Metformin induces apoptosis of lung cancer cells through activating JNK/p38 MAPK pathway and GADD153

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There are epidemiological and experimental evidences that metformin, an insulin-sensitizer agent widely used for diabetes treatment, has inhibitory effects on the growth of various human cancers. However, the underlying molecular mechanisms for its anti-neoplastic activity has not been yet clarified and the effect of metformin on human lung cancer remains unknown. In this study we revealed for the first time that metformin treatment led to increased apoptosis in human lung cancer cell lines A549 and NCI-H1299 and significantly inhibited the cells proliferation in a dose- and time-dependent manner, which was further demonstrated by the data obtained from A549 tumor xenografts in nude mice. We also found that metformin treatment can activate AMP-activated protein kinase, JNK/p38 MAPK signaling pathway and caspases, as well as upregulate the expression of growth arrest and DNA damage inducible gene 153 (GADD153). Either blockade of JNK/p38 MAPK pathway or knockdown of GADD153 gene abrogated the apoptosis-inducing effect of metformin. Taken together, our data suggest that metformin inhibits the growth of lung cancer cells and induces apoptosis through activating JNK/p38 MAPK pathway and GADD153.

Key words: Metformin; lung cancer; apoptosis; JNK; p38 MAPK; GADD153

The biguanide metformin is one of the mostly used drugs for treatment of type 2 diabetes [1]. By reducing hepatic gluconeogenesis and increasing glucose uptake in skeletal muscles, metformin can lower blood glucose levels while inducing no risk of hypoglycemia and very rarely causing lactic acidosis [2-4]. Recent clinical studies have revealed that metformin treatment is associated with reduced cancer risk and improved prognosis [5,6]. The effects of metformin on breast, prostate, colorectal, ovarian cancer and glioma cells have been experimentally investigated and its antineoplastic activity has been proven in these cancers [7-12]. The growth inhibitory effects of metformin are generally believed to involve the activation of AMP-activated protein kinase (AMPK), a serine/threonine protein kinase which serves as an energy sensor in all eukaryotic cell types [13-15]. According to several published studies, AMPK activation strongly suppresses cell proliferation in both malignant and nonmalignant cells through inhibition of mTOR signalling, inhibition of protein and fatty acid synthesis, and stimulation of p53-p21 axis [8-10, 16-18]. However, a recent study showed that metformin inhibited the growth

of human prostate cancer cells in an AMPK-independent manner, indicating cell-specific effects and mechanisms of metformin [11].

Lung cancer is the most common cancer in the world both in terms of incidence and total mortality. Although combined-modality therapy with radiotherapy and chemotherapy has been applied, about 86% of the patients with lung cancer die within 5 years [19]. New therapeutic modalities are therefore necessary to improve the response to treatment. In this study, we show for the first time that metformin can efficiently induce growth inhibition and apoptosis of lung cancer cells. The mechanisms involved in metformin-induced apoptosis are also investigated.

Materials and methods

Cell lines and mice. A549 and NCI-H1299 human lung cancer cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L
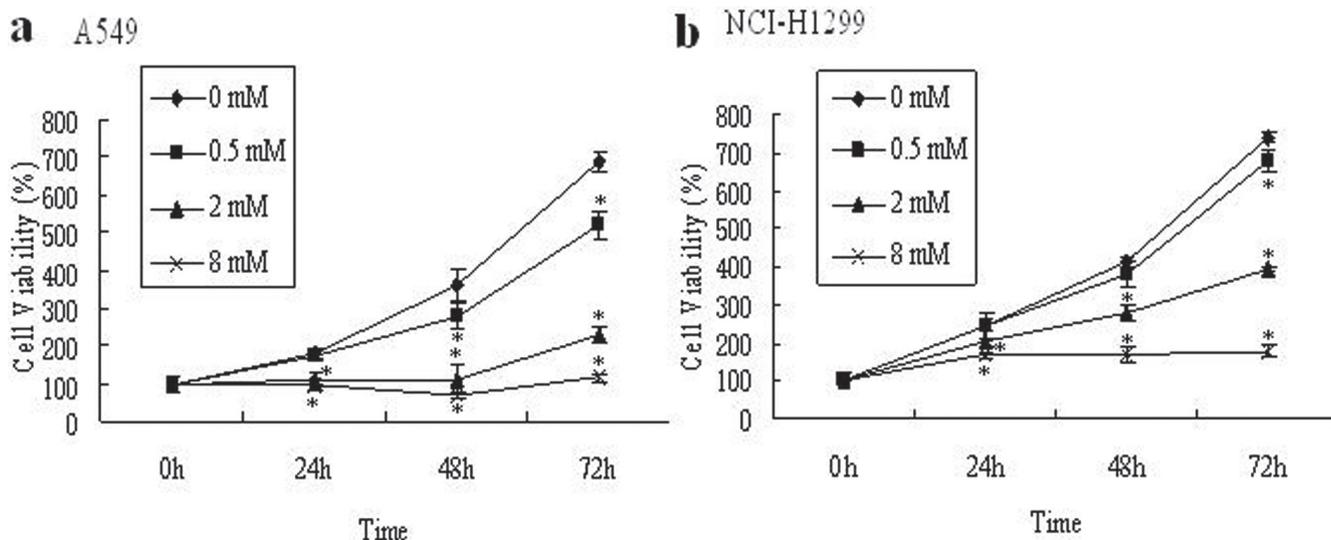
Figure 1. Effects of metformin on lung cancer cell growth in vitro. A549 (a) or NCI-H1299 (b) cells were incubated with metformin (0.5, 2 and 8 mM) or PBS (0 mM), and cell viabilities were measured by MTT assay at indicated time points (*$P<0.05$ refers to untreated cells).

L-glutamine, and 50 mg/ml penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Four- to 6-week-old female BALB/c nude mice were obtained from the Animal Experimental Center of the Second Military Medical University (SMMU). All animals in this study were housed in pathogen-free conditions and were maintained in accordance with guidelines of the Committee on Animals of the SMMU.

Chemicals and reagents. Metformin, SP600125 and SB202190 were obtained from Sigma (St. Louis, MO, USA). Phospho- and total-AMPK, p38 mitogen-activated protein kinase (MAPK), and c-Jun N terminal kinase (JNK) antibodies were all purchased from CST (Danvers, MA, USA). Growth arrest and DNA damage inducible gene 153 (*GADD153*), cleaved caspase-3, -8, -9 antibodies, and Ki67 antibodies (mouse specific) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The siGENOME SMARTpool short interfering RNAs (siRNAs) targeting *GADD153* (siGADD153), along with a negative control siRNA pool (siNEG) were purchased from Dharmacon (Lafayette, CO, USA).

Cell proliferation assays. A549 (5000 cells/well) and NCI-H1299 cells (4000 cells/well) were seeded in 6 wells in a 96-well plate and cultured. Metformin was added 24 h later to give a final concentration of 0.5, 2 or 8 mM. Cell viability was determined at 24 hour time points for a total of 72 hours using MTT assay (Sigma). The percentage of cell viability is plotted for each cell line ± one standard deviation of the reading from six wells, relative to 0 h readings.

Annexin V/PI staining assay. Cells were cultured in 6-well plates and incubated with metformin (0.5, 2 or 8 mM) or PBS for 48 h. Apoptotic cells were detected by double staining with fluoresceinisothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). The samples were analyzed by flow cytometry.

Western blot analysis. Total cell lysate was prepared in 1×SDS buffer. Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were then blotted with individual antibodies. Antigen-antibody complexes were visualized with the enhanced chemiluminescence reagent Supersigal (Pierce, Rockford, IL, USA).

Real-time PCR. mRNA expression of *GADD153* was determined by real-time PCR using SYBR Premix Ex Taq (TaKaRa, Dalian, China). β-actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The results were expressed relative to the control condition, which was arbitrary assigned a value of 1.

RNAi knockdown of *GADD153*. A549 cells were transfected with siGADD153 or siNEG with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, using a final concentration of 50 nM siRNA for 8 h. After 48 h posttransfection real-time PCR was performed to confirm siRNA-mediated knockdown of *GADD153*.

Mice xenografts. Two×10[7] A549 cells were suspended in PBS before being inoculated s.c. on the right flanks of BALB/c nude mice (15 mice/group). Treatment with metformin was started when the average tumor volumes reached 100 mm[3]. Metformin was dissolved in 200 ul PBS and administered daily intraperitoneally at a dose of 40 mg/kg/day or 200 mg/kg/day for a month. The control group received an equal volume of vehicle only. Animal weight and tumor volume were measured every 4 days and estimated from caliper measurements using the formula $\pi/6 \times A \times B^2$ (A is larger diameter, B is smaller
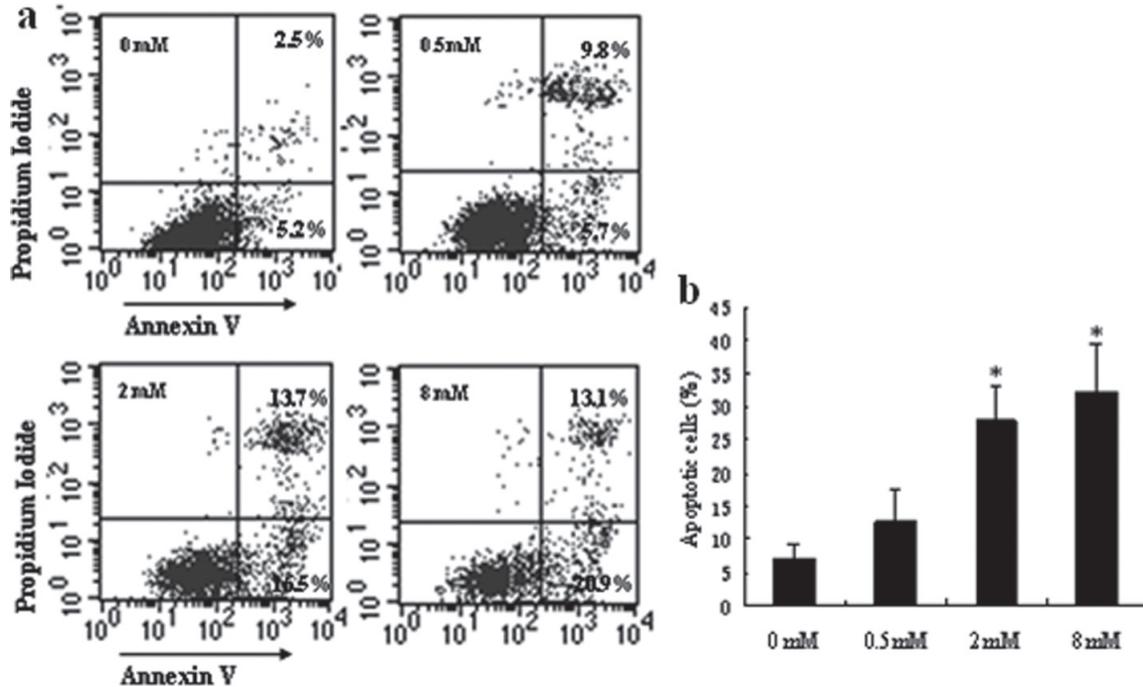
Figure 2. Effects of metformin on lung cancer cells apoptosis. (a)The numbers of apoptotic A549 cells labeled with annexin V were analyzed using flow cytometry after treatment with metformin (0.5, 2 and 8 mM) or PBS (0 mM) for 48 h. The percentages of cells (annexin+PI- and annexin+PI+) are indicated for each condition. (b) The percentages of apoptotic cells (annexin+) treated with 2 or 8 mM metformin were significantly increased compared to control (0 mM). Data on NCI-H1299 are not shown. (*P<0.05)

diameter). At the end of treatment, animals were euthanized, and tumors were harvested for histopathological analysis.

Immunostaining. Tumor tissue samples from mice subjected to different treatments were taken and prepared with a routine pathological procedure. Paraffin sections and post-fixed slides were subjected to immunohistochemical analysis with antibody to Ki67, and in situ apoptosis assay with the DeadEnd colorimetric TUNEL system (Promega, Madison, WI, USA).

Statistics. Data are presented as the mean ± SEM of at least three independent experiments unless otherwise stated. Differences between groups were analyzed using the Student t test or ANOVA. Values of $P < 0.05$ were considered significant.

Results

Metformin inhibits the growth of human lung cancer cell lines in a dose- and time-dependent manner. A549 or NCI-H1299 cells were incubated with metformin or PBS for 24, 48 or 72 h and cell viability was assessed using MTT assay. As shown in Fig. 1, metformin treatment significantly inhibited tumor cell growth in a dose- and time-dependent manner. After treated with 8 mM metformin for 72 h, the viabilities of A549 and NCI-H1299 cells were 17% and 24% of untreated controls, respectively.

Induction of apoptosis by metformin. Metformin has been shown to induce apoptosis in some cell types while have no

effect of apoptosis induction on others [8,11,20]. In order to determine the effect of metformin on apoptosis in human lung cancer cells, A549 or NCI-H1299 cells were incubated with metformin for 48 h and then an annexin V/PI staining assay was performed. The numbers of apoptotic (annexin+) lung cancer cells were significantly increased by metformin at 2 or 8 mM (Fig. 2). For cells treated with 0.5 mM metformin, the amount of annexin+PI+ cells which presumably underwent secondary necrosis significantly increased ($P<0.05$, Fig. 2a), though the total number of apoptotic cells was similar to that of control.

Activation of JNK/p38 MAPK pathway are involved in metformin-induced apoptosis in lung cancer cells. Metformin has been proven an AMPK activator in many epithelial cells. We found that metformin induced AMPK phosphorylation early in A549 cells (Fig. 3a). Since AMPK has been implicated as an upstream element of MAPKs signaling pathways which are important in apoptosis in various cell types [20-22], we explored the involvements of JNK/p38 MAPK pathway and caspases in metformin-induced apoptosis in lung cancer cells and found that both p-p38 and p-JNK expression were significantly increased in an early time in metformin-treated A549 cells (Fig. 3a). Accordingly, a significant time-dependent activation of caspases, including caspase-8, -9 and -3 were also observed (Fig. 3b). In contrast, the expressions of total JNK and p38 MAPK proteins had no detectable changes after met-
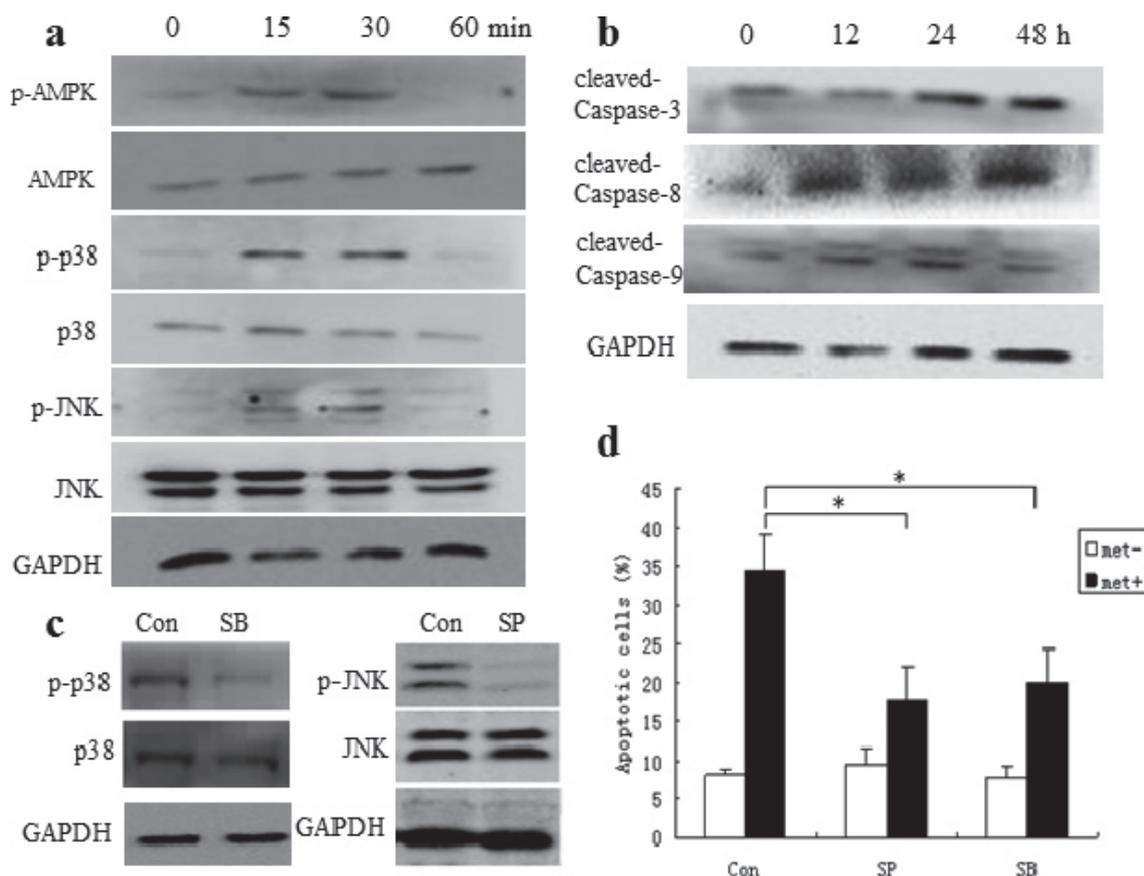
Figure 3. Activation of AMPK, JNK/p38 MAPK pathway and caspases are involved in metformin-induced apoptosis in lung cancer cells. (a and b) Expression of p-AMPK, AMPK, p-p38, p38, p-JNK, JNK and cleaved caspase-3, 8, 9 proteins were detected by western blot analysis after the indicated exposure time of A549 cells to metformin (5 mM). Samples were probed for GAPDH as a loading control. (c and d) A549 cells were pre-treated with vehicle (Con), SP600125 (SP, 100nM) or SB202190 (SB, 100mM) for 1 h before addition of metformin (5 mM) or an equal volume of PBS for a further 48 h. The phosphorylated and total p38 or JNK levels in SB or SP-treated cells and control cells were detected by western blot analysis. The number of apoptotic cells (annexin+) was analyzed using flow cytometry. (*$P<0.05$)

formin treatment. A specific p38 MAPK inhibitor SB202190 and a specific JNK inhibitor SP600125 were then used to pre-treat A549 cells for 1 h before metformin was added. Western blot analysis showed either p-p38 or p-JNK expression was significantly decreased in SB202190 or SP600125-treated cells. The specific inhibitors had not any effect on the expression of total p38 or JNK protein (Fig. 3c). As shown in Fig. 3d, both the numbers of apoptotic cells pre-treated with SB202190 and SP600125 significantly decreased after metformin treatment compared to the control. These results suggested that met-formin-induced apoptosis of A549 cells is partly mediated by the activation of JNK/p38 MAPK pathway and caspases.

Upregulation of *GADD153* gene contributes to metform-in-induced apoptosis in lung cancer cells. Metformin triggers production of reactive oxygen species as well as reduction of blood glucose, either of which has a strong positive impact on the expression of GADD153, a transcription factor contribu-ting to cell apoptosis when upregulated [23-26]. To further explore the possible mechanisms underlying the apoptosis-

inducing ability of metformin, mRNA expression of *GADD153* in A549 cells after metformin treatment were determined. As expected, mRNA level of *GADD153* was significantly raised by metformin in a time-dependent manner (Fig. 4a). To assess the functional significance of elevated GADD153 expression, we used RNA interference to knockdown *GADD153* gene in A549 cells. Though the apoptosis-inducing effect of met-formin was not entirely obviated, transfection of a Dharmacon siGENOME SMARTpool of four different siRNAs led to sup-pressed *GADD153* expression and significantly decreased cell apoptosis compared to a negative control siRNA pool (Fig. 4b and 4c). The results indicated that GADD153 upregulation plays an important role in metformin-induced apoptosis in lung cancer cells.

Metformin inhibits tumor growth in vivo. To determine if metformin could affect tumor growth in vivo, a tumor xenograft model was implemented with A549 cells. Intraperitoneal injec-tion with metformin (40 or 200 mg/kg/day) or PBS was started when the average tumor volumes reached 100 mm³ (11th day
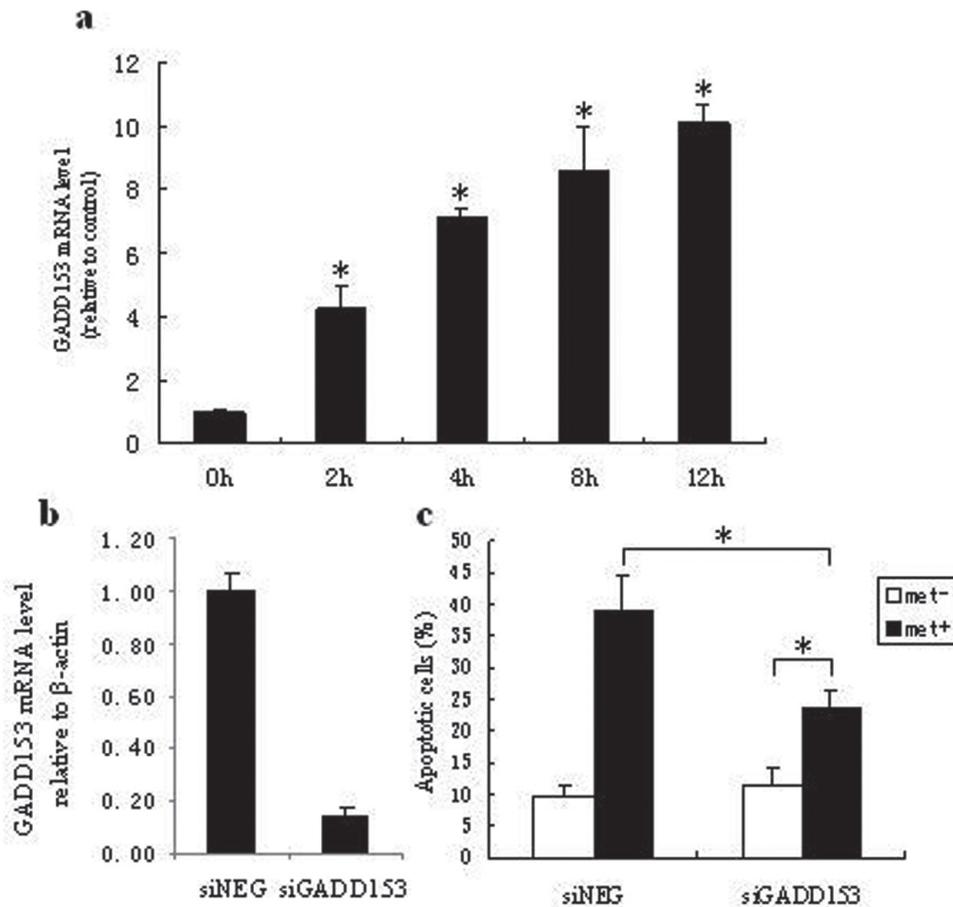
Figure 4. GADD153 amplification induced by metformin contributes to cell apoptosis. (a) Real-time PCR showed that *GADD153* mRNA level in A549 cells was significantly elevated by metformin (5 mM) in a time-dependent manner. (b) Confirmation of siRNA-mediated knockdown of *GADD153* by real-time PCR. After 48 h posttransfection, the level of *GADD153* mRNA in A549 cells transfected with siGADD153 was 86% lower than that in cells transfected with siNEG. (c) After 12 h posttransfection, the cells were treated with metformin (5 mM) or not, incubated for an additional 48 h and then harvested for apoptosis analysis by annexin V/PI staining and quantified by flow cytometry. (*$P<0.05$)

after xenograft). Neither visible toxic effect on mice nor change of animal weight was observed during treatment (data not shown). Metformin administration led to significant inhibition of tumor growth in mice compared to the control. After 1 month of treatment, the average tumor volumes of xenografts from mice treated with 40 mg/kg/day and 200 mg/kg/day metformin were 20% and 41% smaller than that of the PBS-treated tumors, respectively (Fig. 5a). Immunostaining of the tumor specimens showed that in tumors treated with metformin the expressions of Ki67, the proliferation marker, significantly decreased (Fig. 5b and 5e) while the percentages of apoptotic cells significantly elevated compared to the control (Fig. 5d and 5c).

Discussion

Recent population-based studies of type 2 diabetes patients have reported that metformin, an insulin-sensitizer agent widely used for diabetes treatment, may reduce cancer risk

and improve prognosis[5,6]. There are also growing experimental evidences that metformin may impede the growth of human cancers by either blocking cell cycle progression or inducing apoptosis, depending on the cell type investigated. In the present study using A549 and NCI-H1299 cell lines, we revealed the inhibitory effects of metformin on the viability and proliferation of human lung cancer cells and its apoptosis-inducing ability, which was further evidenced by the data obtained from A549 tumor xenografts in nude mice.

As reported, there are at least two possible mechanisms underlying the anti-proliferative effect of metformin on neoplastic cells in vivo: reduction of systemic insulin levels, which may reduce insulin-stimulated cancer cell growth [27,28], and a direct action involving AMPK activation within neoplastic cells [12]. Activation of AMPK can induce apoptosis and/or cause G1 cell cycle arrest in both malignant and nonmalignant cells [17,18]. Phosphorylated AMPK by metformin treatment observed in this study highly indicated the direct action of
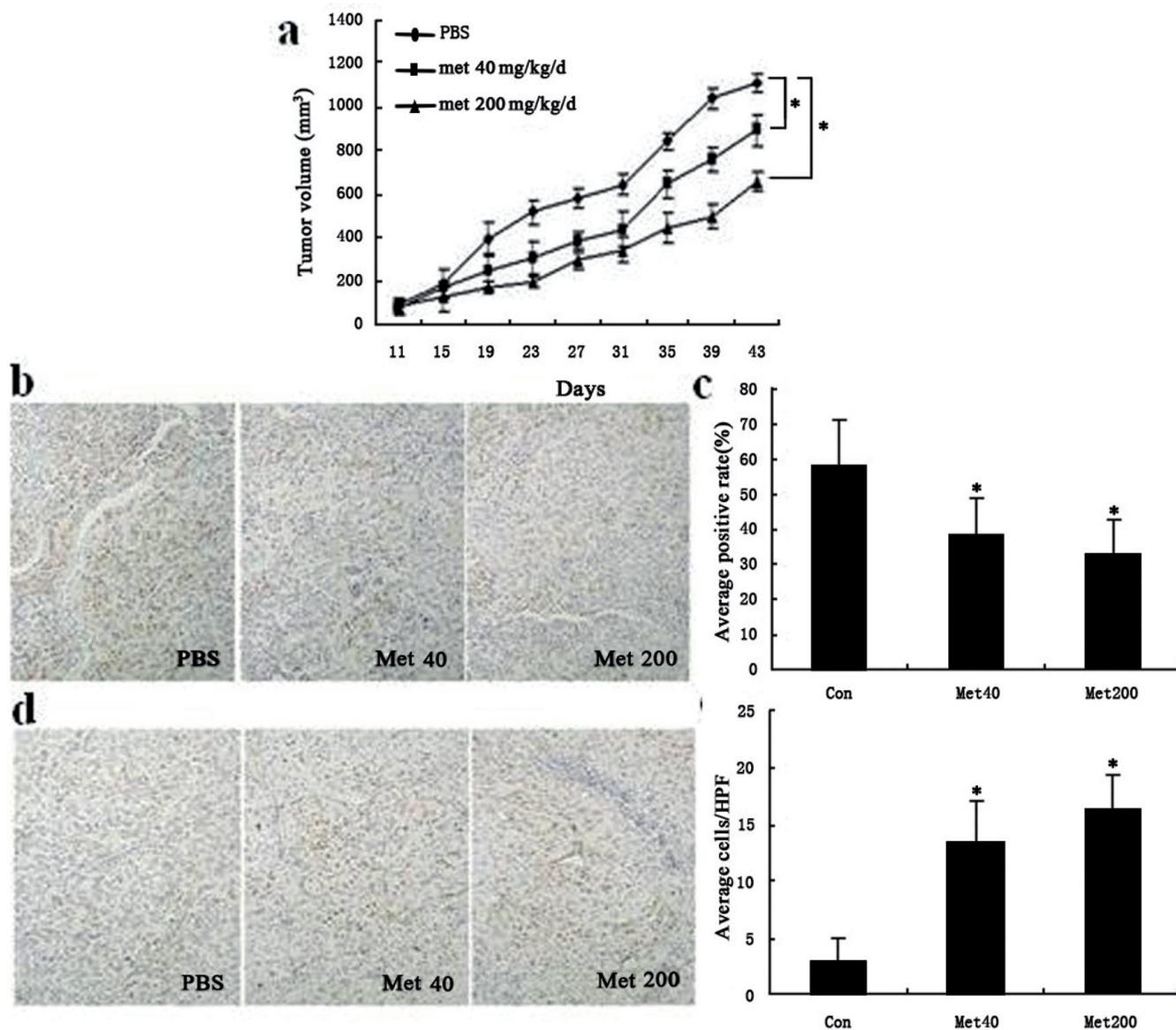
Figure 5. Effects of systemic metformin administration on tumor growth, cells proliferation and apoptosis of human lung A549 tumor xenografts in nude mice. (a) Significant growth inhibition was observed in tumors treated with metformin at a dose of either 40 or 200 mg/kg/day compared to the control. Data are the mean±SD derived from each group. (b and c) Immunohistochemistry of the tumor sections derived from mice receiving different treatments for 1 month was determined by reaction with anti-Ki67 antibody. The nuclei of cells positive for Ki67 expression were stained brown (original magnification ×100). (d and e) In situ TUNEL apoptosis assay of the same tumor sections. Apoptotic nuclei were stained dark brown (original magnification ×100). Percentage of positive cells was calculated by counting>200 cells from five randomly chosen fields in each section. (*$P<0.05$).

metformin on lung cancer cells. Using specific inhibitors, we demonstrated that activation of two MAPK family members, JNK and p38 MAPK, were involved in metformin-induced apoptosis, presumably in a caspase-dependent manner. Similar result has been seen in confluent rat glioma cell line C6 [20]. In that study, metformin was found to cause massive cell apoptosis associated with JNK activation, mitochondrial depolarization and oxidative stress. Since both JNK and p38

MAPK have been indicated the downstream mediators of AMPK-initiated apoptotic program [20-22], we suppose that activation of AMPK may contribute to lung cancer cells apoptosis induced by metformin partly through activating JNK/p38 MAPK signaling pathway.

Previous studies have reported that metformin treatment can trigger production of reactive oxygen species and reduction of blood glucose, either of which has a strong positive

impact on the expression of GADD153 [24-26]. GADD153, also known as CHOP (CEBP homology protein), is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. It is known to be rapidly induced following a variety of stresses, such as nutrient deprivation, anticancer agents treatment, and endoplasmic reticulum (ER) stress [24,26,29-34]. Overexpression of GADD153 can initiate inhibitory effect of cell growth and induce apoptosis [23,33,35,36] in many cell types. A recent study by Satoh et al [37] demonstrated that the level of GADD153 was increased in non-small cell lung cancer cells after exposure to troglitazone, an sulfonylurea that has been associated with growth inhibition of a variety of malignancies. We herein showed that metformin had a similar induction effect on GADD153 expression in lung cancer cells. Additional experiments with *GADD153* siRNA knockdown strongly suggested the involvement of increased GADD153 expression in metformin-induced cell apoptosis. Indeed, mediating ER stress [38] is believed one of the main functions of GADD153. The induction of GADD153 in response to metformin and its requirement for metformin-induced cell apoptosis suggests, therefore, that metformin possibly induces apoptosis at least partly as a result of ER stress.

Previous studies have reported association between various genes or signallings and GADD153-induced apoptosis, including Bcl-2 family members, NF-κB, p53-p21 axis, and MAPKs [34,36,39,40]. GADD153 can be post-translationally activated by p38 MAPK and has been implicated as a downstream element of MAPKs pathways [41-45]. However, MAPKs are not absolutely required for the apoptosis-inducing activity of GADD153 [46]. Our results showed that activation of JNK/p38 MAPK and GADD153 were both involved in metformin-induced apoptosis in lung cancer cells. Further studies are needed to clarify the concrete connection between them.

It is important to be noted that in most studies the antineoplastic effects of metformin were observed in pharmacological doses. For cultured cells, metformin was used at levels of 0.5-20 mM, whereas in mice the usually adopted doses were 50-750 mg/kg/day [8,47,48], which all far exceeded the recommended therapeutic doses for diabetic patients (1000-2550 mg/day based on an average patient body weight of 60 kg). Since extremely high plasma metformin levels are rarely seen in the clinical setting, it has been considered that the results obtained from cells and the model systems can not be directly extrapolated to potential effects in a clinical trial evaluating standard clinical metformin doses for cancer treatment [49]. We tried to approximate the clinical use of metformin in our study and the results showed that metformin efficiently inhibited the growth of the xenograft tumors in vivo by 20% at a lower dose (40 mg/kg/day), though not so strong as that at a higher dose (200 mg/kg/day, 41%). No visible toxic effect on mice was observed during metformin treatment, which indicated a relatively favorable toxicity profile of metformin. Our data are consistent with results obtained by Sahra et al [11]

who showed that intraperitoneal treatment with metformin at a dose of 1 mg/day led to a 35% reduction of tumor growth in mice bearing xenografts of LNCaP human prostate cancer cells. The discrepancy of drug toxicity between the two studies may be due to a cell specific effect.

In conclusion, we demonstrate for the first time that metformin inhibits the growth of lung cancer cells and induces apoptosis both in vitro and in vivo. Phosphorylation of AMPK, JNK/p38 MAPK and upregulation of GADD153 may be involved in metformin-induced cell apoptosis. Considering the low cost and satisfactory safety profile of metformin, further investigation should be designed to evaluate its potential role in treatment of lung cancer and its detailed mechanisms.

References

[1] WITTERS LA. The blooming of the French lilac. J Clin Invest 2001; 108:1105-1107.

[2] HUNDAL HS, RAMLAL T, REYES R, LEITER LA, KLIP A. Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. Endocrinology 1992; 131: 1165-1173. doi:10.1210/en.131.3.1165

[3] STUMVOLL M, NURJHAN N, PERRIELLO G, DAILEY G, GERICH JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. N Engl J Med 1995; 333: 550-554. doi:10.1056/NEJM199508313330903

[4] SHAW RJ, LAMIA KA, VASQUEZ D, KOO SH, BARDEESY N, et al. The Kinase LKB1 Mediates Glucose Homeostasis in Liver and Therapeutic Effects of Metformin. Science 2005; 310: 1642-1646. doi:10.1126/science.1120781

[5] EVANS JM, DONNELLY LA, EMSLIE-SMITH AM, ALESSI DR, MORRIS AD, Metformin and reduced risk of cancer in diabetic patients. BMJ 2005; 330: 1304-1305. doi:10.1136/bmj.38415.708634.F7

[6] BOWKER SL, MAJUMDAR SR, VEUGELERS P, JOHNSON JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. Diabetes Care 2006; 29: 254-258. doi:10.2337/diacare.29.02.06.dc05-1558

[7] ANISIMOV VN, BERSTEIN LM, EGORMIN PA, PISKU-NOVA TS, POPOVICH IG, et al. Effect of metformin on life span and on the development of spontaneousmammary tumors in HER-2/neu transgenic mice. Exp Gerontol 2005; 40: 685-693. doi:10.1016/j.exger.2005.07.007

[8] BUZZAI M, JONES RG, AMARAVADI RK, LUM JJ, DEBER-ARDINIS RJ, et al. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res 2007; 67: 6745-6752. doi:10.1158/0008-5472.CAN-06-4447

[9]   GOTLIEB WH, SAUMET J, BEAUCHAMP MC, GU J, LAU S, et al. In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. Gynecol Oncol 2008; 110: 246-250. doi:10.1016/j.ygyno.2008.04.008

[10]   DOWLING RJ, ZAKIKHANI M, FANTUS IG, POLLAK M, SONENBERG N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res 2007; 67: 10804-10812. doi:10.1158/0008-5472.CAN-07-2310

[11]   BEN SAHRA I, LAURENT K, LOUBAT A, GIORGETTI-PERALDI S, COLOSETTI P, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 2008; 27: 3576-3586. doi:10.1038/sj.onc.1211024

[12]   ZAKIKHANI M, DOWLING R, FANTUS IG, SONENBERG N, POLLAK M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 2006; 66: 10269-10273. doi:10.1158/0008-5472.CAN-06-1500

[13]   FRYER LG, PARBU-PATEL A, CARLING D. The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem 2002; 277: 25226-25232. doi:10.1074/jbc.M202489200

[14]   HAWLEY SA, GADALLA AE, OLSEN GS, HARDIE DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes 2002; 51: 2420-2425. doi:10.2337/diabetes.51.8.2420

[15]   ZHOU G, MYERS R, LI Y, CHEN Y, SHEN X, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001; 108: 1167-1174.

[16]   BOLSTER DR, CROZIER SJ, KIMBALL SR, JEFFERSON LS. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J Biol Chem 2002; 277: 23977-23980. doi:10.1074/jbc.C200171200

[17]   HADAD SM, FLEMING S, THOMPSON AM. Targeting AMPK: A new therapeutic opportunity in breast cancer. Crit Rev Oncol Hematol 2008; 67: 1-7. doi:10.1016/j.critrevonc.2008.01.007

[18]   MOTOSHIMA H, GOLDSTEIN BJ, IGATA M, ARAKI E. AMPK and cell proliferation—AMPK as a therapeutic target for atherosclerosis and cancer. J Physiol 2006; 574: 63-71. doi:10.1113/jphysiol.2006.108324

[19]   JEMAL A, SIEGEL R, WARD E, MURRAY T, XU J, et al. Cancer statistics, 2007. CA Cancer J Clin 2007; 57: 43-66. doi:10.3322/canjclin.57.1.43

[20]   ISAKOVIC A, HARHAJI L, STEVANOVIC D, MARKOVIC Z, SUMARAC-DUMANOVIC M, et al. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. Cell Mol Life Sci 2007; 64: 1290-1302. doi:10.1007/s00018-007-7080-4

[21]   KEFAS BA, CAI Y, LING Z, HEIMBERG H, HUE L, et al. AMP-activated protein kinase can induce apoptosis of insulin-producing MIN6 cells through stimulation of c-Jun-N-terminal kinase. J Mol Endocrinol 2003; 30: 151-161. doi:10.1677/jme.0.0300151

[22]   MEISSE D, VAN DE CASTEELE M, BEAULOYE C, HAINAULT I, KEFAS BA, et al. Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. FEBS Lett 2002; 526: 38-42. doi:10.1016/S0014-5793(02)03110-1

[23]   FRIEDMAN AD. GADD153/CHOP, a DNA damage-inducible protein, reduced CAAT/enhancer binding protein activities and increased apoptosis in 32D c13 myeloid cells. Cancer Res 1996; 56: 3250-3256.

[24]   LUETHY JD, HOLBROOK NJ. Activation of the gadd153 promoter by genotoxic agents: a rapid and specific response to DNA damage. Cancer Res 1992; 52: 5-10.

[25]   GUYTON KZ, XU Q, HOLBROOK NJ. Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element. Biochem. J 1996; 314: 547-554.

[26]   JOHNSSON A, STRAND C, LOS G. Expression of GADD153 in tumor cells and stromal cells from xenografted tumors in nude mice treated with cisplatin: correlations with cisplatin-DNA adducts. Cancer Chemother Pharmacol 1999; 43: 348-352. doi:10.1007/s002800050906

[27]   OSBORNE CK, BOLAN G, MONACO ME, LIPPMAN ME. Hormone responsive human breast cancer in longterm tissue culture: effect of insulin. Proc Natl Acad Sci U S A 1976; 73: 4536-4540. doi:10.1073/pnas.73.12.4536

[28]   BELFIORE A. The role of insulin receptor isoforms andhybri-dinsulin/IGF-I receptors in human cancer. Curr Pharm Des 2007; 13: 671-686. doi:10.2174/138161207780249173

[29]   KIM DG, YOU KR, LIU MJ, CHOI YK, WON YS. GADD153-mediated anticancer effects of N-(4-hydroxyphe-nyl)-retinamide on human hepatoma cells. J Biol Chem 2002; 277: 38930-38938. doi:10.1074/jbc.M205941200

[30]   CARLSON SG, FAWCETT TW, BARTLETT JD, BERNIER M, HOLBROOK NJ. Regulation of the C/EBP-related gene GADD153 by glucose deprivation. Mol Cell Biol 1993; 13: 4736-4744.

[31]   ABCOUWER SF, SCHWARZ C, MEGUID RA. Glutamine deprivation induces the expression of GADD 45 and GADD 153 primarily by mRNA stabilization. J Biol Chem 1999; 274: 28645-28651.

[32]   O'REILLY MA, STARVERSKY RJ, WATKINS RH, MANIS-CALCO WM, KENG PC. p53-independent induction of GADD45 and GADD153 in mouse lungs exposed to hypoxia. Am J Physiol Lung Cell Mol Physiol 2000; 278: 552-559.

[33]   ZINSZNER H, KURODA M, WANG X, BATCHVAROVA N, LIGHTFOOT RT, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 1998; 12: 982-995. doi:10.1101/gad.12.7.982

[34]   McCULLOUGH KD, MARTINDALE JL, KLOTZ LO, AW TY, HOLBROOK NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001; 21: 1249-1259. doi:10.1128/MCB.21.4.1249-1259.2001

[35]   LOVAT PE, OLIVERIO S, RANALLI M, CORAZZARI M, RODOLFO C, et al. GADD153 and 12-lipoxygenase mediate fenretinide-induced apoptosis of neuroblastoma. Cancer Res 2002; 62: 5158-5167.

[36] KIM SH, HWANG CI, JUHNN YS, LEE JH, PARK WY, et al. GADD153 mediates celecoxib-induced apoptosis in cervical cancer cells. Carcinogenesis 2007; 28: 223-231. doi:10.1093/carcin/bgl227

[37] SATOH T, TOYODA M, HOSHINO H, MONDEN T, YAMADA M, et al. Activation of peroxisome proliferator-activated receptor-stimulates the growth arrest and DNA-damage inducible 153 gene in non-small cell lung carcinoma cells. Oncogene 2002; 21: 2171-2180. doi:10.1038/sj.onc.1205279

[38] RON D, HABENER JF. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev 1992; 6: 439-453. doi:10.1101/gad.6.3.439

[39] NOZAKI S, SLEDGE Jr GW, NAKSHATRI H. Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death. Oncogene 2001; 20: 2178-2185. doi:10.1038/sj.onc.1204292

[40] ZU K, BIHANI T, LIN A, PARK YM, MORI K, et al. Enhanced selenium effect on growth arrest by BiP/GRP78 knockdown in p53-null human prostate cancer cells. Oncogene 2006; 25: 546-554.

[41] SARKAR D, SU ZZ, LEBEDEVA IV, SAUANE M, GOPALKRISHNAN RV, et al. mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. Proc Natl Acad Sci USA 2002; 99: 10054-10059. doi:10.1073/pnas.152327199

[42] OH-HASHI K, MARUYAMA W, ISOBE K. Peroxynitrite induces GADD34, 45, and 153 VIA p38 MAPK in human neuroblastoma SH-SY5Y cells. Free Radical Biol Med 2001; 30: 213-221. doi:10.1016/S0891-5849(00)00461-5

[43] KULTZ D, MADHANY S, BURG MB. Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. J Biol Chem 1998; 273: 13645-13651. doi:10.1074/jbc.273.22.13645

[44] BRENNER B, KOPPENHOEFER U, WEINSTOCK C, LINDERKAMP O, LANG F, et al. Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. J Biol Chem 1997; 35: 22173-22181. doi:10.1074/jbc.272.35.22173

[45] WANG XZ, RON D. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. Science 1996; 272: 1347-1349. doi:10.1126/science.272.5266.1347

[46] MAYTIN EV, UBEDA M, LIN JC, HABENER JF. Stress-inducible transcription factor CHOP/GADD153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. Exp Cell Res 2001; 267: 193-204. doi:10.1006/excr.2001.5248

[47] ALGIRE C, ZAKIKHANI M, BLOUIN MJ, SHUAI JH, POLLAK M. Metformin Attenuates the Stimulatory Effect of a High Energy Diet on in vivo LLC1 Carcinoma Growth. Endocr Relat Cancer 2008;15: 833-839. doi:10.1677/ERC-08-0038

[48] PHOENIX KN, VUMBACA F, CLAFFEY KP. Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model. Breast Cancer Res Treat 2009; 113: 101-111. doi:10.1007/s10549-008-9916-5

[49] STAMBOLIC V, WOODGETT JR, FANTUS IG, PRITCHARD KI, GOODWIN PJ. Utility of metformin in breast cancer treatment, is neoangiogenesis a risk factor? Breast Cancer Res Treat 2009; 114: 387-389. doi:10.1007/s10549-008-0015-4