in vitro* and in nude mouse xenografts *in vivo*, but further study is required to determine whether AMPK activation is essential for the antitumor effects of metformin.

Several lines of evidence indicate that AMPK activation may be involved in metformin-induced cell cycle arrest of tumor cells (22,28). Metformin was found to exert its antitumor activity through the AMPK-dependent pathway (29). HeLa cells are deficient in the liver kinase B1 (LKB1; the upstream activator of AMPK) allele and are insensitive to metformin-induced antitumor effects (25). Our current data showed that metformin treatment increased the level of AMPK phosphorylation *in vivo* and *in vitro*. Furthermore, AICAR, an AMPK activator, was able to replicate the other antipro-
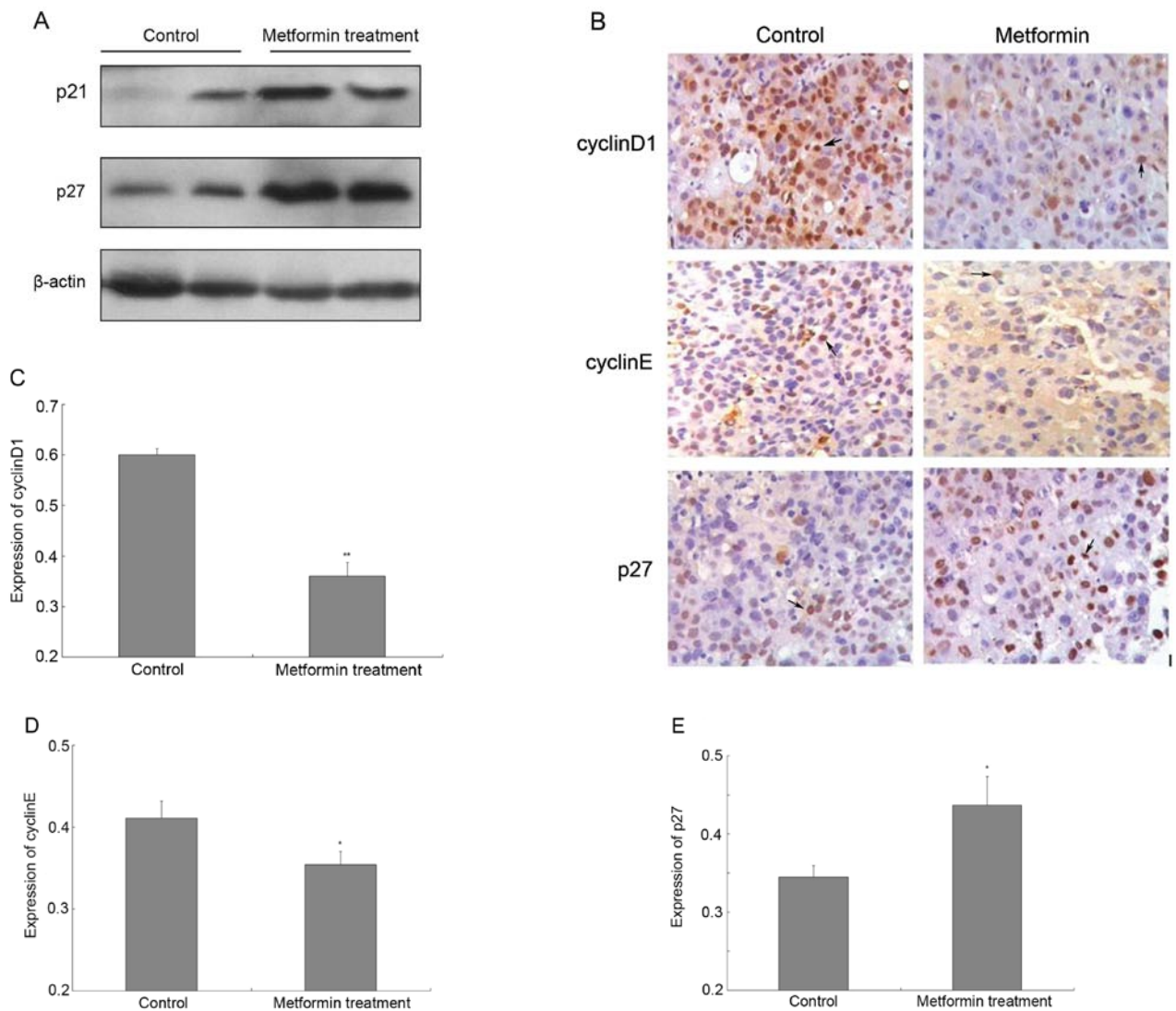
Figure 6. Effects of metformin treatment on the regulation of cell cycle-related gene expression *in vivo*. (A) Xenograft tissues were subjected to protein extraction and western blot analysis of p21^{CIP} and p27^{KIP}. β-actin was used as a loading control. (B) Immunohistochemistry analysis of cyclin D1, cyclin E and p27^{KIP}. The nuclear staining (arrow) represents positive gene expression. (C-E) Summarized data for the IHC results of B. Detailed description of the calculation is provided in Materials and methods. Data are shown as means ± SEM; *P<0.05 and **P<0.01 compared with the control.
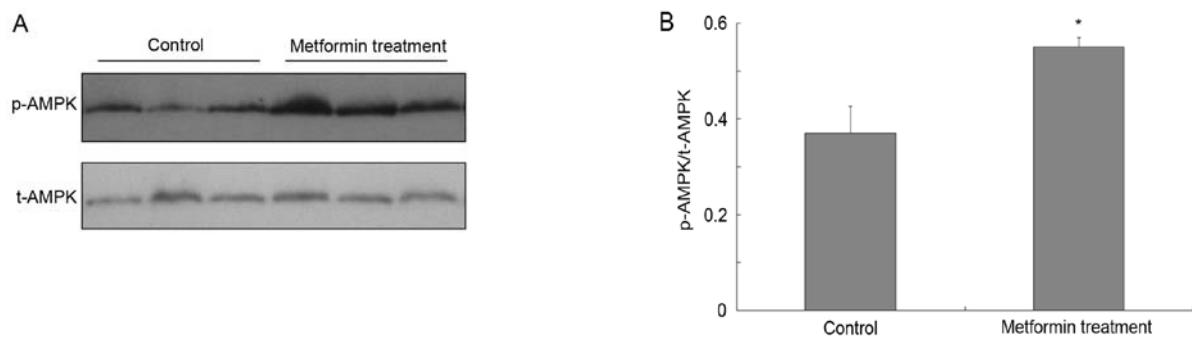

Figure 7. Metformin induces activation of p-AMPK *in vivo*. (A) Tumor xenograft tissues were subjected to protein extraction and western blot analysis of p-AMPK and t-AMPK expression. (B) The graph shows the summarized data of western blotting for 3 independent experiments as means ± SEM; *P<0.05 compared with the control. AMPK, AMP-activated protein kinase.

liferative effects of metformin in HCC cells *in vitro*. These data indicate that metformin-activated AMPK contributes to the inhibitory effects of metformin in HCC cells. However, data that contradicts this finding has been observed (26,30), suggesting that the effects of metformin on tumor cells are AMPK-independent. The reason for this may be because

different experimental conditions were used to test the effects of metformin (28,31).

We recognize that metformin is not only an AMPK activator, but also possesses antiproliferative effects, which may be involved in other gene pathways and multiple regulators. Therefore, we propose that AMPK-dependent and AMPK-independent pathways may coexist. In any case, the most destructive feature of tumor cells is uncontrolled cell cycle progression, and as a central metabolic sensor AMPK can influence cell proliferation via the cell cycle, in addition to mediating the metabolism of fatty acids, glycogen, and proteins (16,28). From this point of view, AMPK may be a target for tumor therapy.

The regulation of cell proliferation is dependent on a balance between cell division and death. In cancer cells, this balance swings toward proliferation, and cancer cells manifest dysregulated cell cycle and immortality. In the present study, metformin arrested HCC cells at the G1 phase of the cell cycle and induced apoptosis. This is consistent with the findings of Qu *et al* (32), and reveals the diversity of the effects of metformin. Therefore, metformin may be useful in the control of HCC progression.

In regular cell cycle progression, the transition from G1 to S phase depends on the regulation of specific cyclins and cyclin-dependent kinase inhibitors (CDKIs), including cyclin D1, cyclin E, p21^{CIP} and p27^{KIP}. Cyclin D1 and cyclin E promote cell DNA synthesis and cell growth. Overexpression of cyclin D1 and cyclin E promotes cancer progression (33,34). In contrast, downregulation of cyclin D1 and cyclin E expression restricts the cell cycle progression to the G0/G1 phase and inhibits tumor cell proliferation (22,35). Our current data showed that metformin treatment decreased the levels of cyclin D1 and cyclin E both in HCC cells *in vitro* and in nude mouse xenografts.

Moreover, p21^{CIP} downregulates the level of phosphorylated retinoblastoma (Rb) to induce cell cycle arrest (36) and combines with proliferating cell nuclear antigen (PCNA) to reduce DNA replication (37). The cell cycle-negative regulator of p27^{KIP} can suppress activation of cyclin-dependent kinase (CDK) (38), inactivate activated cyclin/CDK complexes (35,39), and reduce Rb phosphorylation and E2F release to inhibit gene transcription and control cell cycle progression (40). Our present *in vivo* and *in vitro* data collectively showed that metformin inhibited the expression of cyclin D1 protein, but upregulated p21^{CIP} and p27^{KIP} expression. These data suggest that metformin can aid in the control of HCC.

From a clinical perspective, evaluation of chemotherapeutics must include, not only their antitumor effects, but also the financial cost and side-effects. As a traditional antidiabetic agent, metformin is popular since it is relatively inexpensive and safe. In our *in vivo* experiment, we found that metformin did not influence the weight and serum glucose level of animals. This may be additional support for the use of metformin in antitumor therapy. However, further investigation is needed to determine the therapeutic dose range of metformin for antitumor therapy, and whether metformin treatment has adverse effects within this range. Recently, there have been several clinical chemoprevention trials using metformin and we await publication of the positive data.

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