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Inhibition of AKT Enhances the Sensitivity of NSCLC Cells to Metformin

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Abstract. *Background/aim: Metformin is an antidiabetic drug that has been reported to have antitumor activity in many cancer types. This study investigated the molecular mechanisms underlying the antitumor effect of metformin. Materials and Methods: We investigated the molecular mechanism of the antitumor effect of metformin alone and in combination with AKT serine/threonine kinase (AKT) inhibition via cell viability and western blot analyses. Results: Notably, metformin increased the phosphorylation of AKT at serine 473 using protein array screening. Metformin-induced AKT activation was markedly suppressed by siRNA targeting activating transcription factor 4 (ATF4) but not AMP-activated protein kinase α. These results indicate that AKT activation by metformin was induced in an ATF4-dependent and AMPKα-independent manner. Treatment using metformin combined with MK-2206, an AKT inhibitor, or a siRNA for AKT markedly reduced the viability of cells compared with those cells treated with these agents alone. In addition, MK-2206 increased cell sensitivity to the combination of metformin with ionizing radiation or cisplatin. Conclusion: Inhibition of AKT can enhance the antitumor effect of metformin and would be a promising strategy to sensitize non-small-cell lung cancer to a combination of metformin with radiation or cisplatin.*

Lung cancer has a high incidence rate and is the main cause of cancer mortality (1). Non-small-cell lung cancer (NSCLC) is the most commonly diagnosed type of lung cancer and accounts for approximately 85% of all cases (2). The majority of patients with NSCLC are diagnosed in advanced disease stages without the opportunity for surgical resection, thus leading to poor prognosis (3). Despite substantial improvements in therapy, the 5-year overall survival for patients with NSCLC does not exceed 25% (4, 5).

Metformin is currently used as a first-line drug in the treatment of type II diabetes mellitus and has shown anticancer effects in both *in vitro* and *in vivo* preclinical studies in lung cancer (6). In addition to monotherapy, the combination of metformin with other treatment strategies was shown to potentiate the anticancer effect synergistically (7, 8). Furthermore, a clinical trial also showed promising results, in which combined treatment with metformin and cisplatin markedly improved overall survival in patients with metastatic NSCLC without a significant increase in toxicity (9). However, the molecular mechanisms underlying these effects of metformin are complex and elusive. The most well-known anticancer effect of metformin is the activation of AMP-activated protein kinase α (AMPKα) *via* inhibition of complex I in the mitochondrial electron transport chain (10). Activated AMPKα regulates multiple signaling pathways involved in cancer cell proliferation and survival, including mechanistic target of rapamycin complex 1 (mTORC1) (11). Metformin-induced suppression of mTORC1 by AMPKα activation inhibits cancer cell proliferation and induces cell death by reducing the phosphorylation of its downstream targets, including ribosomal S6 kinase 1 and eIF-4E-binding protein 1 (12).

In this study, we aimed to investigate the molecular mechanisms underlying the antitumor effect of metformin. Moreover, we investigated the efficacy of metformin combined with AKT serine/threonine kinase (AKT) inhibition.

Materials and Methods

Cell culture, reagents and γ-ionizing radiation. H1299 NSCLC cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Roswell Park Memorial Institute 1640 medium (#LM011-01; Welgene, Gyeongsangbuk-do,

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Republic of Korea) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) at 37˚C in a humidified incubator with CO_2. Metformin, phenformin, thiazolyl blue tetrazolium bromide (MTT) and *cis*-diammineplatinum (II) dichloride were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). AKT inhibitor MK-2206 was obtained from Selleckchem (Houston, TX, USA), and the stock solution was prepared in DMSO at 10 mM at −20˚C. For irradiation (IR) treatment, H1299 cells were irradiated with 5 Gy using a Gammacell 3000 Elan irradiator with a ^{137}Cs γ-radiation source (Atomic Energy of Canada Limited, Chalk River, ON, Canada).

Measurement of cell viability. H1299 cells were seeded in a 6-well plate and grown overnight until they reached approximately 50% cell confluence. The cells were then treated with and without 5 Gy IR with 10 mM metformin alone and in combination with 10 μM MK-2206 for 30 h. Cell viability was subsequently assessed by measuring the mitochondrial conversion of MTT. The proportion of converted MTT was calculated by measuring the absorbance at 570 nm. The results are expressed as the percentage reduction in MTT, assuming that the absorbance of the control cells was 100%. The MTT experiments were repeated three times.

Phospho-kinase array experiments. The phosphorylation levels of 43 kinases were measured using Human Phospho-Kinase Array Kit (ARY003, Proteome Profiler™; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, H1299 cells were treated with 10 mM metformin for 6 h to obtain cell lysate. The arrays were blocked with blocking buffer and then incubated with 200 μg of cell lysate overnight at 4˚C. The arrays were washed, incubated with horseradish peroxidase-conjugated phospho-kinase antibody, and treated with SuperSignal West Pico chemiluminescent substrates (Pierce; Thermo Fisher Scientific) to produce a chemiluminescence signal which was captured on X-ray film. The phospho-kinase array experiments were repeated twice.

Transient transfection. *AKT* (#6211S) and control (#6568) siRNAs were obtained from Cell Signaling Technology (Beverly, MA, USA). Activating transcription factor 4 *(ATF4)* (#sc-35112), *AMPKA1/2* (#sc-45313) and control (#sc-37007) siRNAs were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). siRNA transfection of H1299 NSCLC cells was performed using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific). For western blot analysis and MTT assay, cells were transfected with siRNA for 24 h and then treated with 10 mM metformin for 24 h or 30 h. The experiments were repeated three times.

Western blot analysis. Cells were lysed with lysis buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Penzberg, Germany) for 30 min at 4˚C. Cell lysates were clarified by centrifugation at 12,000 × *g* for 20 min at 4˚C, and the protein concentrations were measured by Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (5-15 μg) were separated using 6-14% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes, followed by immunoblotting with specific primary and horseradish peroxidase-conjugated secondary antibodies. The blots were developed using SuperSignal West Pico chemiluminescent substrates (Pierce; Thermo Fisher Scientific) to produce a chemiluminescence signal which was captured on X-ray film. Antibodies to AKT, phospho (p)-AKT, AMPKα, and p-AMPKα (Thr172) were obtained from Cell Signaling Technology. ATF4 antibody was obtained from Santa Cruz Biotechnology. Antibody to β-actin was obtained from Sigma-Aldrich (Merck KGaA). All the western blot analysis were repeated three times.

Statistical analysis. The results are expressed as the mean±standard deviation of three independent experiments. Statistical analyses were performed *via* a one-way analysis of variance followed by Tukey's *post hoc* test with GraphPad Prism software (Version 5.0; GraphPad Software Inc., San Diego, CA, USA). Significance was accepted at $p<0.05$.

Results

Metformin induced AKT activation in H1299 NSCLC cells. To investigate the effects of metformin on the activation of protein kinases, we employed a phosphokinase array which detects the levels of phosphorylation of 43 kinase phosphorylation sites. Interestingly, the phosphorylation of AKT at serine 473 was increased in H1299 cells treated with metformin (Figure 1A). Western blot analysis demonstrated that metformin induced AKT phosphorylation at serine 473 in a dose- and time-dependent manner (Figure 1B and 1C). Phenformin, a bi-guanide derivative similar to metformin, also increased the phosphorylation of AKT at serine 473 (Figure 1B and 1C). Full activation of AKT was reported to require phosphorylation of serine 473 in the hydrophobic motif (13). These data suggest that metformin can induce AKT activation in H1299 NSCLC cells.

Metformin induced AKT activation by ATF4 in H1299 NSCLC cells. ATF4 has been reported as a downstream mediator responsible for the metabolic effects of metformin (14, 15), and we also found that metformin induced ATF4 expression (Figure 2A). Thus, we examined whether ATF4 is involved in metformin-induced AKT activation. Knockdown of *ATF4* by siRNA attenuated phosphorylation of AKT serine 473 induced by metformin, suggesting that metformin induces AKT activation through ATF4 (Figure 2B). Since metformin activates AMPKα, the cellular energy sensor (16), we investigated whether AMPKα is involved in metformin-induced AKT activation. Metformin increased the phosphorylation of AMPKα at threonine 172 (Figure 2B). However, the down-regulation of AMPKα did not alter AKT phosphorylation induced by metformin (Figure 2C) and the knockdown of *ATF4* did not affect metformin-induced AMPK phosphorylation (Figure 2B). These data suggest that metformin induced AKT activation in an ATF4-dependent and AMPKα-independent manner.

Inhibition of AKT enhanced the sensitivity of NSCLC cells to metformin. Next, we investigated whether inhibition of AKT enhanced cell sensitivity to metformin. H1299 cells were transfected with *AKT* siRNA and then treated with metformin.

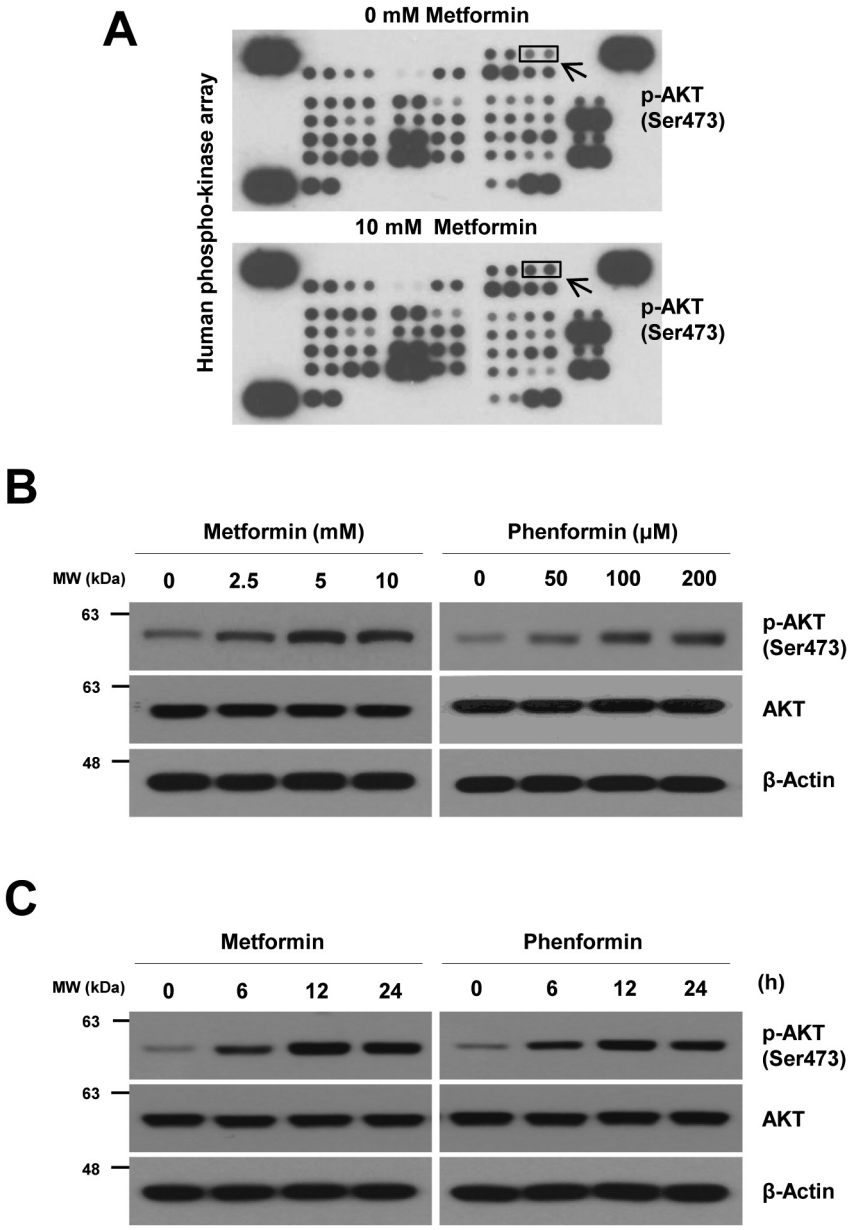

Figure 1. *Metformin induces activation of AKT serine/threonine kinase (AKT) in H1299 cells. A: H1299 cells were treated with 10 mM metformin for 6 h then cell lysates were prepared and analyzed using a human phosphokinase array kit. B: H1299 cells were treated with the indicated concentrations of metformin or phenformin for 24 h then assayed for AKT activation by phosphorylation. C: H1299 cells were treated with 10 mM metformin or 200 μM phenformin for the indicated time then assayed for AKT activation by (phosphorylation) by western blot analysis. β-Actin was used as a loading control. The blot is representative of three independent experiments.*

AKT siRNA reduced AKT expression and metformin-induced AKT phosphorylation (Figure 3A, upper panel). *AKT* siRNA did not affect metformin-induced AMPKα phosphorylation nor ATF4 expression (Figure 3A, upper panel). Knockdown of *AKT* with siRNA increased H1299 NSCLC cell sensitivity to metformin (Figure 3A, lower panel).

Similarly, MK-2206, an allosteric AKT inhibitor, reduced metformin-induced phosphorylation of AKT (Figure 3B, upper panel) but did not affect metformin-induced AMPKα phosphorylation nor ATF4 expression (Figure 3B, upper panel). MK-2206 also enhanced the sensitivity of these cells to metformin (Figure 3B, lower panel). This suggests that

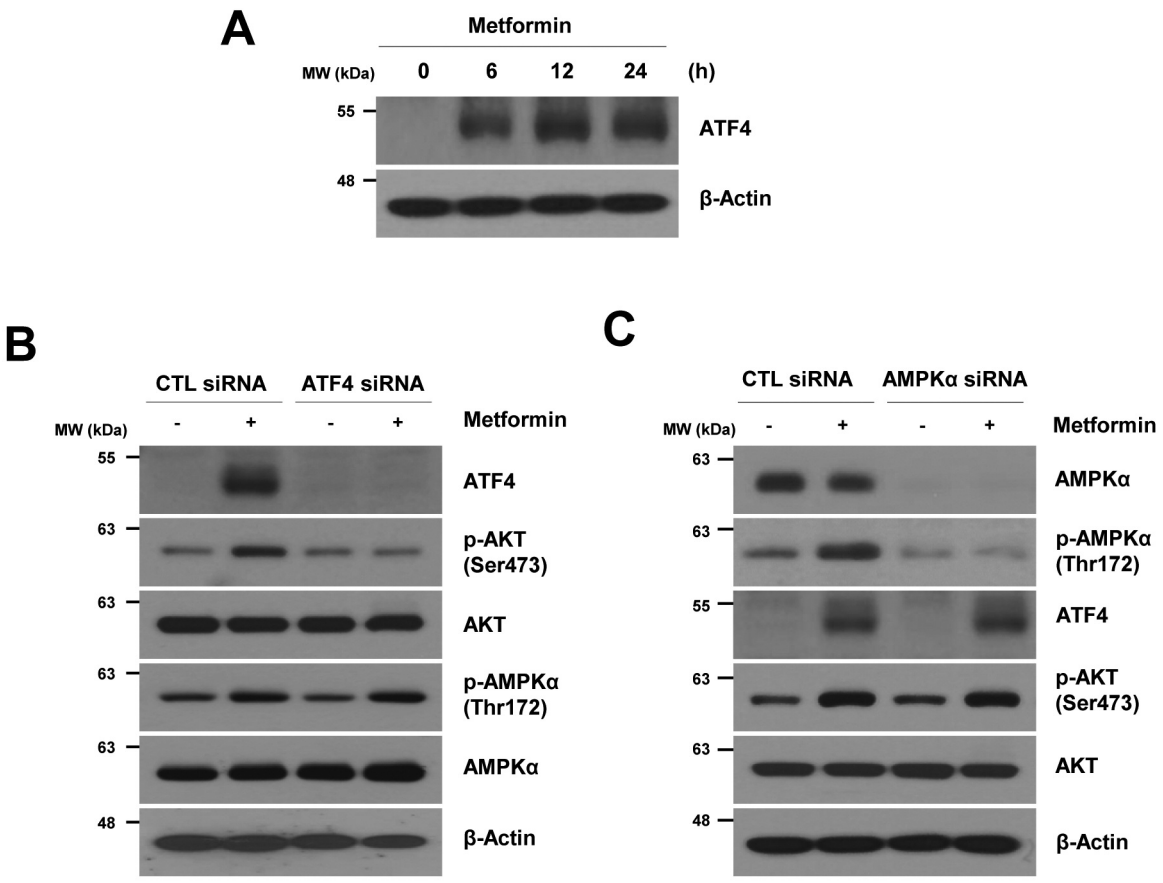

Figure 2. *Metformin induces activation of AKT serine/threonine kinase (AKT) by activating transcription factor 4 (ATF4) in H1299 cells. A: H1299 cells were treated with 10 mM metformin for the indicated time. B, C: H1299 cells were transfected with control, ATF4 or AMP-activated protein kinase (AMPKα) small interfering RNA (siRNA) for 24 h and then treated with 10 mM metformin for 24 h. The indicated protein levels were estimated by western blot analysis. β-Actin was used as a loading control. The blot is representative of three independent experiments. CTL: Control.*

inhibition of AKT improves the efficacy of metformin against H1299 NSCLC cells.

MK-2206 increased sensitivity of H1299 NSCLC cells to the combination of metformin with IR or cisplatin. Conventional chemotherapy and radiotherapy represent the mainstay treatments for patients with NSCLC (17, 18), however, resistance to chemo-/radiotherapy is the main cause of therapeutic failure (19). As shown in Figure 4, a less than 20% decrease in cell viability was observed in cells treated with 5 Gy IR or 5 μM cisplatin for 24 h. The combination of metformin with IR or cisplatin reduced cell viability more than each treatment alone. AKT inhibitor MK-2206 effectively increased sensitivity of cells to the combination of metformin with IR or cisplatin. These data suggest that inhibition of AKT increases the sensitivity of H1299 NSCLC cells to the combination of metformin with IR or cisplatin.

Discussion

Metformin is a widely used antidiabetic drug that has been reported to have antitumor activity in many cancer types, including NSCLC (20, 21). Interest in the potential antitumor and cancer-prevention effects of metformin is based on numerous clinical trials that showed a significant reduction in cancer incidence and mortality in diabetic patients treated with metformin (22). Interestingly, we found that metformin induced AKT activation in a dose- and time-dependent manner by phosphokinase array and western blot analysis (Figure 1). Phenformin, a bi-guanide derivative similar to metformin, also increased AKT activation (Figure 1).

ATF4 is the master regulator of the integrated stress response, which is an adaptive pathway that is triggered by multiple stressors (23). ATF4 has been reported to be a

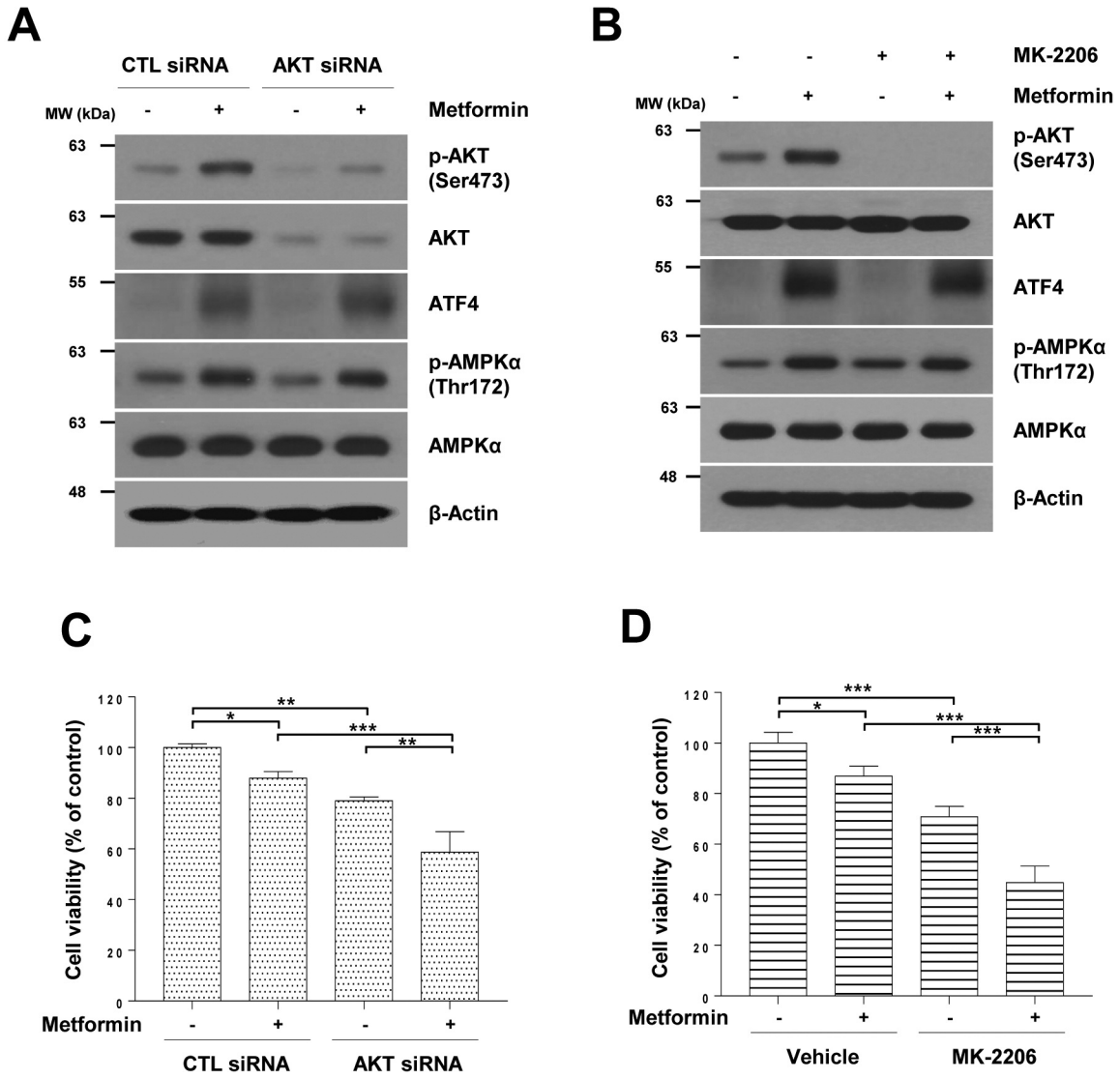

Figure 3. *Inhibition of AKT serine/threonine kinase 1 (AKT) increased cell sensitivity to metformin. A: H1299 cells were transfected with control or AKT small interfering RNA (siRNA) for 24 h followed by treatment with 10 mM metformin for 30 h then levels of AKT, activating transcription factor 4 (ATF4) and AMP-activated protein kinase (AMPKα) were estimated by western blot analysis (upper panel) and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (lower panel). B: H1299 cells were treated with 10 mM metformin with/without 10 μM AKT inhibitor MK-2206 for 30 h then levels of AKT, ATF4 and AMPKα were estimated by western blot analysis (upper panel) and cell viability was determined (lower panel). β-Actin was used as a loading control. The blots are representative of three independent experiments. Data are presented as the mean±SD. Significantly different at: *p<0.5, **p<0.01, ***p<0.001. CTL: Control.*

downstream mediator responsible for the metabolic effects of metformin (14, 15). We found that metformin also induced ATF4 expression (Figure 2A). Knockdown of *ATF4* by siRNA attenuated AKT serine 473 phosphorylation induced by metformin (Figure 2B), suggesting that metformin induces AKT activation through ATF4.

A major mechanism of action of the antitumor effect of metformin is linked to AMPK activation, which in turn inhibits the mTOR signaling pathway (24, 25). As shown in Figure 2B and C, metformin induced AMPK activation, apparently without the involvement of AMPK. These data suggest that metformin induces AKT activation in an AMPK-independent manner.

Accumulating evidence has indicated that the induction of AKT activation promotes acquired resistance to treatment with radiation, chemotherapy, and targeted therapy (26).

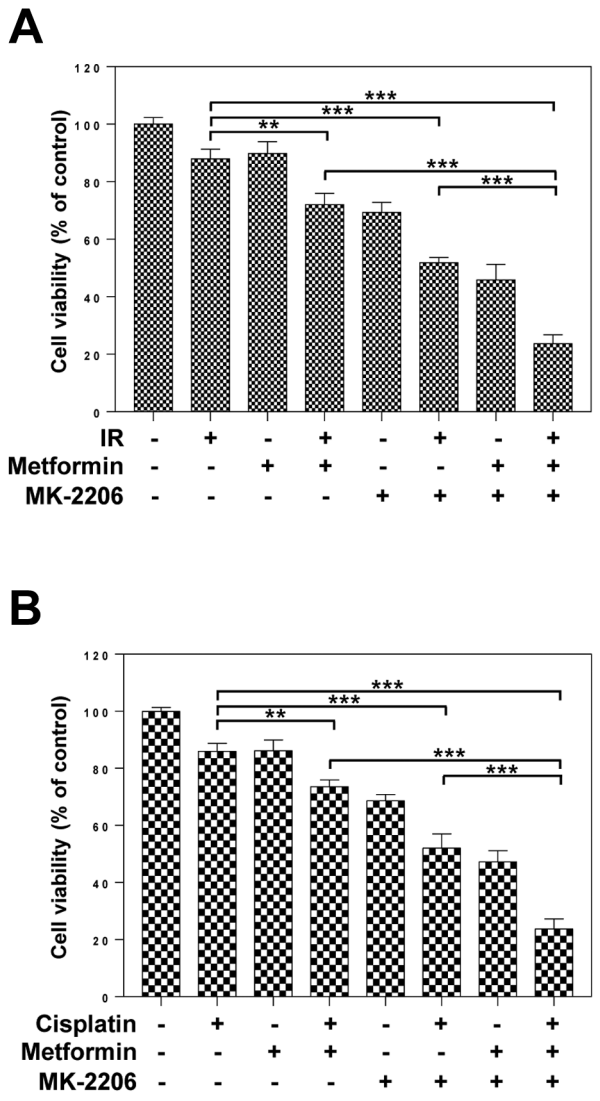

Figure 4. *MK-2206, an allosteric AKT serine/threonine kinase 1 (AKT) inhibitor, increased sensitivity to the combination of metformin with ionizing radiation (IR) or cisplatin. A: H1299 cells were treated with 5 Gy IR with/without 10 mM metformin alone and in combination with 10 μM MK-2206 for 30 h. B: H1299 cells were treated with 5 μM cisplatin alone and in combination with 10 mM metformin or 10 μM MK-2206 for 30 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Significantly different at: **p<0.01, ***p<0.001.*

Therefore, inhibiting AKT activation may be a valid approach to treating cancer and improving the efficacy of anticancer therapy. Treatment with the AKT inhibitor MK-2206 or *AKT* siRNA enhanced cell sensitivity to metformin (Figure 3), suggesting that the inhibition of AKT improves the efficacy of metformin. The combination of metformin with IR or cisplatin reduced cell viability more than IR or cisplatin alone (Figure 4). Cisplatin and IR were also reported to induce AKT activation in several cancer types (27, 28). Inhibition of AKT effectively increased cell sensitivity to the combination of metformin with IR or cisplatin (Figure 4).

Our results suggest that inhibition of AKT can enhance the antitumor efficacy of metformin and would be a promising strategy for sensitizing NSCLC to a combination of metformin with radiation or platinum-based chemotherapeutic drugs.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

Hyeon-Ok Jin and In-Chul Park developed the concept and designed the study. Se-Kyeong Jang, Sung-Eun Hong, Da-Hee Lee, Ji Yea Kim, Ji-Young Kim and Hyeon-Ok Jin carried out the experiments. Jungil Hong provided technical support and conceptual advice. Hyeon-Ok Jin and In-Chul Park wrote the article. All Authors read and approved the final article.

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