DEPTOR-related mTOR suppression is involved in metformin's anti-cancer action in human liver cancer cells

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A B S T R A C T

Metformin, one of the most commonly used drugs for patients with type 2 diabetes, recently has received much attention regarding its anti-cancer action. It is thought that the suppression of mTOR signaling is involved in metformin's anti-cancer action. Although liver cancer is one of the most responsive types of cancer for reduction of incidence by metformin, the molecular mechanism of the suppression of mTOR in liver remains unknown. In this study, we investigated the mechanism of the suppressing effect of metformin on mTOR signaling and cell proliferation using human liver cancer cells. Metformin suppressed phosphorylation of p70-S6 kinase, and ribosome protein S6, downstream targets of mTOR, and suppressed cell proliferation. We found that DEPTOR, an endogenous substrate of mTOR suppression, is involved in the suppressing effect of metformin on mTOR signaling and cell proliferation in human liver cancer cells. Metformin increases the protein levels of DEPTOR, intensifies binding to mTOR, and exerts a suppressing effect on mTOR signaling. This increasing effect of DEPTOR by metformin is regulated by the proteasome degradation system; the suppressing effect of metformin on mTOR signaling and cell proliferation is in a DEPTOR-dependent manner. Furthermore, metformin exerts a suppressing effect on proteasome activity, DEPTOR-related mTOR signaling, and cell proliferation in an AMPK-dependent manner. We conclude that DEPTOR-related mTOR suppression is involved in metformin's anti-cancer action in liver, and could be a novel target for anti-cancer therapy.

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1. Introduction

Metformin is one of the most commonly used oral glucose lowering drugs for type 2 diabetes and is recommended as a first-line drug in recent treatment guidelines of the American Diabetes Association and European Association for the Study of Diabetes [1]. The main target tissue of metformin is liver and its major effect is the suppression of hepatic gluconeogenesis, leading to lowering fasting blood glucose levels without stimulation of insulin secretion and weight gain [2,3]. Recently, metformin has received much attention regarding its anti-cancer effect [4]. Epidemiologic evidence suggests that metformin reduces the risk of cancers in patients with diabetes [5,6]. Hepatocellular carcinoma (HCC) is one of the most responsive types of cancer for reduction of incidence by

metformin [7]. The anti-cancer effect of metformin has been verified *in vivo* as well as *in vitro* [8—11]. Following these findings of basic research, metformin has been tried in clinical use in chemotherapy [12,13].

It is thought that the suppression of mTOR signaling is involved in metformin's anti-cancer effect. Several studies have been undertaken to elucidate the molecular mechanism of the suppression of mTOR signaling. Phosphorylation of tuberous sclerosis complex 2 (TSC2), an upstream inhibitor of mTOR complex (mTORC) 1, was thought to be involved in the suppression of mTOR signaling by metformin in breast cancer cells [9]. On the other hand, there has been a report that metformin suppresses mTOR signaling via the TSC2-independent pathway through phosphorylation of raptor, an mTORC1 constituent [14]. In addition, AMPK phosphorylation by metformin was thought to lead to the inhibition of mTORC1. TSC2 or raptor pathways also were thought to be regulated by phosphorylation of AMPK [9,14]. In contrast, there has been a report that metformin suppresses mTOR signaling in an AMPK-independent manner [15]. Thus, the results obtained so far are controversial.

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Although the main target tissue of metformin's anti-diabetic action is liver, in which the drug reduces the risk of cancer and lowers mortality of patients with diabetes, there are few reports available on this mechanism in liver cancer cells.

In the current study, we investigated the mechanism of the suppressing effect of metformin on mTOR signaling and cell proliferation in liver. We found that DEPTOR, an endogenous substrate of mTOR suppression [16], is involved in the suppressing effect of metformin on mTOR signaling and cell proliferation in human liver cancer cells. We also demonstrate that metformin increases the protein levels of DEPTOR via suppression of proteasome activity in an AMPK-dependent manner. Our study thus reveals a novel pathway of metformin's anti-cancer actions in liver.

2. Materials and methods

2.1. Cell line, culture and reagents

Human liver cancer cell line HepG2 and HuH-7 cells were obtained from RIKEN bioresource center (Ibaraki, Japan). HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, USA) and contained 5.5 mM glucose with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C with 5% CO_2. HuH-7 cells were cultured in RPMI-1640 medium (Sigma, USA) and contained 5.5 mM glucose with 10% FBS at 37 °C with 5% CO_2.

2.2. Colony formation assays

Cells were treated with or without metformin (Sigma) at 2 mM for 96 h. The colonies on the dish were fixed with 4% paraformaldehyde and stained with crystal violet (Sigma), counted by HG Colony™ colony counter (HYPER GEAR, Japan).

2.3. MTT assays

Cell proliferation rate was determined by Cell-titer 96 aqueous one solution cell proliferation assay kit (Promega, USA). The absorbance at 490 nm (Reference at 630 nm) was recorded with iMark™ Microplate Reader (Bio-Rad, USA).

2.4. Immunoblottings

Immunoblotting was performed as described previously [17]. Primary antibodies used were anti-phosphorylated (phospho) p70-S6 kinase (p70S6K) (Thr389), anti-p70S6K, anti-phospho ribosome protein S6 (rpS6) (Ser240/244), anti-rpS6, anti-phospho TSC2 (Ser939, Thr1462), anti-TSC2, anti-phospho AMPK (Thr172), anti-AMPKα, anti-phospho raptor (Ser792), anti-raptor, anti-β-actin (Cell Signaling Technology, USA), and anti-DEPTOR antibody (Millipore). Secondary antibodies used were horseradish peroxidase—conjugated anti-rabbit and anti-mouse antibody (GE Healthcare, U.K.). The fluorescent bands were visualized using a detection system (Amersham ECL Prime; GE Healthcare) and quantified by densitometry using Image J software from National Institutes of Health.

2.5. Proteasome activity assays

Proteasome activity was assessed as Pajonk F et al. described previously [18]. Cells were treated with metformin (2 mM) and MG132 (0.5 μM) (Calbiochem, USA) for 72 h. Cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl2, 2 mM ATP), and pelleted by centrifugation. 20 μg protein of each sample was diluted with buffer I to a final volume of 200 μl and the fluorogenic proteasome substrate SucLLVY-7-amido-4-methylcoumarin (chymotrypsin-like, Sigma) was added in a final concentration of 80 μM in 1% DMSO. Cleavage activity was monitored by detection of free 7-amido-4-methylcoumarin using PowerScan HT™ fluorescence plate reader (DS Pharma Biomedical, Japan) at 360/460 nm and 37 °C. As a control for the assay, 7-amido-4-methylcoumarin (AMC, 2 μM, Sigma) was incubated with the drugs in buffer I without cell extracts and measurements of proteasome function were corrected when necessary.

2.6. Isolation of total RNA and quantitative RT-PCR

Total RNA was prepared by Trizol reagent (Invitrogen) as previously described [19]. The human sequences of forward and reverse primers to evaluate DEPTOR expression were 5′-TTTGTGGTGCGAGGAAGTAA-3′ and 5′-CATTGCTTTGTGTCATTCTGG-3′; respectively. The human sequences of forward and reverse primers to evaluate GAPDH expression were 5′-GAGTCAACG-GATTTGGTCGT-3′ and 5′-TTGATTTTGGAGGGATCTCG-3′; respectively.

2.7. Cell transfection and short interfering RNA of TSC2, DEPTOR and AMPK

Cell transfection was performed as previously described [19]. Stealth short interfering RNA (siRNA) of TSC2, DEPTOR and AMPKα1 were purchased from Invitrogen (Carlsbad, USA). The sequences of siRNAs for TSC2 were 5′-CCUGUCUGGACAUGAUGGCUCGAUA-3′ and 5′-UAUCGAGCCAUCAUGUCCAGACAGG-3′, 5′-GCUGGUGAA-CUUGGUCAAAUUCAAU-3′ and 5′-AUUGAAUUUGACCAAGUU-CACCAGC-3′, respectively. The sequences of siRNA for DEPTOR were 5′-GGACCGUGAGCAAUCUGAUUCUGAC-3′ and 5′-GUCAGAAUCA-GAUUGGUCACGGUCC-3′. The sequences of AMPKα1 siRNAs were 5′-CCCUCAAUAUUUAAAUCCUUCUGUG-3′ and 5′-CACA-GAAGGAUUUAAAUAUUGAGGG-3′. The sequences of control siRNAs were 5′-ACCAACAACAGUUUGGGAAUAGGGA-3′ and 5′-UCC-CUAUUCCCAAACUGUUGUUGGU-3′.

2.8. Statistics

Comparison between two groups was performed using unpaired Student's t test (not noted) and paired student's t test. For more than two groups, one-way or two-way ANOVA followed by post hoc Bonferroni testing was performed. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Suppressing effect of metformin on cell proliferation is in a TSC2-independent manner in human liver cancer cells

To confirm that metformin suppresses cell proliferation rate in human liver cancer cells, HepG2 cells were incubated with or without metformin (2 mM) for 96 h. By colony formation assay, the cell proliferation rate was significantly suppressed with metformin after 48-h incubation (Fig. 1A). We also performed MTT assay. The increase in absorbance was significantly suppressed after 72-h incubation with metformin (Fig. 1B), as was found also in HuH-7 cells (Suppl. Fig. 1A). We then examined whether mTOR signaling is involved in the suppressing effect of metformin on cell proliferation. We measured the phosphorylation of p70S6K, a downstream target of mTOR. After 72-h exposure to metformin, phosphorylation of p70S6K was decreased in HepG2 cells (Fig. 1C and D) and HuH-7 cells (Suppl. Fig. 1B and C). Furthermore, we measured the phosphorylation of ribosome protein S6 (rpS6), a downstream target of p70S6K. After 72-h exposure to metformin,
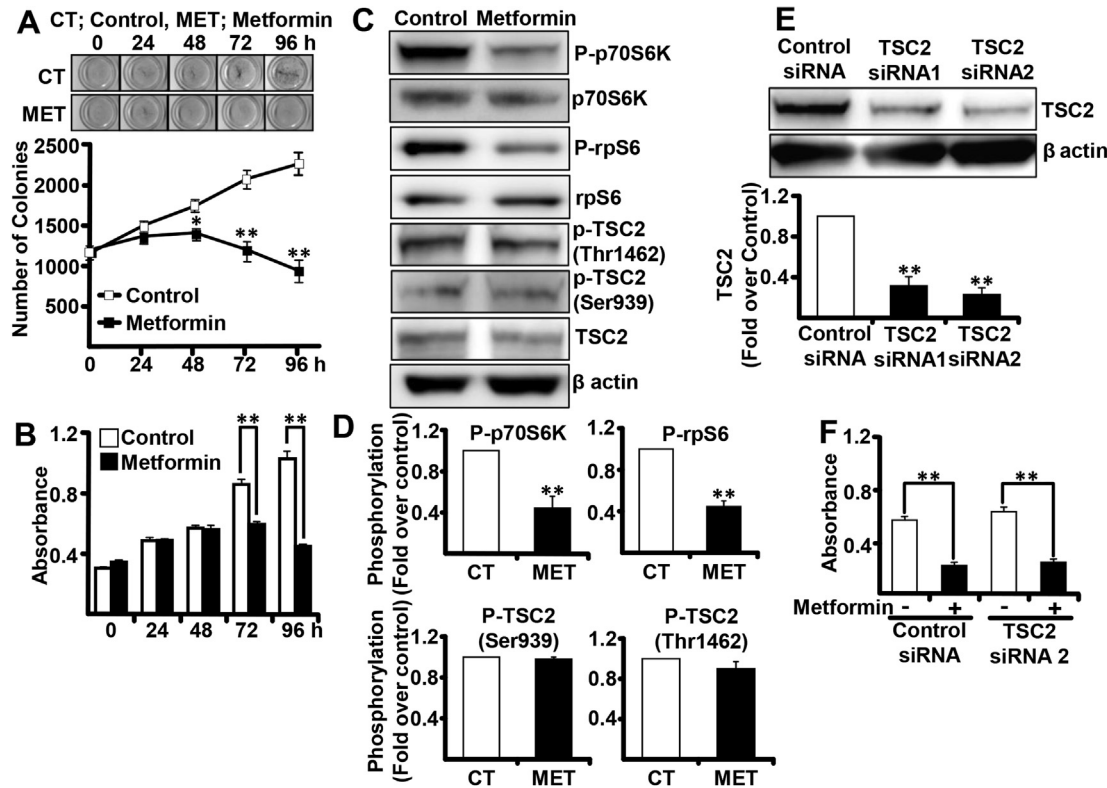
Fig. 1. Suppressing effect of metformin on cell proliferation is in a TSC2-independent manner in human liver cancer cells. (A) Colony formation assays. HepG2 cells were treated with or without metformin (2 mM) for indicated time. (B) MTT assays. HepG2 cells were treated with or without metformin (2 mM) for indicated time. (C) (D) Effect of metformin on phosphorylation of p70S6K, rpS6, and TSC2. HepG2 cells were treated with or without metformin (2 mM) for 72 h. Data is expressed as fold stimulation over control. (E) siRNA silencing of TSC2. Total cell extracts were subjected to immunoblotting after 48 h of treatment with either TSC2 siRNA or control siRNA transfection. Data is expressed as fold stimulation over control. (F) MTT assay after TSC2 siRNA transfection. HepG2 cells were treated with or without metformin (2 mM) for 72 h. Data are depicted as mean ± SE from at least three separate experiments. $^{*}P < 0.05$, and $^{**}P < 0.01$, versus control.

phosphorylation of rpS6 was decreased in HepG2 cells (Fig. 1C and D) and HuH-7 cells (Suppl. Fig. 1B and C), suggesting that metformin suppressed mTOR signaling in human liver cancer cells. Although there are reports of the involvement of TSC2 phosphorylation in the suppression of mTOR signaling [8,9], we found that metformin did not increase the phosphorylation of TSC2 in HepG2 cells (Fig. 1C and D). Furthermore, the suppressing effect of metformin on cell proliferation was not eliminated by TSC2 silencing (Fig. 1E and F). These results indicate that metformin suppresses cell proliferation of human liver cancer cells in a TSC2-independent manner. There may also be pathways other than TSC2 involved in the anti-cancer action of metformin in human liver cancer cells.

3.2. Metformin increases the protein levels of DEPTOR, a suppressing effector of mTOR signaling

We then examined DEPTOR, an inhibitory protein of mTOR. DEPTOR was recently identified as an mTOR-binding protein that functions as an endogenous inhibitor of the kinase activity of both mTORC1 and 2 [16]. We evaluated the protein levels of DEPTOR with or without metformin. After 48-h exposure to metformin, the protein levels of DEPTOR were significantly increased in HepG2 cells (48 h; 2.45 ± 0.24, 72 h; 3.04 ± 0.23, fold over control) (Fig. 2A and B) and HuH-7 cells (72 h; 1.67 ± 0.02, fold over control) (Suppl. Fig. 1D). Furthermore, as the protein levels of DEPTOR are likely to be regulated by the proteasome degradation system [16], we measured proteasome activity. After 48-h exposure to metformin, proteasome activity was significantly suppressed (24 h; 91.7 ± 2.0, 48 h; 56.0 ± 6.6, 72 h; 51.8 ± 3.5, % of control) (Fig. 2C) and HuH-7 cells (72 h; 58.2 ± 1.8, % of control) (Suppl. Fig. 1E). We

confirmed that MG132 (0.5 μM), a potent proteasome inhibitor, also suppressed proteasome activity to the same level as metformin (2 mM) (72 h; 47.9 ± 2.2, % of control). The mRNA levels of DEPTOR were not changed (48 h) or decreased (72 h) with metformin (Fig. 2D), indicating that the increase in protein levels of DEPTOR was not affected by the change of mRNA levels of DEPTOR. These results indicate that the degradation of DEPTOR by the proteasome system is inhibited by metformin via the suppression of proteasome activity, allowing protein levels of DEPTOR to be increased in human liver cancer cells.

3.3. Suppressing effect of metformin on mTOR signaling is in a DEPTOR-dependent manner

To verify that metformin suppresses cell proliferation in a DEPTOR-dependent manner, the effect of silencing DEPTOR was examined (Fig. 3A and B). By transfection of DEPTOR siRNA, the suppressing effects of metformin on the phosphorylation of p70S6K and rpS6 were eliminated (Fig. 3A, C and D). The suppressing effect of metformin on cell proliferation was also eliminated by DEPTOR silencing (Fig. 3E). Taken together, these findings indicate that metformin inhibits mTOR signaling and suppresses cell proliferation in human liver cancer cells in a DEPTOR-dependent manner.

3.4. Metformin suppresses proteasome activity and increases the protein levels of DEPTOR in an AMPK-dependent manner

Participation of AMPK in the anti-cancer effect of metformin remains controversial [8,10,11]. We therefore examined the involvement of AMPK in the suppression of mTOR signaling and cell
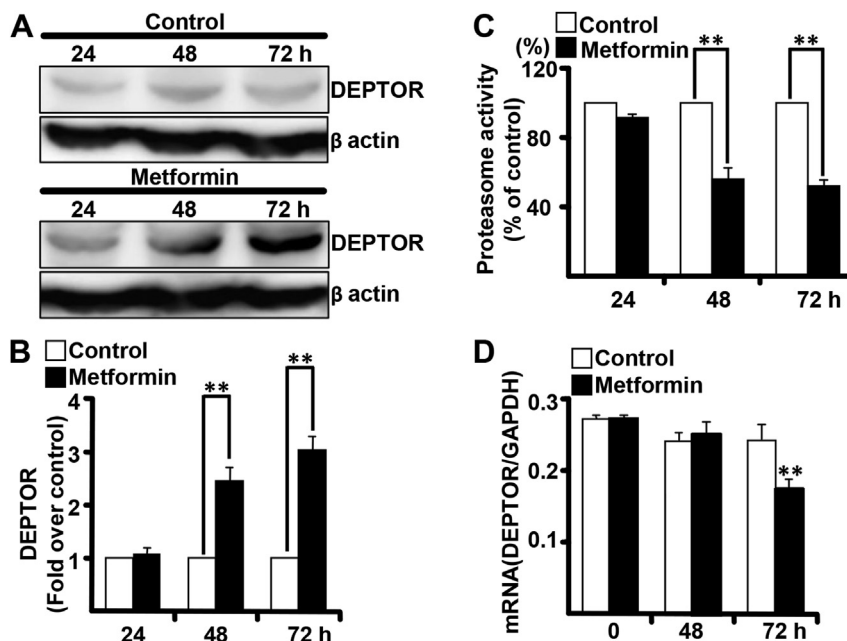
Fig. 2. Metformin increases the protein levels of DEPTOR, a suppressing effector of mTOR signaling. (A) (B) Effect of metformin on the protein levels of DEPTOR. HepG2 cells were treated with or without metformin (2 mM) for indicated times. Beta actin served as internal control. Data is expressed as fold stimulation over control. (C) Proteasome activity assay. HepG2 cells were treated with or without metformin (2 mM) for indicated times. (D) mRNA expression levels of DEPTOR. HepG2 cells were treated with or without metformin at 2 mM for indicated times. Data are depicted as mean ± SE from at least three separate experiments. **$P < 0.01$, versus control (B) (C) and versus 0 h (D).

proliferation by metformin in HepG2 cells. By transfection of AMPKα1 siRNA (Fig. 4A and B), the suppressing effects of metformin on the phosphorylation of p70S6K and rpS6 were eliminated (Fig. 4A, C and D). The suppressing effect of metformin on cell proliferation was also eliminated by AMPKα1 silencing (Fig. 4E), indicating that metformin suppresses mTOR signaling in an AMPK-dependent manner in human liver cancer cells. We then examined the participation of AMPK in the regulation of the protein levels of
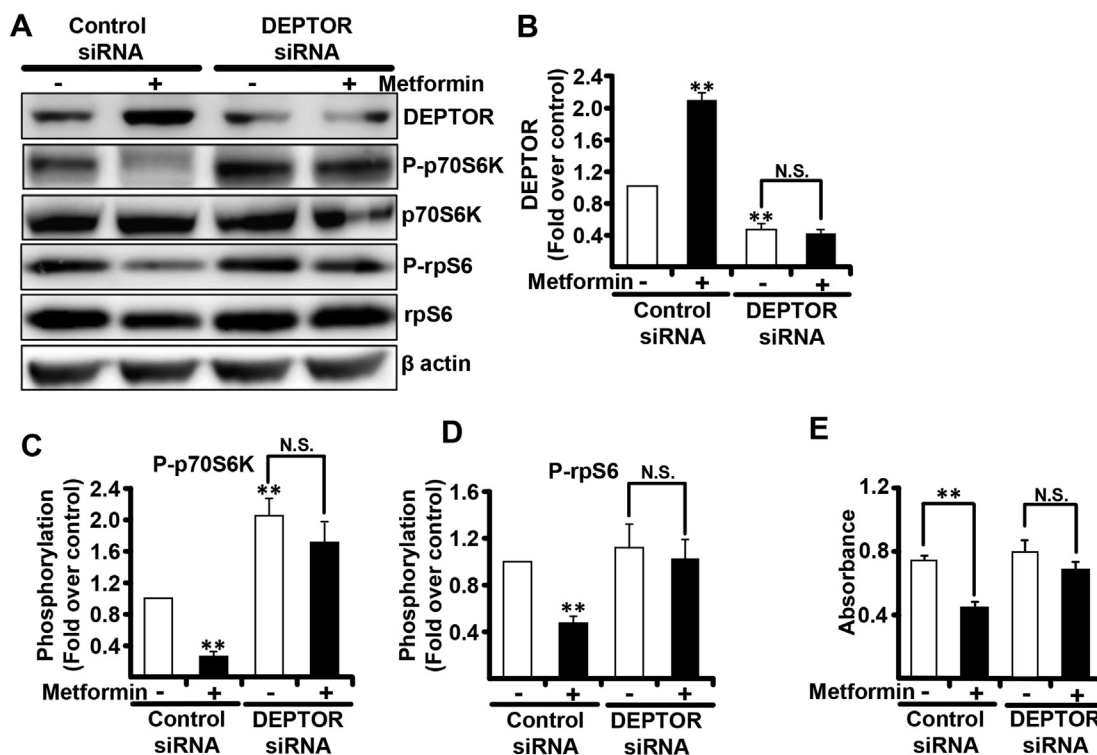
Fig. 3. Suppressing effect of metformin on mTOR signaling is in a DEPTOR-dependent manner. (A) Immunoblotting analysis after DEPTOR siRNA transfection. HepG2 cells were treated with or without metformin (2 mM) for 72 h. (B) Protein levels of DEPTOR. Data is expressed as fold stimulation over control. (C) Phosphorylation of p70S6K. Data is expressed as fold stimulation over control. (D) Phosphorylation of rpS6. Data is expressed as fold stimulation over control. (E) MTT assay after DEPTOR siRNA transfection. HepG2 cells were treated with or without metformin (2 mM) for 72 h. Data are depicted as mean ± SE from at least three separate experiments. **$P < 0.01$, versus control.

DEPTOR by metformin. The protein levels of DEPTOR were decreased and the increasing effect of metformin on the protein levels of DEPTOR was eliminated by AMPKα1 silencing (Fig. 4A and F). The suppressing effect of metformin on proteasome activity also was eliminated by AMPKα1 silencing (Fig. 4G). Phosphorylation of raptor, a component of mTORC1 that inhibits mTORC1 signaling, was not increased by metformin (Fig. 4A and H).

4. Discussion

In the current study, we found that DEPTOR is involved in the suppressing effect of metformin on mTOR signaling and cell proliferation in human liver cancer cells. DEPTOR is an mTOR-binding protein that functions as an endogenous inhibitor of kinase activity for both mTORC1 and 2, and was recently identified by Peterson TR et al. [16]. The protein levels of DEPTOR are known to be regulated by a proteasome-mediated proteolytic degradation [16]. We have shown that metformin exerts a suppressing effect on proteasome activity and an increasing effect on the protein levels of DEPTOR. The suppressing effects of metformin on mTOR signaling and cell proliferation in human liver cancer cells are DEPTOR-dependent. Furthermore, metformin exerts a suppressing effect on proteasome activity, DEPTOR-related mTOR signaling, and cell proliferation in an AMPK-dependent manner.

The suppression of mTOR signaling is thought to be involved in the anti-cancer effect of metformin [20,21]. In the present study, we have shown that metformin suppresses mTOR signaling via increase in the protein levels of DEPTOR in human liver cancer cells.

Several studies have reported that metformin suppresses mTOR signaling via the phosphorylation of TSC2 by AMPK activation in breast cancer cells [8,9]. In our data, the suppressing effect of metformin on the cell proliferation was found to be in a TSC2-independent manner in human liver cancer cells. Raptor, a component of mTORC1, is also known to be a suppressor of mTOR signaling. Gwinn DM et al. reported that metformin phosphorylates raptor and suppresses mTOR signaling in HEK293 cells [14]; however, we could not confirm that raptor is involved in the suppressing effect of metformin on mTOR signaling in human liver cancer cells. There are several possibilities regarding these inconsistencies. One is the difference of dosage of metformin. In contrast with the previous studies in which the dosage of metformin was 10–20 mM [8,9,14], our experiments were performed at 2 mM; a more physiological condition that is used for clinical administration. Another possibility is the difference of cell species. Previous studies were performed using breast cancer cells [8,9] or HEK 293 cells [14], our study was performed using HepG2 and HuH-7 cells derived from liver. Our findings show a novel mechanism of metformin's anti-cancer action in human liver cancer cells at a similar dosage range used to treat patients with type 2 diabetes.

In the current study, we show that metformin suppresses proteasome activity in human liver cancer cells. It is well known that the proteasome activity is increased in cancer cells, including HCC [22,23]. Proteasome inhibitors are currently used as anti-cancer agents in the treatment of hematological malignancies [24]. Indeed, clinical trials are currently underway to test the efficacy of proteasome inhibitor in HCC [25]. We have demonstrated that the
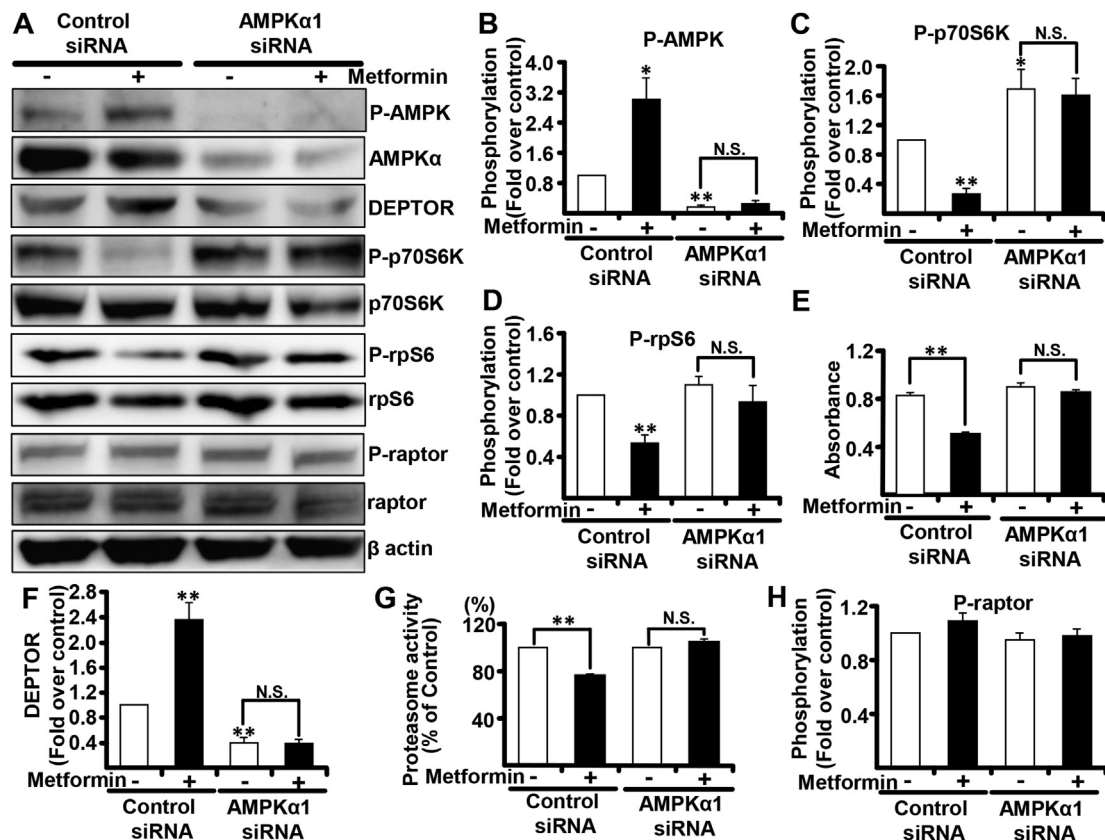
Fig. 4. Metformin suppresses proteasome activity and increases the protein levels of DEPTOR in an AMPK-dependent manner. (A) Immunoblotting analysis after AMPKα1 siRNA transfection. HepG2 cells were treated with or without metformin (2 mM) for 72 h. (B) Phosphorylation of AMPK. Data is expressed as fold stimulation over control. (C) Phosphorylation of p70S6K. Data is expressed as fold stimulation over control. (D) Phosphorylation of rpS6. Data is expressed as fold stimulation over control. (E) MTT assay after AMPKα1 siRNA transfection. HepG2 cells were treated with or without metformin (2 mM) for 72 h. (F) Protein levels of DEPTOR. Data is expressed as fold stimulation over control. (G) Proteasome activity assay after AMPKα1 siRNA transfection. HepG2 cells were treated with or without metformin (2 mM) for 72 h. (H) Phosphorylation of raptor. Data is expressed as fold stimulation over control. Data are depicted as mean ± SE from at least three separate experiments. *$P < 0.05$, and **$P < 0.01$, versus control.

suppressing effect of metformin on proteasome activity is in an AMPK-dependent manner in human liver cancer cells. In this study, we did not investigate the mechanism of regulation of metformin on proteasome activity by AMPK activation. There is a possibility of direct involvement of AMPK in proteasome activity; a binding-site of AMPK has been detected in the proteasome complex [26]. Further research is required for elucidation of the involvement of AMPK in the proteasome system.

Recently, it was noticed that non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) in patients with diabetes is related to the occurrence of HCC in patients with diabetes; the prevalence of NAFLD/NASH being approximately 70% in patients with diabetes [27]. It is now believed that up-regulation of mTOR signaling is involved in the pathogenesis of NAFLD/NASH, which may suggest that mTOR inhibition by metformin might ameliorate the disease [28—31]. Further investigation is required using DEPTOR-deficient model animals or NASH-associated HCC model animals to clarify these issues.

In conclusion, metformin exerts its anti-cancer effect through the AMPK-DEPTOR-mTOR pathway in human liver cancer cells. DEPTOR-related mTOR suppression could therefore be a novel target for anti-cancer therapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.148.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.148.

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