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Inhibition of p38 MAPK-dependent MutS homologue-2 (MSH2) expression by metformin enhances gefitinib-induced cytotoxicity in human squamous lung cancer cells

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ABSTRACT

Objectives: Gefitinib, a quinazoline-derived tyrosine kinase inhibitor, has anti-tumor activity *in vivo* and *in vitro*. Human MutS homologue-2 (MSH2) plays a central role in promoting genetic stability by correcting DNA replication errors. The present study investigated the effects of p38 mitogen-activated protein kinase (MAPK) signal on gefitinib-induced MSH2 expression in two human non-small cell lung squamous cancer cell lines.

Materials and methods: After the gefitinib treatment, the expressions of MSH2 mRNA were determined by real-time PCR and RT-PCR analysis. Protein levels of MSH2, phospho-MKK3/6, phospho-p38 MAPK were determined by Western blot analysis. We used specific MSH2, and p38 MAPK small interfering RNA to examine the role of p38 MAPK-MSH2 signal in regulating the chemosensitivity of gefitinib. Cell viability was assessed by MTS assay, trypan blue exclusion, and colony-forming ability assay.

Results: Exposure of gefitinib increased MSH2 protein and mRNA levels, which was accompanied by MKK3/6-p38 MAPK activation in H520 and H1703 cells. Moreover, blocking p38 MAPK activation by SB202190 significantly decreased gefitinib-induced MSH2 expression by increasing mRNA and protein instability. In contrast, enhancing p38 activation using constitutively active MKK6 (MKK6E) increased MSH2 protein and mRNA levels. Specific inhibition of MSH2 expression by siRNA enhanced gefitinib-induced cytotoxicity. Metformin, an anti-diabetic drug, might reduce cancer risk. In human lung squamous cancer cells, metformin decreased gefitinib-induced MSH2 expression and augmented the cytotoxic effect and growth inhibition by gefitinib. Transient expression of MKK6E or HA-p38 MAPK vector could abrogate metformin and gefitinib-induced synergistic cytotoxic effect in H520 and H1703 cells.

Conclusion: Together, down-regulation of MSH2 expression can be a possible strategy to enhance the sensitivity of gefitinib to human lung squamous cancer cells.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide and non-small-cell lung cancer (NSCLC) is the most common type, accounting for approximately 85% of all cases [1,2]. Over-expression of epidermal growth factor receptor (EGFR) reportedly occurs in 40–80% of NSCLC cases [3] and is most commonly reported in squamous cell, followed by large cell and adenocarcinoma [4,5]. Several studies have indicated that high EGFR expression level correlates with poor disease prognosis and reduced survival [3,4,6,7].

Gefitinib (Iressa, ZD1839) is a selective EGFR tyrosine kinase inhibitor (TKI) and is the first EGFR-directed small-molecule drug approved as treatment of NSCLC [8,9]. Gefitinib inhibits EGFR signaling by interfering with the ATP-binding site on the catalytic domain of the receptor [10,11]. It shows anti-tumor activity in mouse xenograft models and tumor cell lines by blocking

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downstream cell growth and survival signaling pathways such as ERK1/2 and PI3-K/AKT [12–14]. However, increased phosphorylation of p38 mitogen-activated protein kinase (MAPK) and JNK by gefitinib has been observed in a dose-dependent manner in keratinocytes [15]. Nevertheless, little is known about the contribution of p38 MAPK signaling cascades on gefitinib-induced cytotoxicity in human lung squamous cells.

The mismatch repair (MMR) system plays a central role in promoting genetic stability by correcting DNA replication errors [16–18]. In mammalian cells, the MMR pathway is initiated by the recognition of a single base mismatch or insertion/deletion loops (IDL) by either the MutSα heterodimer, consisting of the MSH2 and MSH6 proteins, or the MutSβ heterodimer consisting of MSH2 and MSH3. MutSα preferentially recognizes base-base mismatches and IDL of 1–2 nucleotides while MutSβ has preference for larger IDL [19]. Increased expression of MSH2 RNA and/or protein has been reported in various malignancies [20–23]. To date, whether gefitinib affects MSH2 expression in NSCLC is unknown and the role of MSH2 in gefitinib-induced cytotoxicity has not been elucidated.

Metformin is an anti-diabetic drug that lowers hyperglycemia by inhibiting hepatic glucose production [24]. It has been shown to inhibit neoplastic growth in experimental animal models, including lung, breast, prostate, and colon cancers [25–28]. In the current study, a putative involvement of MSH2 in response to gefitinib in human lung squamous cancer cells was identified and characterized. Results showed that MSH2 up-regulation by gefitinib was *via* MKK3/6-p38 MAPK signaling activation. Moreover, knockdown of MSH2 expression enhanced gefitinib-induced cytotoxicity in H520 and H1703 cells. In the NSCLC cells with reduced MSH2 protein and mRNA by metformin, the gefitinib-induced cytotoxicity was augmented.

2. Materials and methods

2.1. Reagents and cell culture

Metformin, cycloheximide, mithramycin A (Sp1-specific chemical inhibitor), and tanshinone IIA (AP-1-specific chemical inhibitor) were purchased from Sigma–Aldrich Inc. (St. Louis, MO). Gefitinib (Iressa®, ZD1839) was purchased from AstraZeneca (London, UK) while SB202190 were purchased from Calbiochem-Novabiochem (San Diego, CA). Human lung squamous cell carcinoma H520 (HTB-182) and H1703 (CRL-5889) were purchased from American Type Culture Collection (Manassas, VA, USA). No EGFR mutation was detected in these two NSCLC cell lines.

The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and fetal calf serum (10%). The cell lines were routinely tested to confirm that they were free of *Mycoplasma*.

2.2. Quantitative real-time polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were performed using an ABI Prism 7900HT according to the manufacturer's instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designed primers were: for MSH2 forward primer, 5′-AAGCCCAGGATGCCATTG-3′, MSH2 reverse primer, 5′-CATTTGACACGTGAGCAAAGC-3′; GAPDH forward primer, 5′-CATGAGAAGTATGACAACAGCCT-3′; GAPDH reverse primer, 5′-AGTCCTTCCACGATACCAAAGT-3′. The real-time PCR was performed in triplicate in a total reaction volume of 25 μL containing 12.5 μL of SYBR Green PCR Master Mix, 300 nM forward and reverse primers, 11 μL of distilled H_2O, and 1 μL of cDNA from each sample. The PCR thermal cycling conditions were 10 min denaturation at 95 °C; 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 20 s. Quantification was performed using the $2-\Delta\Delta Ct$ method, where Ct value was defined as threshold cycle of PCR at which amplified product was detected. The ΔCt was obtained by subtracting the housekeeping gene (GAPDH) Ct value from the Ct value of the gene of interest (MSH2). The present study used ΔCt of control subjects as the calibrator. The fold change was calculated according to the formula $2-\Delta\Delta Ct$, where $\Delta\Delta Ct$ was the difference between ΔCt and the ΔCt calibrator value (which was assigned a value of 1 arbitrary unit).

2.3. Reverse transcription-PCR (RT-PCR)

RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed with 2 μg of total RNA using random hexamers following the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. MSH2 was amplified by primers 5′-AAGCCCAGGATGCCATTG-3′ (forward) and 5′-CATTTGACACGTGAGCAAAGC-3′ (reverse) in conjunction with a thermal cycling program consisting of 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s.

The gene expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as an internal control. The GAPDH was amplified by primers 5′-CTACATGGTTTACATGTTCC-3′ (forward) and 5′-GTGAGCTTCCCGTTCAGCTCA-3′ (reverse) in conjunction with a thermal cycling program consisting of 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. Expression of GAPDH was used as a control to measure the integrity of the RNA samples.

2.4. Western blot analysis

Equal amounts of proteins from each set of experiments were subjected to Western blot analysis as previously described [29]. The specific phospho-p38 MAPK (Thr180/Tyr182), phospho-MKK3 (Ser189)/MKK6 (Ser207), phospho-EGFR (Tyr1068), and EGFR antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies against MSH2(N-20) (sc-494), p38alpha (C-20) (sc-535), HA(F-7) (sc-7392), MKK3(N-20) (sc-959), and Actin(I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.5. Transient transfection of small interfering RNA

The sense-strand sequences of siRNA duplexes were as follows: for MSH2, 5′-GCAGAUGAAUAGUGCUGUA-3′, 5′-GAAGAGACCUUAACUAUGC-3′, 5′-CAACAUAUAUUCGACAAAC-3′, 5′-GAGAAUGAUUGGUAUUUGG-3′; for p38alpha, 5′-GAACUGCGGUUACUUAAAC-3′ (#1), 5′-AUGAAUGAUGGACUGAAAUGGUCUG-3′ (#2), and for the control, 5′-UUCUCCGAACGUGUCACGUTT-3′ (Dharmacon Research, Lafayette, CO). Cells were transfected with siRNA duplexes (200 nM) using Lipofectamine 2000 (Invitrogen) for 24 h.

2.6. Enforced expression of MKK6E and HA-p38 MAPK vectors

Plasmids transfection of MKK6E (constitutively active form of MKK6) and HA-p38 MAPK was achieved as previously described [29]. Exponentially growing human lung cancer cells (10^6) were plated for 18 h and were transfected with MKK6E or HA-p38 MAPK expression vectors using Lipofectamine (Invitrogen).

2.7. Cell viability assay

In vitro 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were cultured at 5000 per well in 96-well tissue culture plates. To assess cell viability, drugs were added after plating. At the end of the culture period, 20 μL of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added and the cells were incubated for another 2 h. Absorbance was measured at 490 nm using an ELISA plate reader (Biorad Technologies, Hercules, CA).

2.8. Combination index analysis

The cytotoxicity induced by the combined treatment of gefitinib and metformin was compared with the cytotoxicity induced by each drug alone, using the combination index (CI), where CI < 0.9, CI = 0.9–1.1, and CI > 1.1 indicated synergistic, additive, and antagonistic effects, respectively. The combination index analysis was performed using the CalcuSyn software (Biosoft, Oxford, UK). The mean of CI values at a fraction affected (FA) of 0.50 was averaged for each experiment and the values were used to calculate the mean of three independent experiments.

2.9. Colony-forming ability assay

Immediately after drug treatment, the cells were washed with phosphate-buffered saline and trypsinized to determine the cell numbers. The cells were plated at a density of 500–1000 cells on a 60 mm-diameter Petri dish in triplicate for each treatment and cultured for 12–14 days. The cell colonies were stained with 1% crystal violet solution in 30% ethanol. Cytotoxicity was determined by the number of colonies in the treated cells divided by the number of colonies in the untreated control.

2.10. Trypan blue dye exclusion assay

Cells were treated with gefitinib and/or metformin for 24 h. Trypan blue dye was excluded by living cells but could penetrate dead cells. The proportion of dead cells was determined using a hemocytometer to count the number of cells stained with trypan blue.

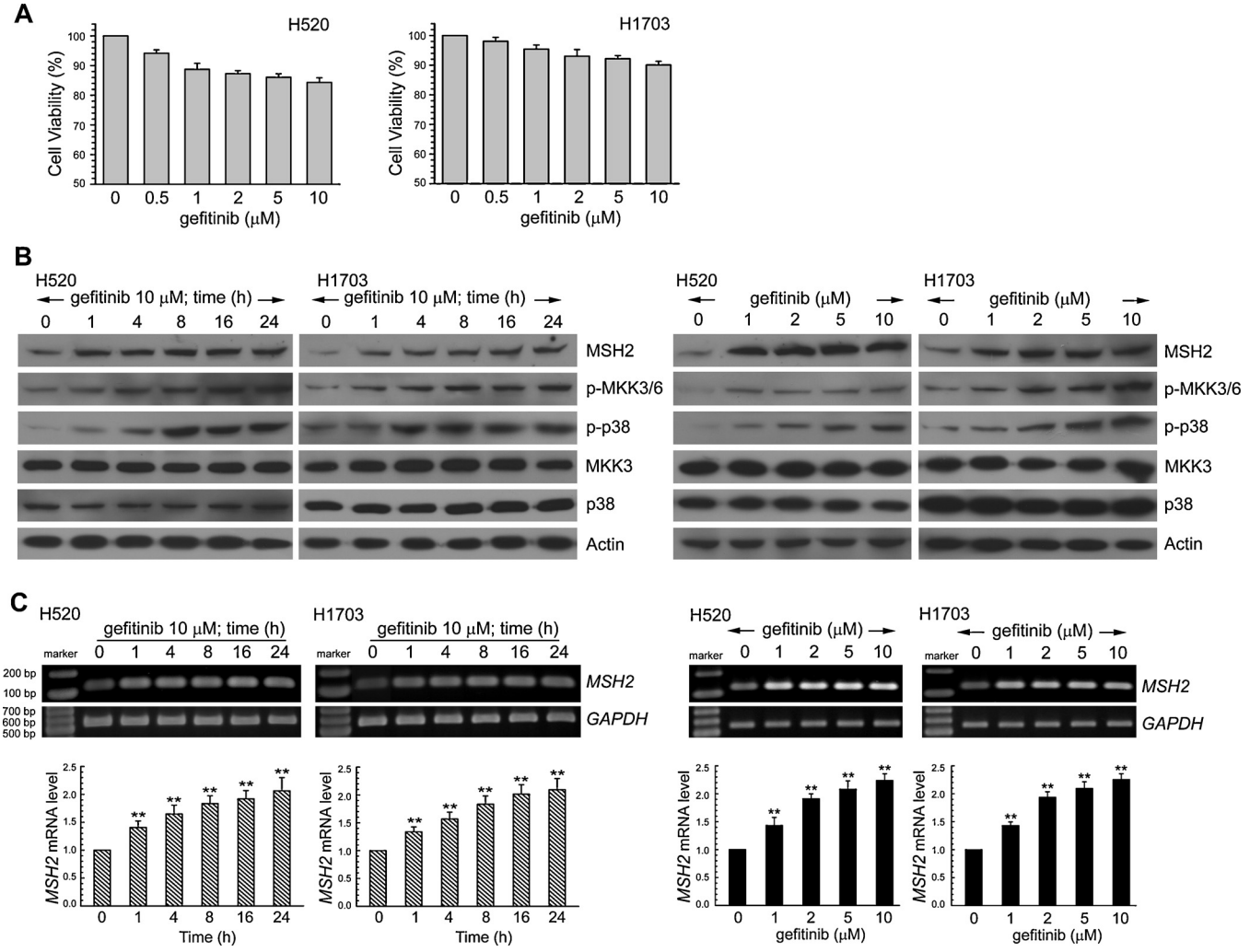

Fig. 1. Gefitinib increased phospho-MKK3/6-p38 MAPK, MSH2 protein and mRNA levels in NSCLC cells. (A) Gefitinib (0.5, 1, 2, 5, and 10 μM) was added to H520 or H1703 cells for 24 h. Cytotoxicity was determined by MTS assay. (B) H520 or H1703 cells (10^6) were cultured in complete medium for 18 h and exposed to various concentrations of gefitinib (1, 2, 5, and 10 μM) for 1–24 h in complete medium. After treatment, the cell extracts were examined by Western blot for MSH2, phospho-MKK3/6, phospho-p38, MKK3, p38, and actin protein levels. (C) After treatment, total RNA was isolated and subjected to RT-PCR (upper panel) and real-time PCR (lower panel) for MSH2 mRNA expression. The results (mean ± SEM) were from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, by Student's *t*-test for comparison between cells treated with and without gefitinib.

2.11. Sp1 transcription activity ELISA

The nuclear fractions from the cells maintained in each culture condition were used to measure Sp1 activity. Sp-1 ELISA was performed using an Sp1 ELISA kit (Panomics Inc., Fremont, CA) according to the manufacturer's protocol. Briefly, 50 μg of nuclear fraction was mixed with transcription factor binding buffer supplied by the manufacturer and then applied to the each well of 96-well plate covered with oligo-DNA fragment containing consensus Sp1 binding sequence. After incubation for 16 h at 4 °C without agitation, the wells were washed five times with 200 μL PBS containing 0.05% Tween 20 (PBS-T).

After the final wash, 100 μL of diluted anti-Sp1 antibody (1:100) solution was added to each well except the blank wells and the plate was incubated for 1 h at room temperature without agitation. Each well was washed again using PBS-T and then incubated with 100 μL of diluted peroxidase conjugated secondary antibody (1:100) for 1 h at room temperature without agitation. Each well was then treated with chemi-luminescence developing solution. After 30-min incubation at room temperature with gentle agitation protected from light, 100 μL of the stop solution was added to each well and absorbance was measured at a wavelength of 450 nm using a spectro-photometric plate reader (Benchmark Plus Micro-plate Spectrophotometer, Bio-Rad Laboratories, Inc.).

2.12. Statistical analyses

For each protocol, three or four independent experiments were performed. Results were expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed by using SigmaPlot 2000 (Systat Software, San Jose, CA). Differences in measured variables between experimental and control groups were assessed by unpaired *t*-test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Gefitinib increased MSH2 protein and mRNA levels via MKK3/6-p38 MAPK activation

To evaluate the effect of gefitinib on the cell viability in 2 NSCLC cell lines, H520 and H1703, we did an MTS assay to determine the sensitivity to gefitinib. In Fig. 1A, H520 and H1703 cells showed a limited sensitivity to gefitinib treatment with about 84% and 90%

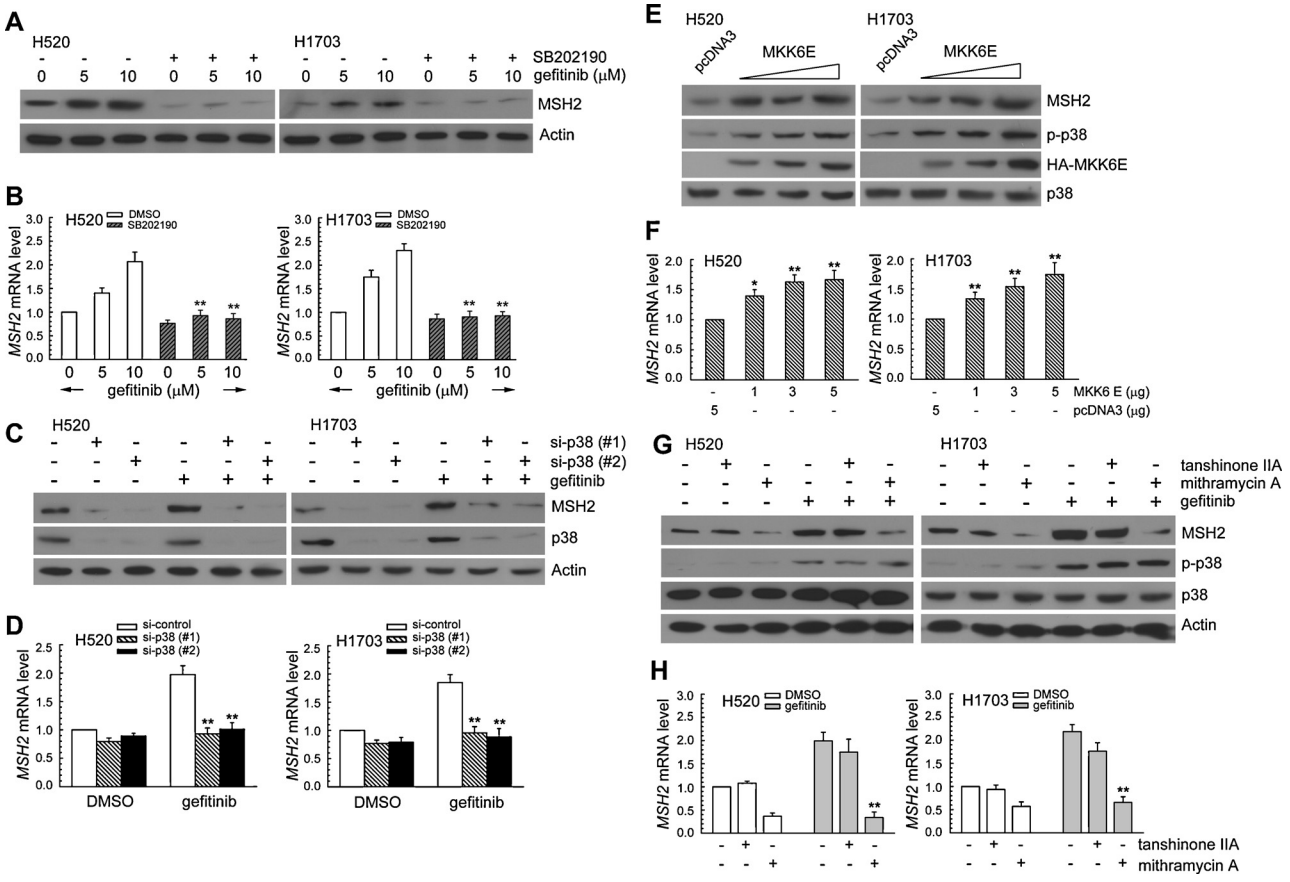

Fig. 2. p38 MAPK signaling pathway was involved in the gefitinib-induced increase in MSH2 protein and mRNA level. (A and B) SB202190 (5 or 10 μM) was added to H520 or H1703 cells for 1 h before gefitinib (10 μM) treatment for 24 h. The results (mean ± SEM) were from four independent experiments. $^{**}p < 0.01$, by Student's *t*-test for comparison between cells treated with gefitinib-DMSO or gefitinib-SB202190 combinations. (C and D) H520 or H1703 cells (5×10^5) were transfected with si-p38 RNA, incubate for 24 h, and treated with 10 μM gefitinib for 24 h. After treatment, the cell extracts were examined by (A and C) Western blot and (B and D) real-time PCR for determination of MSH2 protein and mRNA levels, respectively. The results (mean ± SEM) were from 3 independent experiments. $^{**}p < 0.01$, by Student's *t*-test for comparison between the cells treated with gefitinib in si-p38 RNA or si-scrambled RNA-transfected cells. (E and F) MKK6E expression vectors (1, 3, and 5 μg) were transferred into cells using lipofection and allowed to express for 2 days. The whole cell extracts were subjected to Western blot analysis and real-time PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, by Student's *t*-test for comparison between the MKK6E or pcDNA3 vector-transfected cells. (G and H) Cells pre-treated with mithramycin A (0.1 μM) or tanshinone IIA (0.1 μM) for 1 h were exposed to gefitinib (10 μM) for an additional 24 h. After treatment, the cell extracts were examined by Western blot and real-time PCR for determination of MSH2 protein and mRNA levels, respectively. The results (mean ± SEM) were from four independent experiments. $^{**}p < 0.01$, by Student's *t*-test for comparison between the cells treated with gefitinib alone or with gefitinib–mithramycin A combination.

cells surviving at 10 μM dose of gefitinib. We then test the effect of gefitinib on MSH2 expression and p38 MAPK signal activation. Fig. 1B and C, gefitinib induced MSH2 protein and mRNA expression in a time- and dose-dependent manner, accompanied by an increase in phospho-MKK3/6-p38 MAPK protein levels. Treatment with the p38 MAPK inhibitor, SB202190, decreased MSH2 protein as well as mRNA levels in gefitinib-exposed H520 or H1703 cells (Fig. 2A and B). Similarly, knockdown of the p38 MAPK expression by specific si-p38 RNA attenuated the gefitinib-induced MSH2 protein and mRNA levels (Fig. 2C and D). When the H520 or H1703 cells were transiently transfected with MKK6E, a constitutively active form of MKK6, cellular p38 MAPK phosphorylation, MSH2 protein, and mRNA expression increased compared with transfection with the control vector, pcDNA3 (Fig. 2E and F). Thus, gefitinib increased MSH2 expression *via* p38 MAPK activation.

Previous study has indicated that specificity factor 1 (Sp1) transcription factor, a well-known downstream effector of the p38 MAPK pathway, modulates MSH2 expression under hypoxia in human colon cancer cells [30]. To determine whether transcription factor Sp1 was also involved in up-regulating MSH2 expression in gefitinib-exposed NSCLC cells, mithramycin A was used as it could inhibit Sp1 activity and had anti-tumor activities [31–33]. Mithramycin A could decrease gefitinib-induced MSH2 protein and mRNA expression (Fig. 2G and H). However, the other transcriptional factor AP1 inhibitor (tanshinone IIA) did not affect the MSH2 expression in gefitinib-exposed H520 and H1703 cells.

3.2. p38 MAPK activation by gefitinib increased MSH2 mRNA and protein stability

This study examined possible mechanisms for post-transcriptional regulation of MSH2 transcripts under gefitinib treatment. In the presence of actinomycin D, gefitinib treatment showed higher levels of *MSH2* mRNA relative to untreated cells (Fig. 3A and B). Moreover, MSH2 protein levels were progressively reduced with time in the presence of cycloheximide (an inhibitor of *de novo* protein synthesis) (Fig. 3C). However, gefitinib treatment significantly prevented MSH2 degradation after cycloheximide treatment compared to untreated cells (Fig. 3C). Therefore, MSH2 protein was more stable after gefitinib treatment in H520 and H1703 cells.

Interestingly, blocking p38 MAPK activation by SB202190 suppressed gefitinib-induced *MSH2* mRNA and protein stability (Fig. 3). This indicated that gefitinib increased MSH2 protein and mRNA levels through the improvement of protein and mRNA stability of MSH2 by p38 MAPK activation.

3.3. Knockdown of MSH2 sensitized NSCLC cells to gefitinib

This study examined the effect of siRNA-mediated MSH2 knockdown on gefitinib-induced cytotoxicity and cell growth inhibition in NSCLC cells. After 24 h post-transfection, *MSH2* mRNA was reduced by more than 90% compared to cells transfected with negative control siRNA (Fig. 4A). Western blot analysis also showed that MSH2 gene knockdown was accompanied by a decrease in MSH2 protein in untreated or gefitinib-treated H1703 and H520 cells, but did not affect the gefitinib-induced p38 MAPK activation (Fig. 4B).

Furthermore, suppression of MSH2 protein expression by si-MSH2 RNA resulted in increased sensitivity to gefitinib compared to si-control transfected cells (Fig. 4C and Supplemental Fig. S1). More inhibition of cell growth was induced by the combination of MSH2 siRNA and gefitinib than gefitinib alone in NSCLC cells (Fig. 4D). Thus, knockdown of MSH2 sensitized human lung squamous cell carcinoma cells to gefitinib.

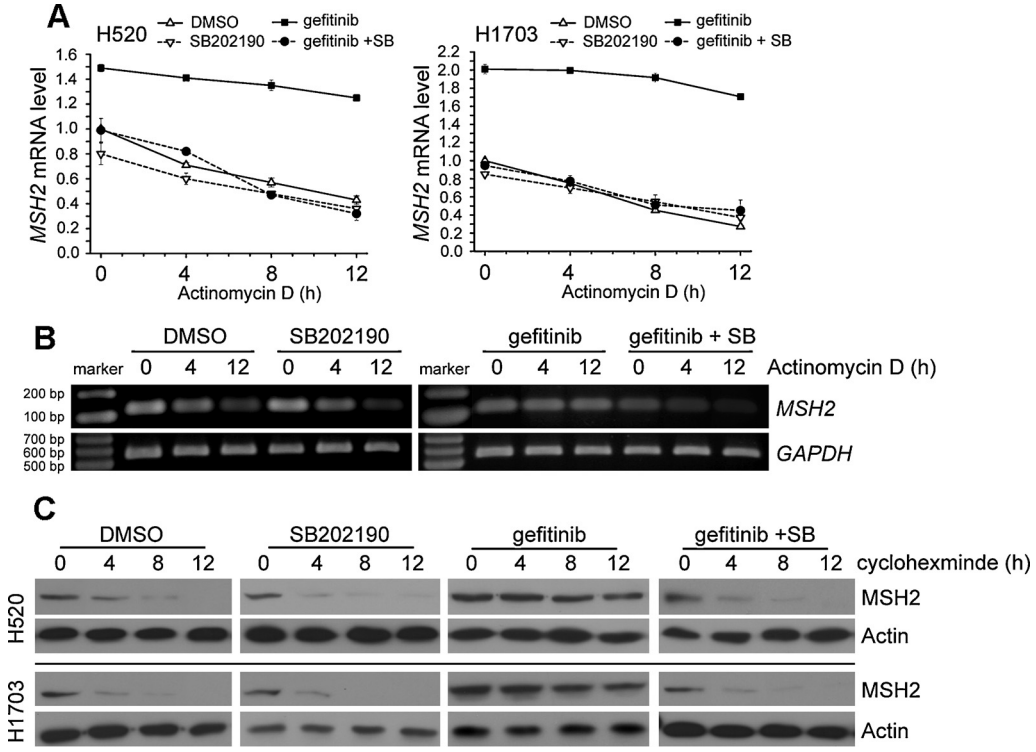

Fig. 3. SB202190 decreased MSH2 mRNA and protein stability in gefitinib-treated NSCLC cells. (A and B) H520 or H1703 cells were exposed to gefitinib (10 μM) with SB202190 (10 μM) or DMSO for 12 h in the presence or absence of actinomycin D (2 μg/mL) for 4, 8, or 12 h. Total RNA was isolated and subjected to real-time PCR and RT-PCR for *MSH2* mRNA expression, which was normalized against *GAPDH* in 3 separate treatments. (C) Cells were exposed to gefitinib (10 μM) and/or SB202190 (10 μM) for 12 h, followed by co-treatment with cycloheximide (0.1 mg/mL) for 4–12 h. Whole-cell extracts were collected for Western blot analysis.

3.4. Blocking p38 MAPK-Sp1 activation enhanced gefitinib-induced cytotoxicity

To examine the role of the p38 MAPK in the cytotoxic effect of gefitinib, si-p38 RNA, or the p38 inhibitor, SB202190, was used to block gefitinib-induced p38 MAPK activation. Co-treatment with p38 MAPK silencing by si-p38 RNA or SB202190 significantly further decreased cell viability in gefitinib-exposed H520 or H1703 cells compared to gefitinib treatment alone (Fig. 4E and F). Moreover, inhibition of Sp1 activity by mithramycin A further decreased cell viability under gefitinib treatment (Fig. 4G and H). However, AP1 inhibition (by tanshinone IIA) did not significantly affect the gefitinib-induced cytotoxicity (Fig. 4G). Taken together, the down-regulation of p38 MAPK activation by p38 MAPK-siRNA, or by pharmacological inhibitor SB202190, or by inactivation of Sp1 enhanced sensitivity to gefitinib in NSCLC cells.

3.5. Metformin abrogated gefitinib-induced MSH2 protein and mRNA level up-regulation

Metformin, a biguanide is among the most commonly prescribed glucose-lowering chemical agents, with proven efficacy and limited side effects [34]. Previous studies have indicated that metformin causes a decrease in the activation of p38 MAPK signaling in human epidermoid carcinoma [35]. This study hypothesized that metformin could enhance gefitinib-induced cytotoxic effect through the down-regulation of p38 MAPK-mediated MSH2 expression in NSCLC cells. Metformin suppressed the endogenous and gefitinib-induced phospho-MKK3/6-p38 MAPK and MSH2 protein levels (Supplemental Fig. S2A and B). Results of real-time PCR analysis also showed that metformin decreased endogenous and gefitinib-induced MSH2 mRNA level in H520 and H1703 cells (Supplemental Fig. S2C and D).

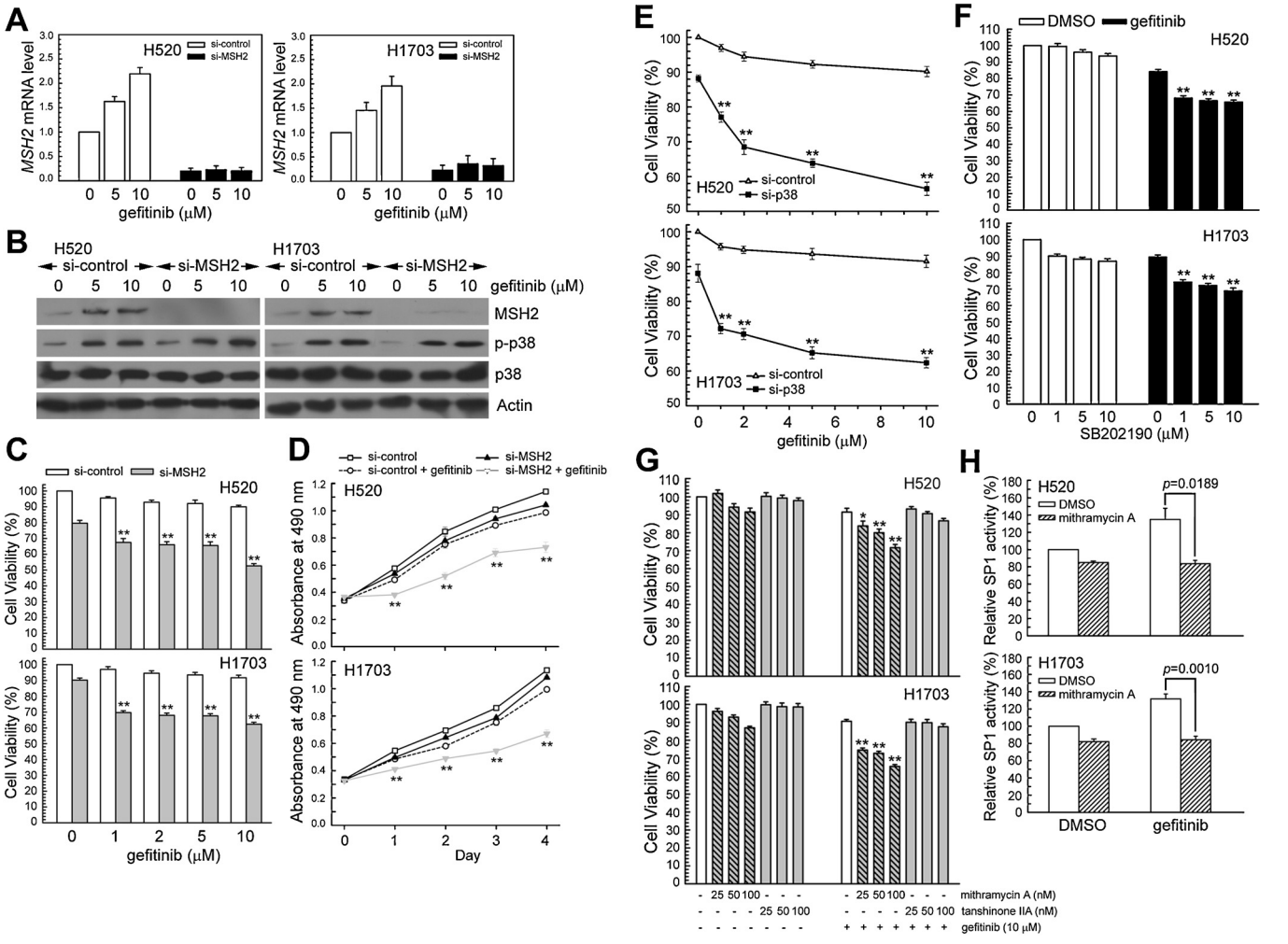

Fig. 4. Knockdown of MSH2 expression by si-RNA transfection or inhibition of p38 MAPK activation by SB202190 or specific si-p38 MAPK RNA transfection enhanced gefitinib-induced cytotoxicity. (A and B) H520 or H1703 cells were transfected with siRNA duplexes (200 nM) specific to MSH2 or scramble (control) in complete medium for 24 h prior to gefitinib treatment (5 or 10 μM) in complete medium for 24 h. (A) Total RNA was isolated and subjected to real-time PCR for *MSH2* mRNA expression. (B) Whole-cell extracts were collected for Western blot analysis using specific antibodies against MSH2, phospho-p38, p38 MAPK, and actin. (C) After treatment, cytotoxicity was determined by MTS assay. (D) After the cells were transfected with si-MSH2 or si-scrambled RNA, the cells were treated with gefitinib (2 μM) for 1–4 days and cell survival was determined by MTS assay. The results (mean ± SEM) were from three independent experiments. $^{**}p < 0.01$, by Student's *t*-test for comparison between the cells treated with gefitinib in si-MSH2 RNA or si-scrambled RNA-transfected cells. (E) The si-p38 RNA-transfected cells were treated with gefitinib for 24 h and cytotoxicity was determined by MTS assay. $^{**}p < 0.01$, by Student's *t*-test for comparison between the cells treated with gefitinib in si-p38 RNA or si-scrambled RNA-transfected cells. (F) Cells were pre-treated with SB202190 (1, 5, 10 μM) for 1 h and then co-treated with gefitinib (10 μM) for 24 h. Cytotoxicity was determined by the MTS assay. $^{**}p < 0.01$, by Student's *t*-test for comparison between the cells treated with gefitinib alone or with gefitinib-SB202190 combination. (G) Mithramycin A (25, 50, or 100 nM) or tanshinone IIA (25, 50, or 100 nM) was added to H520 or H1703 cells for 1 h before gefitinib (10 μM) treatment for 24 h. Cytotoxicity was determined as above. $^{**}p < 0.01$, for comparison between gefitinib alone and gefitinib/mithramycin A combination. (H) Mithramycin A (100 nM) was added to H520 or H1703 cells for 1 h before gefitinib (10 μM) treatment for 24 h. Sp1 activity was determined by Sp1 transcription activity ELISA.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2013.09.011.

3.6. Metformin enhanced the cytotoxicity and growth inhibition of gefitinib in NSCLC cells

Next, the effect of the combination of metformin and gefitinib on cell viability using MTS and trypan blue exclusion assays were investigated. The combination of metformin plus gefitinib for 24 h resulted in greater loss of cell viability than that caused by either metformin or gefitinib alone in H520 and H1703 cells (Fig. 5A and B). Setting the ratio at which metformin and gefitinib combination was 100:1 and analyzing cell viability by MTS assay, the combination index values for metformin and gefitinib were <1, indicating synergism (Fig. 5C and D).

Colony-forming assays were also conducted to investigate whether metformin affected long-term clonogenic cell survival in gefitinib exposed lung cancer cells. However, the colony-forming ability of combined gefitinib–metformin treatment was lower than that of gefitinib treatment alone (50.74% *vs.* 73.91%, $p < 0.05$) (Fig. 5E). Metformin and gefitinib co-treatment had more effective cell growth inhibition than either treatment alone (Fig. 5F). Thus, metformin could sensitize human lung tumor cells to gefitinib and enhanced gefitinib-elicited growth inhibition.

3.7. Combination of metformin and gefitinib-induced synergistic cytotoxicity was abrogated in lung cancer cells with MKK6E or HA-p38 MAPK expression vector transfection

This study explored if the p38 MAPK pathway was directly affected by metformin in terms of cellular response to gefitinib. Transfection with either MKK6E or HA-p38 MAPK enhanced MSH2

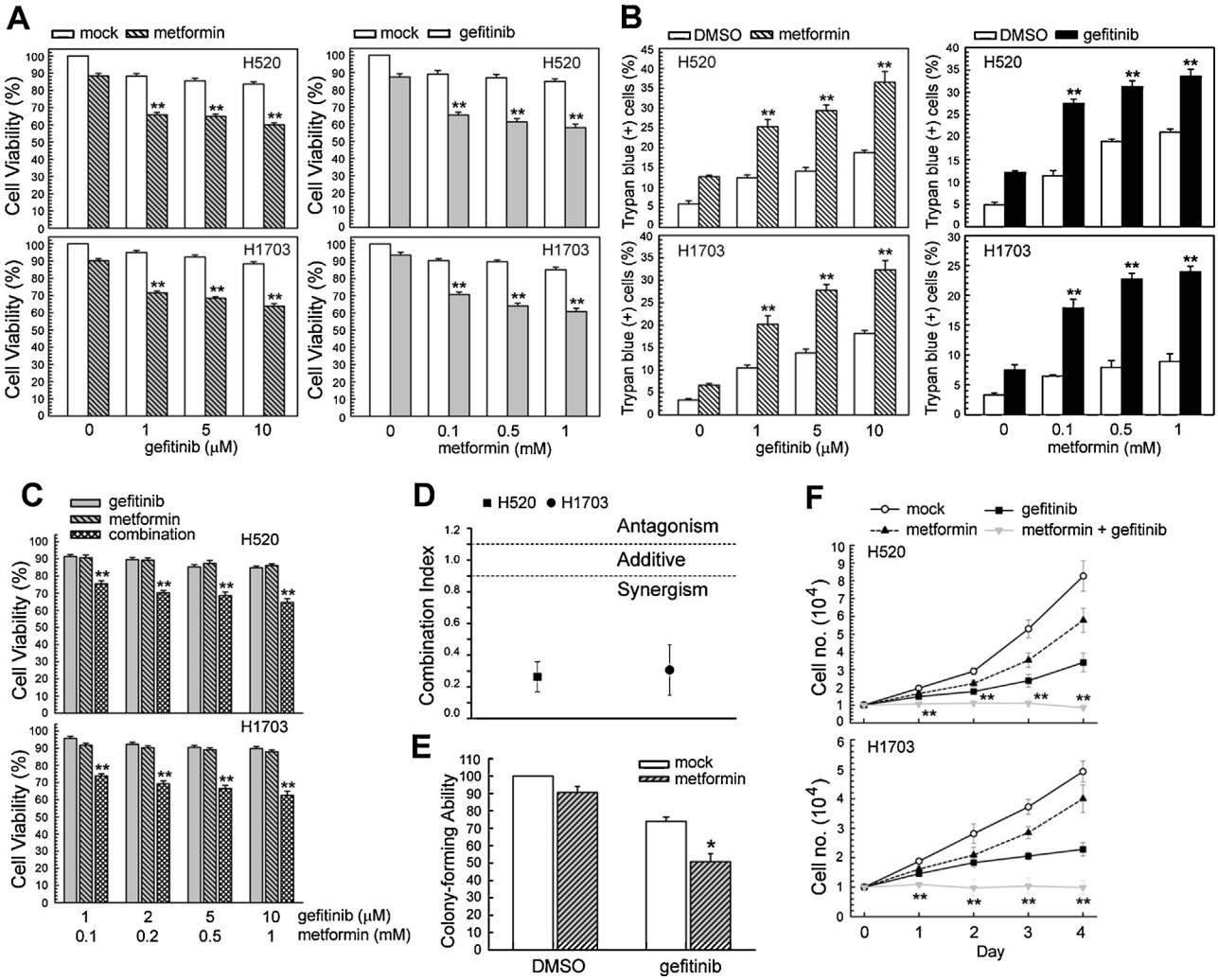

Fig. 5. Metformin co-treatment with gefitinib synergistically enhanced cytotoxicity. (A) Left panel, gefitinib (1, 5, and 10 μM) and metformin (0.1 mM) were added to H520 or H1703 cells for 24 h. Right panel, gefitinib (1 μM) and metformin (0.1, 0.5, and 1 mM) were added to the cells for 24 h. Cytotoxicity was determined by MTS assay. (B) At the end of treatment, unattached and attached cells were collected and stained with trypan blue dye. The numbers of stained cells (dead) were manually counted. Columns, percentage of trypan blue-positive cells representing dead cells; bar, SD from three independent experiments. $^{**}p < 0.01$, by Student's *t*-test for comparison between the cells treated with gefitinib/metformin alone or gefitinib–metformin combination. (C) Gefitinib (1, 2, 5, and 10 μM) and metformin (0.1, 0.2, 0.5, and 1 mM) were added to H520 or H1703 cells for 24 h. Cytotoxicity was determined by MTS assay. (D) The mean CI values at a fraction affected (FA) of 0.50 for gefitinib and metformin combination treatment were averaged for each experiment and used to calculate the mean between experiments. Points and columns, mean values obtained from three independent experiments; bars, standard error (SE). (E) Gefitinib (10 μM) and/or metformin (0.1 mM) were added to H1703 cells for 24 h, cytotoxicity was determined by colony-forming ability assay. $^{*}p < 0.05$, by Student's *t*-test for comparison of cells treated with gefitinib alone or with gefitinib–metformin combination. (F) Cells were treated with gefitinib (1 μM) and/or metformin (0.1 mM) for 1–4 days cell survival was determined by trypan blue dye exclusion assay. $^{**}p < 0.01$, by Student's *t*-test for comparison between cells treated with drug alone or with gefitinib–metformin combination.

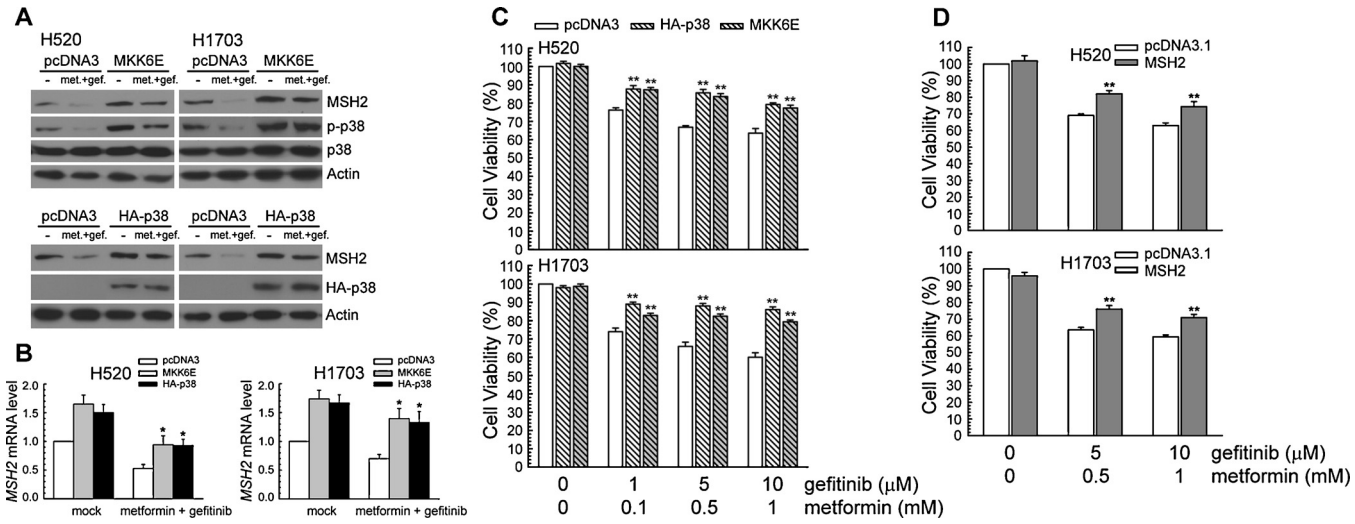

Fig. 6. Over-expression of MKK6E or HA-p38 MAPK restored the metformin-suppressed p38 MAPK activation and MSH2 protein expression in gefitinib-exposed H520 and H1703 cells. (A and B) MKK6E or HA-p38 MAPK expression plasmids were transfected into cells using lipofectamine. After expression for 24 h, the cells were treated with gefitinib (5 μM) and metformin (0.1 mM) for another 24 h and whole-cell extracts were collected for Western blot analysis and real-time PCR assay. (C) After MKK6E or HA-p38 MAPK expression plasmids transfection, cells were treated with gefitinib (1, 5, and 10 μM) and metformin (0.1, 0.5, and 1 mM) for 24 h. Cytotoxicity was determined by MTS assay. $^{**}p < 0.01$, $^{*}p < 0.05$, by Student's *t*-test for comparing cells treated with gefitinib and metformin in MKK6E or HA-p38 MAPK *vs.* pcDNA3-transfected H520 or H1703 cells. (D) After MSH2 expression plasmids transfection, cells were treated with gefitinib (5, and 10 μM) and metformin (0.5, and 1 mM) for 24 h. Cytotoxicity was determined by MTS assay. $^{**}p < 0.01$, by Student's *t*-test for comparing cells treated with gefitinib and metformin in MSH2 *vs.* pcDNA3.1 vector-transfected H520 or H1703 cells.

protein and mRNA level as well as cell survival, which was suppressed by metformin–gefitinib co-treatment (Fig. 6A–C). Furthermore, overexpression of MSH2 could suppress the cytotoxicity of gefitinib–metformin combination (Fig. 6D). Thus, metformin inhibited the p38 MAPK-mediated MSH2 expression in NSCLC cells and consequently, increased gefitinib-induced cytotoxicity.

4. Discussion

The results of the present study provide new insights into the mechanism underlying the role of p38 MAPK-mediated MSH2 expression in reduction gefitinib-induced cytotoxicity. Down-regulation of MSH2 expression can be a possible strategy to enhance the sensitivity of gefitinib to human lung squamous cancer cells. In the human MMR system, MSH2 plays a crucial role in safeguarding genomic integrity by correcting DNA replication errors [36,37]. Despite the functional importance of MMR in maintaining genetic stability, little is known about the regulation of the mechanisms underlying MSH2 gene expression. Under oxidative stress, both p38 MAPK and c-Jun N-terminal kinase (JNK) pathways can mediate the ectopic expression of MSH2 in renal carcinoma cell [38].

Previous studies indicate that p53 binds to the MSH2 promoter for transcriptional activation in ovarian cancer cells [39,40]. Myc has also been implicated as a positive transcriptional regulator of MSH2 expression [41]. In human sporadic colon cancers, HIF-1α over-expression is associated with loss of MSH2 expression, especially when p53 is immunochemically undetectable [30]. In addition, HIF-1α displaces the transcriptional activator Myc from Sp1 binding to repress MutS expression in a p53-dependent manner. Sp1 serves as a molecular switch by recruiting HIF-1α to the gene promoter under hypoxia [30]. In the present study, both p38 MAPK activation and Sp1 activity is required for gefitinib-elicited MSH2 expression in human lung squamous cell carcinoma. Interestingly, down-regulation of MSH2 expression can be a possible strategy to enhance the sensitivity of gefitinib to human lung squamous cancer cells.

Metformin is an anti-diabetic drug that has gained significant attention as an anti-cancer drug in the last decade [42]. It inhibits the growth of various types of cancer cells both *in vitro* and *in vivo* [43–45]. Metformin activates the AMP-activated protein kinase (AMPK) pathway, which leads to a strong reduction of cyclin D1 protein level in human prostate cancer cell, providing evidence for a mechanism that may contribute to the anti-neoplastic effects of metformin [44]. Metformin inhibition of the human epidermoid carcinoma A431 cells tumor growth is accompanied by an increase in Bax/Bcl2-regulated apoptosis signaling and inhibition of activated ERK1/2 and p38 MAPKs [35]. Metformin inhibits pancreatic cancer cell line cell proliferation and dramatically reduces EGFR activation [46]. Wu et al. found that metformin inhibits the growth of human lung cancer cell lines A549 and NCI-H1299 by activating the JNK/p38 MAPK pathway and the DNA damage-inducible gene 153 (GADD153) [47]. In contrast, in the present study is the first to show that the inhibition by metformin of MKK3/6-p38 MAPK-MSH2 pathways in NSCLC cell lines exerts synergistic effects on gefitinib-induced cytotoxicity. Previous studies have demonstrated that the p38 isoforms in different cell compartment may have opposing effects [48]. For example, Ferrari et al. indicated that gene silencing of p38alpha blocks TGF-β1 induction of apoptosis, whereas downregulation of p38beta or p38gamma expression results in massive apoptosis. Thus, in endothelial cells p38alpha mediates apoptotic signaling, whereas p38beta and p38gamma induces survival signaling [49]. Therefore, the coordinated mechanism of control of human lung cancer cells apoptosis, survival, and proliferation by gefitinib-mediated activation of p38 isoforms need further been examined.

A recently study indicated that the combination of metformin with gefitinib has been shown to be particularly synergistic, *in vitro* and *in vivo*, in those NSCLC cell lines harboring an LKB1 wild-type gene [50]. Furthermore, the H1299 NSCLC cell line, in which LKB1 expression was inhibited by siRNA transfection, a significant reduction in the antiproliferative effects of metformin on H1299 cells was observed [50]. On the other hand, the phenformin, another diabetes therapeutic biguanide compound, selectively induces apoptosis in LKB1-deficient NSCLC cell lines. In addition, therapeutic

trials in Kras-dependent mouse models of NSCLC revealed that tumors with Kras and Lkb1 mutations, but not those with Kras and p53 mutations, showed selective response to phenformin, resulting in prolonged survival [51]. Taken together, metformin triggers antiproliferative responses, which may vary depending on LKB1 mutation status. In this study, we used the two EGFR wild type cell lines (H520 and H1703) to examine the role of MSH2 in regulating the cytotoxicity response of gefitinib and metformin. In Suppl. Fig. S2, Western blot analysis of EGFR expression after exposure to metformin and/or gefitinib for 24 h revealed a gefitinib dose-dependent decrease in phospho-EGFR expression in H1703 cells, while no EGFR expression was detected in H520 cells. Therefore, it may suggest the EGFR-independent mechanism of the cytotoxic effect of the gefitinib and metformin combination, which may reduce the specificity of this combination therapy and concurrently expand its possible application range. However, activating mutations of EGFR have been correlated to an increased response rate and survival in patients treated with gefitinib [52]. Therefore, it is valuable to understand whether LKB1 and EGFR gene status in NSCLC cells involve in metformin as a single agent or combined with gefitinib-induced cytotoxic effect and MSH2 expression and those studies are progress in our laboratory.

Taken together, this work is the first to identify MSH2 induction by gefitinib through the p38 MAPK pathway, and that this phenomenon is required for NSCLC survival. Combination treatment with metformin significantly decreases the expression of MSH2, which is associated with enhanced chemo-sensitivity to gefitinib in NSCLC cells. Based on these observations, various approaches targeting MKK3/6-p38 MAPK-MSH2 signaling can be developed to overcome gefitinib resistance in human lung squamous cell carcinoma in the future.

Conflict of interest statements

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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