

p53 deficiency resulted in enhanced IGF-1R signaling pathway inhibition by metformin in lung adenocarcinoma cells

Wei Dong¹, Yunxia Zhao², Hongxin Cao³, Zhinan Wu¹ and Jiajun Du¹

¹Department of Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China

²Department of Neurology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China

³Department of Oncology, Shandong University Qilu Hospital, Shandong University, Jinan, China

Correspondence to: Jiajun Du, email: dujiajun@sdu.edu.cn
Wei Dong, email: dongwei318@163.com

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ABSTRACT

Research in recent years revealed that the antidiabetic drug metformin has some anticancer properties, and p53 plays an important role in the metformin-induced tumor inhibition. But less clear is the mechanism by which p53 status affects metformin mediated cancer cell responses. In this study, the effect of metformin on cancer cells with different p53 genotypes was investigated, and we found metformin could inhibit cancer cell proliferation especially in p53-deficient cells. So a paired isogenic non-small cell lung cancer cell lines H1299 p53^{+/+} and H1299 p53^{-/-} was involved to explore the possible mechanisms. We detected the differences in cell proliferation and solid tumor formation induced by metformin, furthermore, the signaling transduction of insulin like growth factor-1 receptor (IGF-1R) was evaluated, which was essential in cancer transformation and progression. Our results indicated that p53 deficiency lead to more efficient inhibition of IGF-1R signaling transduction, receptor degradation and ubiquitination induced by metformin, which resulted in enhanced cell apoptosis and proliferation inhibition *in vitro*, and more significant solid tumor growth retardation and cancer cell death *in vivo*. Although more cell lines and multiple experiments were required to make a general conclusion, our study suggested the enhanced inhibition of IGF-1R signaling pathway contribute to the metformin's selective toxicity in p53-deficient lung adenocarcinoma cells.

INTRODUCTION

The biguanide metformin is the most widely used treatment drug for type 2 diabetes. It is believed to exert its effect by reducing hepatic glucose production and increasing insulin sensitivity as well as glucose use by peripheral tissues [1]. Recent studies showed encouraging findings that metformin could reduce cancer risk or improve cancer prognosis both *in vitro* and *in vivo* [2]. Two types of distinct but not exclusive anticancer effect were concluded: first, by decreasing insulinemia and glycaemia, metformin could block PI3K/MAPK signaling pathway implicated in cell growth; second, through the activation of the AMPK pathway, metformin could directly act on

cancer cells by targeting various pathways including tumor metabolism, inflammation, angiogenesis and cancer stem cells [3]. The AMPK pathway was agreed to play the major part in the anticancer mechanisms of metformin.

The TP53 tumor suppressor gene is one of the most frequently (>50%) mutated genes in lung cancer [4], which has also been associated with poor progress and therapy resistance [5]. Generally, functional p53 was crucial in the regulation of cell cycle, apoptosis and senescence in response to DNA damage, hypoxia, and oncogenic activation [6]. Also as an important regulator of cancer cell metabolism [7], the loss of p53 in cancer cells might be a main force behind the acquisition of glycolytic phenotype [8], lead to the abnormality in cancer cell glucose metabolism. Recent reports have revealed

that p53 plays an important role in the metformin-induced inhibition of proliferation and AMPK activation [3, 9, 10]. And data from present study demonstrated p53 knockdown or mutation has a negative effect on metformin-induced growth inhibition, senescence and apoptosis [11]. However, the possible mechanism by which p53 status influenced metformin mediated cellular responses is still an important question to be addressed.

The insulin like growth factor-1 receptor (IGF-1R) is an important upstream regulator of AMPK pathway [12], and IGF-1R signaling plays a critical role in cancer transformation and progression [13]. Overexpression of IGF-1R is observed in wide range of human cancers including lung cancer, and it is becoming one of the most intensively investigated molecular targets in oncology [14]. Recently, there are researches on the emerging effects of metformin on IGF-1R system. In our previous studies, we found metformin could inhibit IGF-1R signaling pathway and have synergistic effects with IGF-1R antibody in both small cell and non-small cell lung cancer cell lines [15, 16]. And similar regulation effect of metformin on IGF signaling was found in endometrial cancer cells [17].

In this study, the proliferation inhibition of metformin on cancer cells with different p53 genotypes was investigated, and a paired isogenic cell lines H1299 p53^{+/+} and H1299 p53^{-/-} was involved to explore the difference caused by p53 deficiency *in vitro* and *in vivo*. Moreover, we detected the changes in IGF-1R signaling pathway to explore the possible mechanisms of p53 status in metformin-induced antitumor effects.

RESULTS

Metformin-induced proliferation inhibition in cancer cells with different p53 status

The p53 expression and transcription level in five different non-small cell lung cancer cell lines were checked by WB and RT-PCR. In accordance with previous researches, p53 protein and mRNA was detected in A549 and H460 cells (p53 wild type), but not in H520, H1975 (mutant type) and H1299 (deleted type). The cell viability and IC₅₀ of metformin were detected by PrestoBlue Cell Viability Assay, and we found that the p53 mutant and deleted type cancer cells (H520, H1975 and H1299) were less resistance to metformin induced antitumor effects, with statistically significant lower metformin half maximal inhibitory concentration (IC₅₀) and cell viability curves (Figure 1A&1B). Then flow cytometry was performed to evaluate the metformin induced apoptosis of cancer cells with different p53 status, and we found both the early and late cell apoptosis of p53 mutant and deleted type cancer cells were significantly increased than p53 wild type cancer cells (Figure 1C).

Cancer cell proliferation and apoptosis of H1299 p53^{+/+} and H1299 p53^{-/-}

Since cancer cells with different p53 status showed significant difference in the treatment reaction to metformin, a paired isogenic H1299 p53^{+/+} and H1299 p53^{-/-} was enlisted in further experiment to confirm the difference caused by p53 deficiency and to explore the possible mechanism.

The cell viability and apoptosis experiments were repeated with new cell lines, and once again, metformin significantly inhibit cancer cells proliferation especially in p53-deficient cells, more significant reduction in cell growth and increased cell death was detected, and dose-dependent inhibition was shown in Figure 2.

Solid tumor formation and cancer cell apoptosis

In order to investigate the possible changes in tumor formation ability of cancer cells with different p53 status, we subcutaneously transplanted H1299 p53^{+/+} and p53^{-/-} cells in BALB/c-nu mice, and the inhibition of solid tumor formation by metformin was detected. The tumor growth in size and weight was recorded and cancer cell apoptosis was evaluated. We found that metformin could significantly inhibit solid tumor growth and induce more cancer cell apoptosis in both H1299 p53^{+/+} and p53^{-/-} cells compared to control groups, however, more significant retardation of tumor development and cancer cell death was found in the tumor model of p53-deficient cells (Figure 3).

IGF-1R tyrosine phosphorylation and signaling transduction

To explore the possible mechanism by which p53 status affect the antitumor reactions induced by metformin, we investigated the difference of H1299 p53^{+/+} and p53^{-/-} cells in IGF-1R signaling pathway. Following serum starvation for 12 h, H1299 p53^{+/+} and p53^{-/-} cell lines were treated with 50 ng/ml IGF-1 or first treated with 3 mM metformin for 30 min and then stimulated with 50 ng/ml IGF-1 for 2, 5, 10, 30 and 60 min. As seen in Figure 4, metformin could significantly decrease the IGF-1 (the IGF-1R ligand) induced IGF-1R tyrosine phosphorylation in both the two cell lines. And also the IGF-1 induced Akt and ERK activation was significantly inhibit, which are the two main proteins of IGF-1R signaling transduction. Moreover, there was significant difference in both IGF-1R phosphorylation and signaling transduction between the two cell lines with or without p53 deficiency (Figure 4C). It could be concluded that metformin could more efficiently inhibit IGF-1R tyrosine phosphorylation and signaling transduction in p53-deficient cancer cells.

IGF-1R degradation and ubiquitination

Receptor degradation and ubiquitination both were important biological behaviors, and could determine the receptor activity to some extent. To detect the IGF-1R degradation, H1299 p53^{+/+} and p53^{-/-} cells were treated with 100 ng/ml IGF-1 or 5 mM metformin in serum free medium for 0, 6, 12, and 24 h. We found that metformin significantly down-regulated the IGF-1R expression as the ligand IGF-1 (Figure 5). For the ubiquitination, cells were treated with 100 ng/ml IGF-1 or 5 mM metformin for 10 min after 24 h serum starvation, and then immunoprecipitation and immunoblotting as above. The results indicated that metformin could induce the IGF-1R

ubiquitination after 10 min stimulation in both the two cell lines. Moreover, ubiquitination induced by metformin was much stronger in H1299 p53^{-/-} cells, which indicated metformin-mediate IGF-1R ubiquitination was enhanced in the absent of p53 protein.

DISCUSSION

Metformin has recently received increased attention for its potential antitumor effects in clinical and experimental studies. The anticarcinogenic effects of metformin have been attributed including activation of AMPK pathway, induction of cell cycle arrest and/

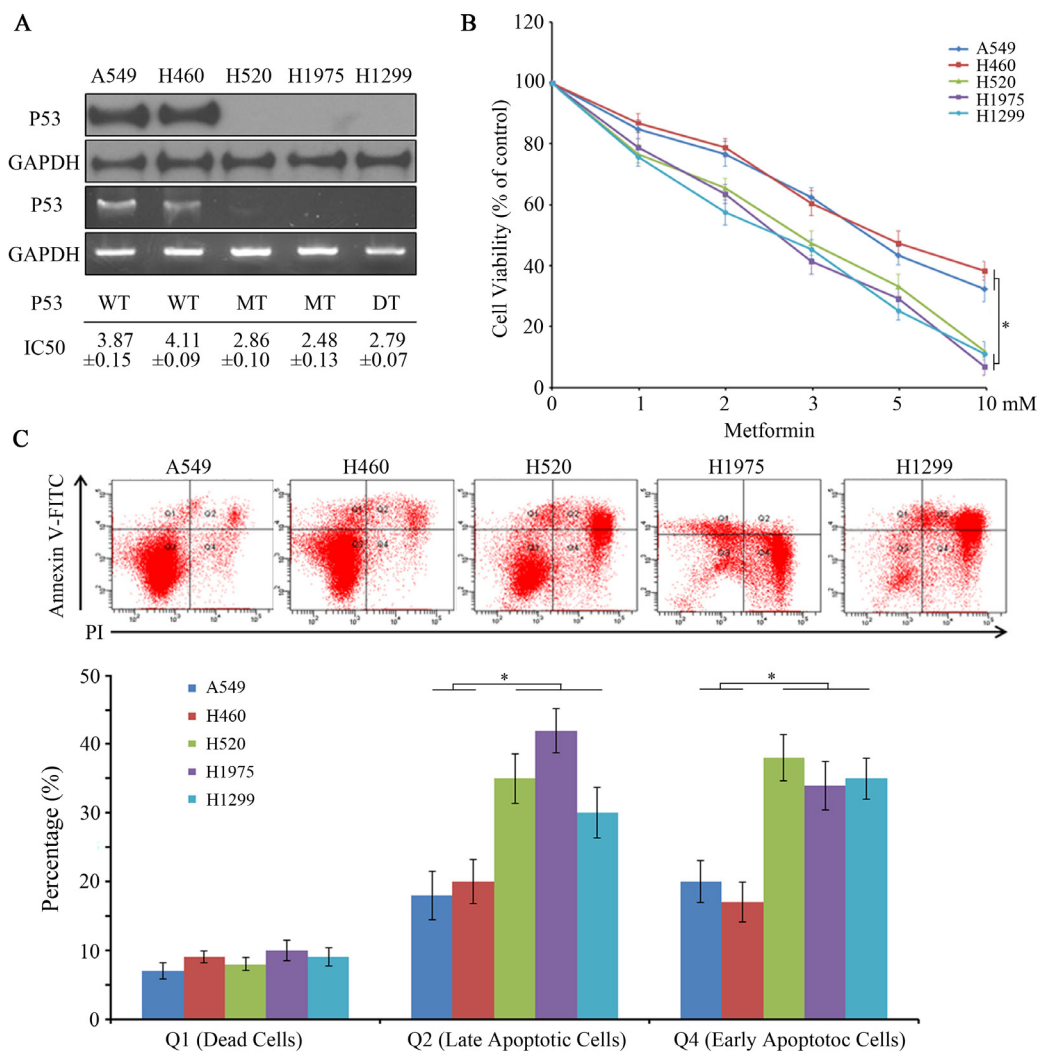


Figure 1: Metformin induced proliferation inhibition in non-small cell lung cancer cell lines. (A) Five different non-small cell lung cancer cells A549, H460, H520, H1975 and H1299 were detected by WB and RT-PCR for p53 expression and transcription, and metformin half maximal inhibitory concentration (IC50) for all cell lines was quantified as listed. (B) Cells were treated with Metformin of different concentration for 24 h and cell viability was measured by PrestoBlue Cell Viability Assay. The number of viable cells following Metformin treatment was presented as percentage of untreated cells. Data were presented as mean ± S.E.M. (C) Cells were cultured in 6 well plates and 3mM metformin was added to the wells for 24 h. After that cells were collected in tubes and treated in accordance with Annexin V-FITC/PI apoptosis detection kit. The percentage of cells labeled as Annexin V (+) PI (-), Annexin V (+) PI (+) and Annexin V (-) PI (+) was investigated. Overall, there were significant differences between p53 wild type cells (A549 and H460) and p53 mutant/deleted type cells (H520, H1975 and H1299) in either cell viability or early/late cell apoptosis (*P<0.05).

or apoptosis, inhibition of protein synthesis, reduction in circulating insulin levels, inhibition of the unfolded protein response (UPR), activation of the immune system, and eradication of cancer stem cell (well reviewed in [1, 3, 18]). Besides these, in our study we found metformin could inhibit phosphorylation of IGF-1R and downstream Akt and ERK signaling, induce significant IGF-1R degradation and ubiquitination, which lead to cancer cell proliferation inhibition *in vitro* and solid tumor growth retardation *in vivo*. Moreover, we found p53 played an important role in these metformin induced IGF-1R dependent tumor suppression.

Recent studies have revealed the undeniable fact that most human cancers display enhanced IGF-1R concentrations and express high IGF-1R mRNA levels [19, 20]. IGF-1R activation initiates downstream cascades which are essential in cancer cell metabolism, mainly including phosphatidylinositol-3-Kinase (PI3K), Akt and mTOR; and Ras, Raf and MAPK/ERK [21]. Activation of these pathways led to oncogenic transformation, proliferation and survival of cancer cells [22]. In our study, we found metformin could significantly inhibit IGF1 (the ligand) induced IGF-1R activation and signaling transduction, and the downstream Akt and ERK activation

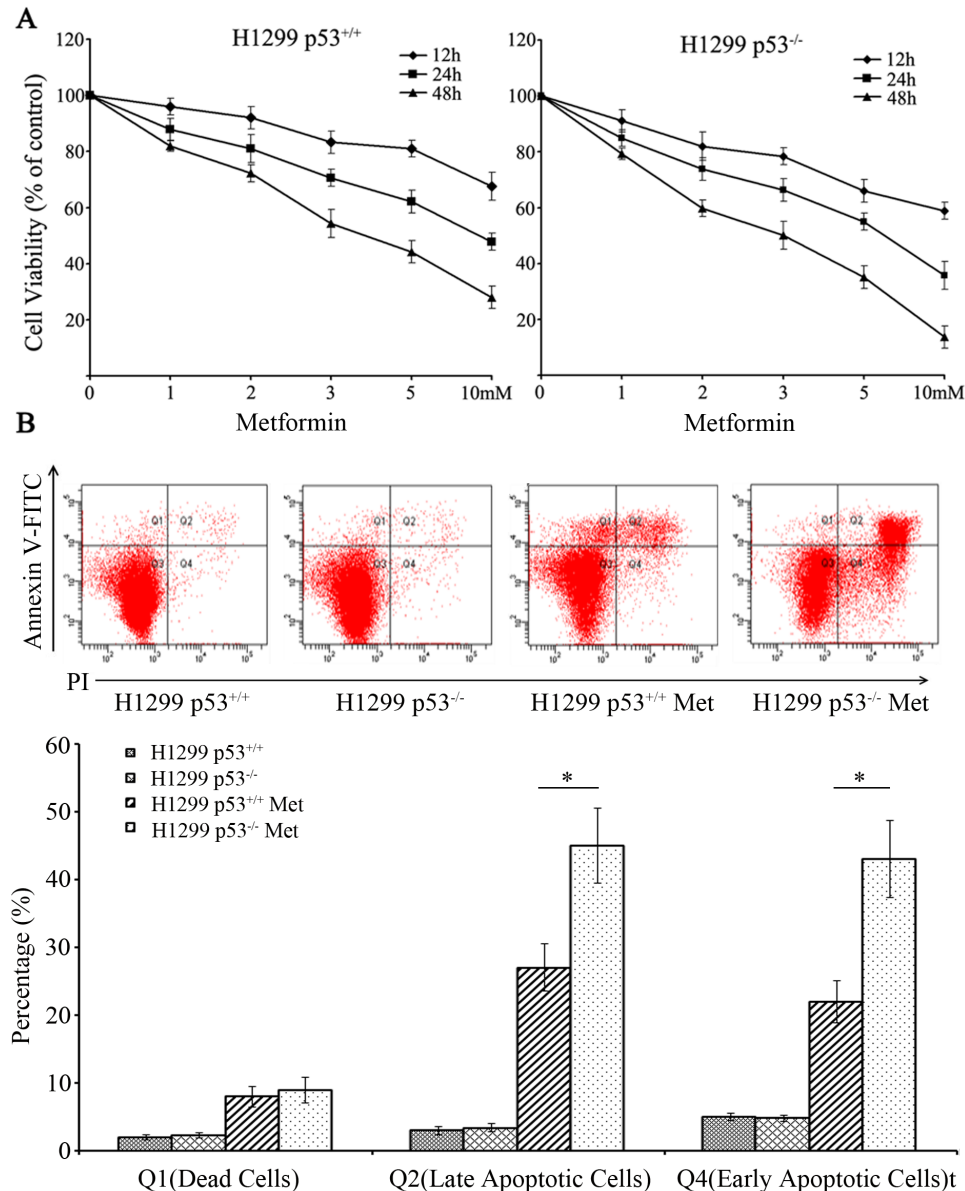


Figure 2: Difference in proliferation inhibition and apoptosis of H1299 p53^{+/+} and H1299 p53^{-/-} induced by metformin. (A) Cells were treated with Metformin of different concentration for 12, 24, and 48 h. Cell viability was measured and data were shown in cell viability curves with mean \pm S.E.M. (B) Cells were treated with 3 mM Metformin for 24 h and then flow cytometry was performed to detect the cell apoptosis. There was significant difference between cancer cells with different p53 status in either cell viability or early/late cell apoptosis (*P<0.05).

was believed to mediate AMPK inhibition. In another word, metformin induced IGF-1R signaling inhibition also paved the way to metformin induced AMPK activation, this feedback loop might be a good supplement for the anticarcinogenic effects of metformin as listed above.

In addition to its role in cell growth-inducing activities, IGF-1R was reported to have important function in metabolic regulation [23]. The PI3K pathway, which is activated following IGF-1R activation, not only provides antiapoptotic and mitogenic signals but also has significant effect on cancer cell metabolism. The downstream proteins of IGF-1R, Akt/protein kinase, mTOR induce energy production in cancer cells and stimulate lipid and protein synthesis, which is responsible for a number of metabolic adaptations [24, 25]. So inhibiting the IGF-1R pathway may induce significant changes in cancer cell metabolism and lead to retardation in proliferation, which is consist with our founding in this study.

p53, which was reported as tumor suppressor, has also an important role in the control of cell metabolism

[26]. Activation of p53 allows cells to respond to glucose deprivation by arresting their proliferation until glucose is restored. And it is conceivable that loss-of-function mutation of p53 in cancer cells may lead to stimulation of the glycolytic pathway [23]. Moreover, recent reports have revealed that p53 acts as a major energy conserving component following metformin-induced AMPK activation [9, 10]. Tumor cells with loss of function of p53 are less able to compensate the metabolic stress induced by metformin and are more likely to undergo an energetic crisis because of their inability to stop proliferation [3], so p53-deficient cells showed an impaired ability to survive when treated with metformin compared with p53^{+/+} cells, which was highly in accordance with our data here.

As an upstream regulator of the IGF-1R gene, p53 effects converge on decreased IGF/IGF1R signaling by reducing both IGF bioavailability and IGF-1R density [27]. Cross-talk and coordination between the p53 and IGF-1R/Akt/TOR pathways have been proposed as means

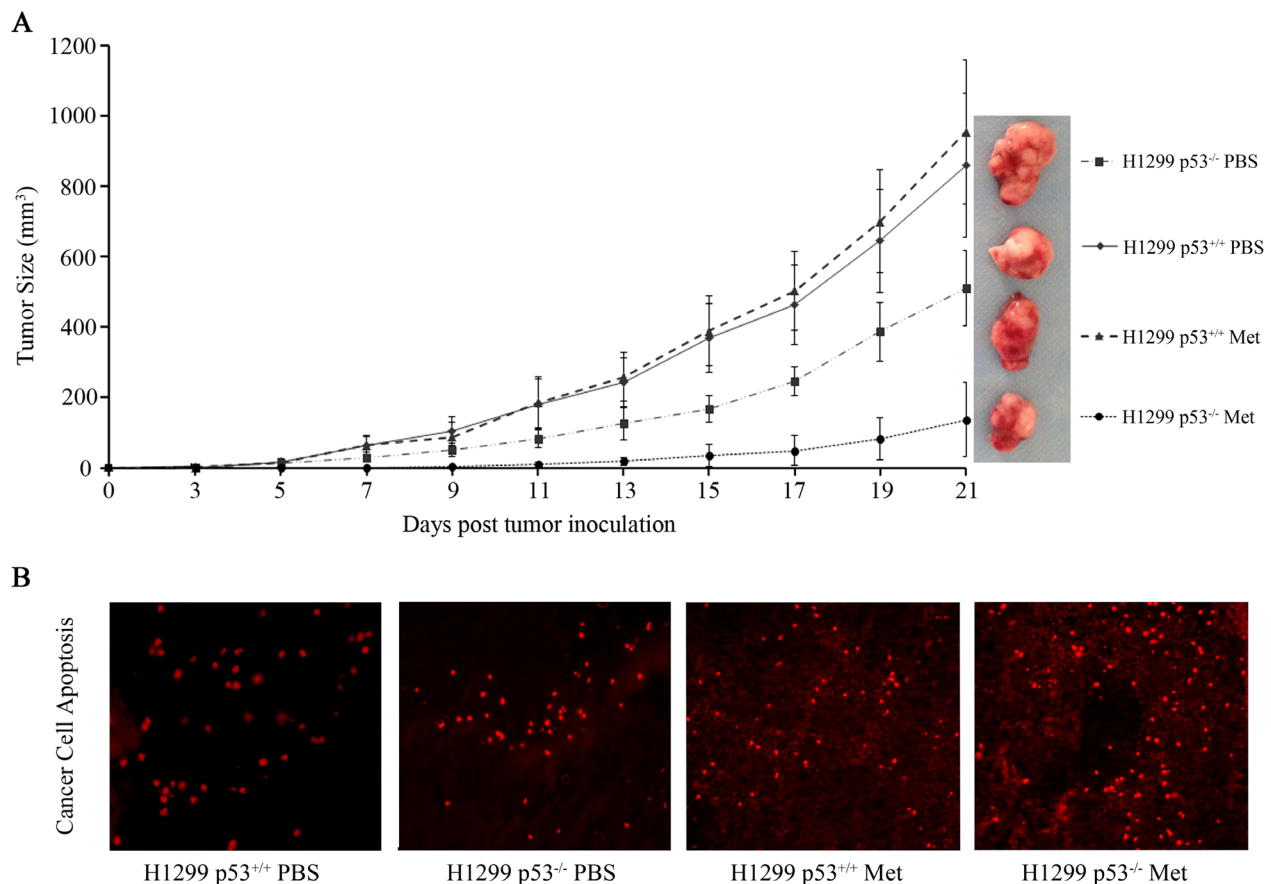


Figure 3: Difference in solid tumor growth retardation and cancer cell death induced by metformin *in vivo*. (A) After subcutaneous transplantation of H1299 cancer cells, the tumor growth in size was detected, tumor volume was calculated, and tumor growth curves were made. Solid tumors from all groups of mice were dissected and measured at the end of animal experiment. The results indicated statistical difference in tumor size between H1299 p53^{+/+} and H1299 p53^{-/-} groups with metformin from day 7 ($p < 0.05$, and $p < 0.01$ from day 9). (B) Cell death caused by metformin in solid tumor sections were detected by TUNEL. We found more significant cancer cell death in p53-deficient cells (H1299 p53^{-/-}) compare to p53 wild groups ($P < 0.05$, MOD for all groups were 0.046 ± 0.0061 , 0.041 ± 0.0073 , 0.137 ± 0.0056 , 0.191 ± 0.0047).

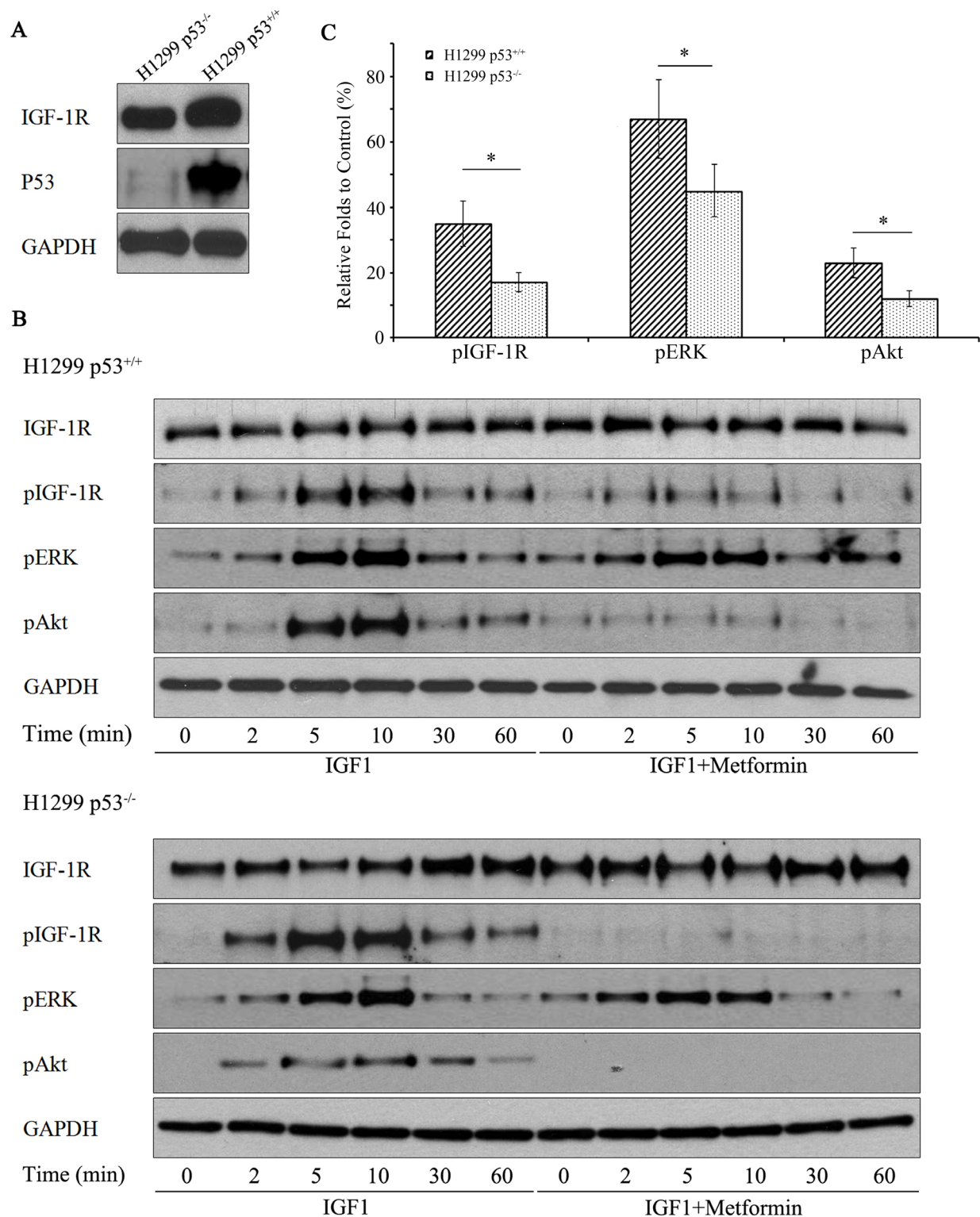


Figure 4: Metformin inhibited IGF-1R signaling pathway transduction. (A) H1299 p53^{+/+} and H1299 p53^{-/-} cell lysates were prepared and IGF-1R, p53 were detected by WB. (B) Cells were treated with 50 ng/ml IGF-1 or first treated with 3 mM metformin for 30 min and then stimulated with 50 ng/ml IGF-1 for 2, 5, 10, 30 and 60 min. Cell lysates were analyzed via WB for IGF-1R, pIGF-1R, pERK, pAKT and GAPDH on 0, 2, 5, 10, 30 and 60 min. Metformin could significantly inhibit pIGF-1R, pERK and pAKT activation in both H1299 p53^{+/+} and H1299 p53^{-/-} cell lines. (C) Intensity of bands at different time points of cells with IGF1+Metformin were quantified by densitometry, normalized to cells with IGF1 as control, and displayed as a percentage of the total intensity. Data were presented as mean \pm S.E.M in histograms. Statistical difference was found in pIGF-1R, pERK and pAKT activation between H1299 p53^{+/+} and H1299 p53^{-/-} cancer cells (*P<0.05).

of integrating growth factor signaling, changes in nutrient levels and stress signals into regulation of cell growth, mitogenesis and apoptosis [28]. The IGF-1R signaling tends to be more malignant due to p53 deficiency, but seemed to be more susceptible to metformin treatment. Our data here indicated that more efficient retardation of IGF-1R signaling pathway and receptor degradation and

ubiquitination were induced by metformin in the absent of p53 protein, resulting in enhanced proliferation inhibition, cell apoptosis *in vitro* and more significant solid tumor growth retardation and cancer cell death *in vivo*.

Although more cell lines and multiple animal experiments were required to make a general conclusion, our study suggested that the change in sensitivity of IGF-

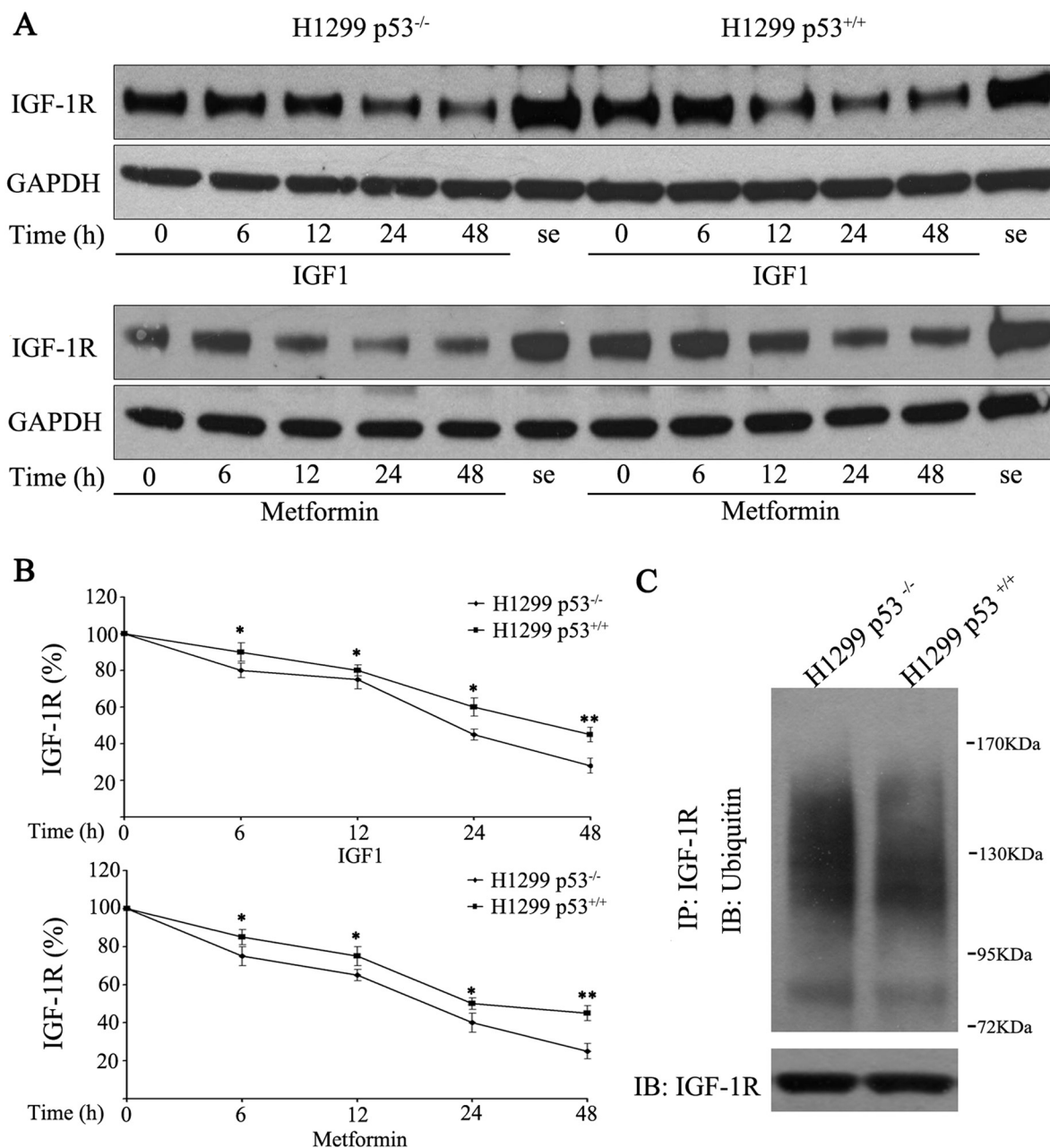


Figure 5: Metformin induced IGF-1R degradation and ubiquitination. (A) 50 ng/ml IGF-1 and 3 mM Metformin was added to cells for a series of time points (0, 6, 12, 24, 48 h, serum as control). The expression level of IGF-1R and GAPDH were detected by WB. (B) Intensity of bands for IGF-1R at different time points were quantified by densitometry, normalized to GAPDH, data were presented as mean \pm S.E.M in the curves. There was significant difference between H1299 p53^{+/+} and H1299 p53^{-/-} cancer cells in both IGF1 and metformin induced receptor degradation (* $P < 0.05$, ** $P < 0.01$). (C) Cells were simulated with 3 mM metformin for 10 min after serum starvation for 24 h. Cells were harvested with IP buffer. The IGF-1R antibody was used for immunoprecipitation, and immunoblotting with ubiquitin antibody. Ubiquitination induced by metformin was significantly stronger in H1299 p53^{-/-} cancer cells ($P < 0.05$).

1R signaling pathway to metformin may be the possible mechanism of metformin's selective toxicity in p53-deficient cancer cells.

However, the clinical use of metformin as an antitumor agent is still awaited, although there were ongoing trails amassing evidence to ascertain if a survival benefits is associated with the use of metformin in various malignancies, the potential use of metformin as an immunotherapy agent needs to be substantiated with further evidence [29].

MATERIALS AND METHODS

Reagents and cell culture

Polyclonal antibodies against phosphorylated-AKT (S473), AKT, pERK1/2, ERK1/2, pIGF-1R and IGF-1R were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-p53 and GAPDH antibody was from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Metformin (150959-5G) was obtained from Sigma (St Louis, MO, USA).

The human non-small cell lung cancer A549, H460, H520 and H1975 cell lines were maintained in RPMI1640 supplemented with 10% fetal bovin serum (FBS, Hyclone); H1299 (H1299 p53^{-/-}, P53 gene deletion) and H1299 p53^{+/+} (wild type P53 DNA transfected) cell lines were maintained in IMDM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin was contained in all cell medium.

Cell viability assay and flow cytometry

Cells were cultured in 96 well plates overnight in the presence or absence of serum. Appropriate substrates were added to the wells for 12, 24, 48h, and then the cell viability was measured by PrestoBlue Cell Viability Assay (Invitrogen, CA, USA). Fluorescence was measured with excitation at 560 nm and emission at 590 nm wavelengths. Cell number was calculated from standard curve of fluorescence measurement.

For flow cytometry assay, cells were cultured in 6 well plates overnight and metformin was added to the wells for 24 h. After that cells were collected in tubes and treated in accordance with Annexin V-FITC/PI apoptosis detection kit (Invitrogen, CA, USA). The cells apoptosis were imaged under a fluorescent microscope using 488 nm excitation, 515 nm emission for FITC and 560 nm emission for PI. Dead cells were labeled with Annexin V (-) propidium iodide (PI) (+) and are shown in the Q1 area; late apoptotic cells were labeled with Annexin V (+) PI (+) and are shown in the Q2 area; live cells were labeled with Annexin V (-) PI (-) and are shown in the Q3 area; early apoptotic cells were labeled with Annexin V (+) PI (-) and are shown in the Q4 area (as shown in Figures 1C&2B).

SDS-PAGE and western blotting

Protein samples were dissolved in LDS sample buffer (Invitrogen, CA, USA) containing 1 mM β-mercaptoethanol, and analyzed by SDS-PAGE with 4-12% Bis-Tris gel (Invitrogen, CA, USA). After separation, the proteins were transferred to nitrocellulose membranes (GE Healthcare) at 4°C for 1 hour and then blocked for 1 hour at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.1% (w/v) Tween 20 in Tris-buffered saline (TBS), pH 7.5 (TBS-T). After Incubated with appropriate primary antibodies overnight at 4°C, membranes were washed three times in TBS-T, and then incubated with a horseradish peroxidase-labeled secondary antibody (Pierce, IL, USA) for 1 hour at room temperature. The detection was made with ECL substrate (Pierce, IL, USA) and exposure to x-ray film.

Real-time PCR and immunoprecipitation (IP)

Total RNA of different cell lines was obtained using TRIzol reagent (Invitrogen), according to manufacturer's instructions. The RNA concentration was determined spectrophotometrically and intensity was detected by gel electrophoresis, and quality was confirmed in an Agilent 2100 Bioanalyzer (Agilent Technologies). The P53 mRNA was quantified by SYBR Green-quantitative real-time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as in our previous study [30]. The PCR primers used were as follows: P53 forward 5'-CCTCAGCATCTTATCCGAGTGG-3' and reverse 5'-TGGATGGTGGTACAGTCAGAGC-3', GAPDH forward 5'-TGACCACATCCTATGCCACT-3' and reverse 5'-CCACCTTCTTGCTGTAGCT-3'.

Cells were lysed with IP lysis buffer supplied by Invitrogen, using 500 µl lysis buffer with 10 mM N-ethylmaleimide, 50 µM MG132, and protease inhibitor cocktail tablet (Roche) per well in the 6-well plates. After incubated with 1 mg antibody for 3 h at 4°C, the samples were added with 15 µl Dynabeads Protein G (Invitrogen, CA, USA), and incubated on a rocker platform at 4°C overnight. Then the immunoprecipitated complexes were collected by magnet, the supernatant discarded, and the beads were washed three times with lysis buffer and dissolved in sample buffer for western blot.

Solid tumor formation and TUNEL

BALB/c-nu mice (female, 5-6 weeks old) were purchased from vital River Laboratory Animal Technology (Beijing, China) and maintained under specific pathogen-free conditions. H1299 p53^{+/+} and p53^{-/-} cells (4×10⁶) were subcutaneously injected. Tumor sizes were measured by longitudinal (*L*) and transverse (*W*) diameters every other day and tumor volumes were calculated as $V=L \times W^2/2$ [31]. The endpoint for this study was set to be three weeks after tumor cells administration, and then all mice were

sacrificed under anesthesia and tumors were exercised and weighed. Tissue samples were embedded in paraffin wax and serially sectioned. All animal experimental protocols were approved by the Ethics Committee of Shandong University.

Tumor sections were deparaffinized and rehydrated, incubated with protease K for 25 min at room temperature for antigen retrieval, and then operated according to the protocol of the TUNEL apoptosis detection kit (Zhongshan jin qiao, China). The red-labeled TUNEL positive cells were imaged under a fluorescent microscope using 488 nm excitation and 530 nm emission. An image analyser (Media Cybernetics) was used to score the staining sections of TUNEL, and results were present in mean optical density (MOD = integrated optical density/area) as in our previous study [32].

Statistical analysis

All experiments were repeated at least three times and the values were expressed as the mean \pm standard error. Statistical significance was determined by Student's t-test using SPSS 17.0 statistical software (SPSS Inc, Chicago, IL). For all tests, a *p* value less than 0.05 was considered to be statistically significant.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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