Original Article
Combinational therapy of crizotinib and afatinib for malignant pleural mesothelioma

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Abstract: Malignant pleural mesothelioma (MPM) is a relative rare but highly aggressive neoplasm which is associated with asbestos exposure in most patients. The majority of patients are diagnosed in advanced stages so patients neither benefit from chemotherapy (e.g. pemetrexed-platinum combination) nor from surgery. It has been reported that cellular-mesenchymal to epithelial transition factor (MET) and epidermal growth factor receptor (EGFR) were critical for MPM cell proliferation. Moreover, targeting MET and EGFR drugs have gained promising results on anti-tumor therapy. Here, a striking difference in overall survival was observed between the MET and EGFR co-expression group (median survival time = 13.5 months) and non-co-expression group (median survival time = 20.5 months). In addition, treatment with combination of crizotinib and afatinib showed stronger inhibition on cell proliferation of MPM than the treatment by either one in vitro and in vivo. In conclusion, our data illustrated that crizotinib combined with afatinib may be a potentially effective strategy for treating MPM patients with over-expression of MET and EGFR.

Keywords: Malignant pleural mesothelioma, MET, EGFR, crizotinib, afatinib

Introduction

Malignant pleural mesothelioma (MPM) is a relatively rare but highly aggressive neoplasm arising from mesothelial cells of the pleural cavity. It is strongly associated with asbestos exposure, with a latency period of 20-40 years [1]. However, the disease deteriorates quickly since the patients become symptomatic. As MPM is resistant to conventional multimodal therapies, the prognosis of the patients with advanced stages is poor, with a median survival of only 11-12 months after diagnosis [2].

Previous multimodality therapy seemed not to be satisfactory enough due to the difficulties of reaching an early diagnosis and drug resistance of MPM [3-6]. Thus new treatment approaches are needed. It has been reported that activation of multiple receptor tyrosine kinases (RTKs) especially MET and EGFR is critical for MPM cell proliferation and/or survival [7]. At the same time, targeted drugs in the list have gained encouraging results on anti-tumor therapy. Thus we further studied the combination of MET and EGFR inhibitors for MPM treatment. In the present study, we first analyzed MET and EGFR expression status in MPM cells and then simultaneously blocked them with MET and EGFR inhibitors.

Crizotinib is an oral small-molecule tyrosine kinase inhibitor of MET, ALK and ROS1 kinases [8, 9]. It was approved by the U.S. Food and Drug Administration (FDA) for treatment of ALK-positive NSCLC in August 2011 [10]. Afatinib (BIBW 2992), which was approved by FDA in July 2013 for NSCLC, is an orally administered irreversible inhibitor of both the EGFR and human epidermal receptor 2 (HER2) tyrosine kinases. Afatinib is also under development in several other solid tumors including head and neck and breast cancer [11-13]. Apart from the study of crizotinib and afatinib in NSCLC, no work evaluating their effect in the MET and EGFR overexpressing MPM has been reported.

The aims of our present study were to evaluate the efficacy of crizotinib in combination with afatinib in therapeutic effect on human MPM tumors.

Our results indicated that treatment with a combination of crizotinib and afatinib showed stronger inhibition on cell proliferation in MPM cells than treatment by either drug alone at both *in vitro* and *in vivo* levels. This represents a promising therapeutic strategy for MPM.

Method and materials

Patient characteristics

A total of 24 MPM tissues and 24 normal pleura tissues as control were collected from the Cancer Center, Sun Yat-sen University between 1999 and 2015. Clinical and pathologic characteristics collected including age, gender, chest pain, dyspnea, pleura effusion, pleura thickening, pathology type and overall time. Follow-up of patients was performed according to rules every 2 months. For the use of these clinical materials with research purposes, the approval from the Institute Research Ethics Committee was obtained.

Immunohistochemistry

Immunohistochemical analysis was carried out on formalin-fixed, paraffin-embedded tissue sections of MPM specimens. Sections (5 μm thick) were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. The slides were rinsed in phosphate-buffered saline (PBS) and blocked for 15 min with 3% H_2O_2 to deprive the endogenous peroxidase activity. After antigen retrieval in citrate buffer (pH 6.0) with microwave, the specimens were incubated with the relevant antibody overnight at 4°C. After washing with PBS, the sections were incubated with the secondary antibodies followed by fast staining with diaminobenzidine (DAB) according to the manufacturer's instructions (Dako Envision + Dual Link System-HRP detection kit). The sections counterstained with hematoxylin. The degree of immunostaining was scored independently by two observers according to both the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as follows: 0 (<25% positive tumor cells), 1 (25-50% positive tumor cells), 2 (50-75% positive tumor cells), and 3 (>75% positive tumor cells).

The intensity of staining was graded as following criteria: 0 (no staining); 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining index was calculated as staining intensity score × proportion of positive tumor cells. Using this method of assessment, we evaluated the expression of proteins by determining the staining index, which scores as 0, 1, 2, 3, 4, 6, and 9. We defined the protein expression levels as follows: - (0-1 point), + (2-3 points), ++ (4-6 points), and +++ (>6 points). Thus, protein expression in specimens was divided into low (- or +) and high expression (++ or +++) groups.

Chemicals and reagents

Crizotinib was provided by Selleckchem (Houston, TX, USA) and was prepared as a 10 mmol/L stock in dimethylsulfoxide (DMSO). Afatinib was obtained from Apexbio (Houston, TX, USA) and was prepared as a 10 mmol/L stock in DMSO. GAPDH antibody and the secondary antibodies were purchased from Kangchen Co. (Shanghai, China). Antibodies against p-MET (#3077), EGFR (#2085) and p-EGFR (#3777) were purchased form Cell Signaling Technology (Danvers, MA, USA). Antibodies against MET, AKT, p-AKT, MAPK1/2 (ERK1/2) and p-ERK1/2 were purchased from Santa Cruz (Dallas, Texas, USA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

The human malignant pleural mesothelioma cell lines NCI-H28, MTSO-211H, NCI-H226, NCI-H2452, NCI-H2052 were a kind gift from Dr. Masaoshi Tagawa (Chiba Cancer Center Research Institute). All cell lines were cultured in RPMI1640 medium supplemented with 10% FBS and with 1% antibiotic solution (penicillin-streptomycin). Immortalized mesothelial cell line (MeT-5A) was purchased from the American Type Culture Collection (Rockville, MD, USA) and was culture in medium199 (sigma, USA).

Western Blotting analysis

After indicated treatment as showed in the text, the cells were harvested and washed twice with ice-cold PBS buffer. Then the cells were collected in cell lysis buffer (1×PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mg/mL phenylmethylsulfonyl fluoride, 10

mg/mL leupeptin, 10 mg/mL aprotinin,). Equal amounts of lysate protein from various treatments were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membrane (Pall, USA). After blocking with 5% skimmed milk, membranes were sequentially incubated with the primary and secondary antibodies. After washing three times with TBST buffer, the protein bands were visualized by the enhanced Phototope TM-HRP Detection Kit (Cell Signaling, USA) and exposed to Kodak medical X-ray processor (Carestream Health, USA).

Transfection of RNAi

Cells were cultured in a 6-well plate in an appropriate seeding density. Then the cells were transfected with MET or EGFR siRNA (GenePharma, Shanghai, China) in lipofectamine 2000 (ThermoFisher, USA) according to the manufacturer's instructions and the final concentration of siRNA in culture media was 50 nM. As a mock transfection control, cells were also transfected under the same condition except that no siRNA was used. All transfections were performed in triplicate and cells were harvested after being transfected for 48 h. Protein lysates were then harvested for Western blotting analysis.

DNA extraction and sequencing

The genomic DNA of the MPM cell lines was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany). All of the extracted DNA samples were quantitated with NanoDrop spectrophotometer 2000c (Thermo Fisher, USA) and stored at -20°C until use. Exons of EGFR were amplified from genomic DNA using the High Fidelity plus PCR System (Roche) and sequenced bidirectionally by Sanger sequencing with the primers specific for EGFR exons 18 to 21.

Cytotoxicity assay

Cytotoxicity was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at the appropriate density. After culturing for 24 h, cells were treated with single crizotinib or afatinib alone, or their combination for another 68 h at 37°C. Afterwards, MTT (5 mg/mL) was added into the cells and were incubat-

ed for another 4 h. Then the medium was removed and it was followed by adding 150 μL of dimethylsulfoxide (DMSO). Cytotoxicity was assessed by the Model 560 Microplate Reader (BIO-RAD, Hercules, CA, USA). Both the fitted sigmoidal dose response curve and IC_{50} were calculated by use of the Bliss method [14].

Drug synergy assays

The cells were treated with a single drug and also with a combination of two drugs for 72 h. The synergy assay results were analyzed by CompuSyn to calculate the combinational index (CI). Using constant ratio, six different dose combinations of drugs were tested. The dose and effect data of three groups was entered into CompuSyn and synergy between the two drugs was determined. The analysis of synergy assay was done by the isobologram and combination index methods, derived from the median-effect principle of Chou and Talalay using CompuSyn software (ComboSyn Inc.) [15].

Colony formation assay

Cells were plated at 500-1000 cells per well in 6-well coated plates in RPMI-1640 supplemented 10% FBS. The culture medium was changed twice per week. After 14 days, the cells were fixed in 4% formaldehyde for 15 minutes and stained with crystal violet. Colonies larger than 1 mm (N = 50 cells/clone) in diameter were counted.

Animal experiments

Our *in vivo* experiments were done in accordance with the guidelines for use of laboratory animals of the Sun Yat-sen University Institutional Animal Care and Use Committee (No. 00118019). MTSO-211H cells (8×10^6) were subcutaneously injected into the right flank of athymic nude mice (STOCK-Foxn1[nu]/Nju, female, 5 to 6 weeks old). When xenograft size reached 5 mm in diameter, mice were randomized into four groups (10 in each group), and then received various treatments: (a) saline (q2d, gavage); (b) crizotinib (q2d, gavage, 20 mg/kg); (c) afatinib (q2d, gavage, 20 mg/kg); (d) crizotinib (q2d, gavage, 20 mg/kg) plus afatinib (q2d, gavage, 20 mg/kg). Tumor size was measured with linear calipers every 3 days. Tumor volumes (V) were calculated using the formula: (length×width^2/2). The mice were euthanized on day 25 and the xenografts were
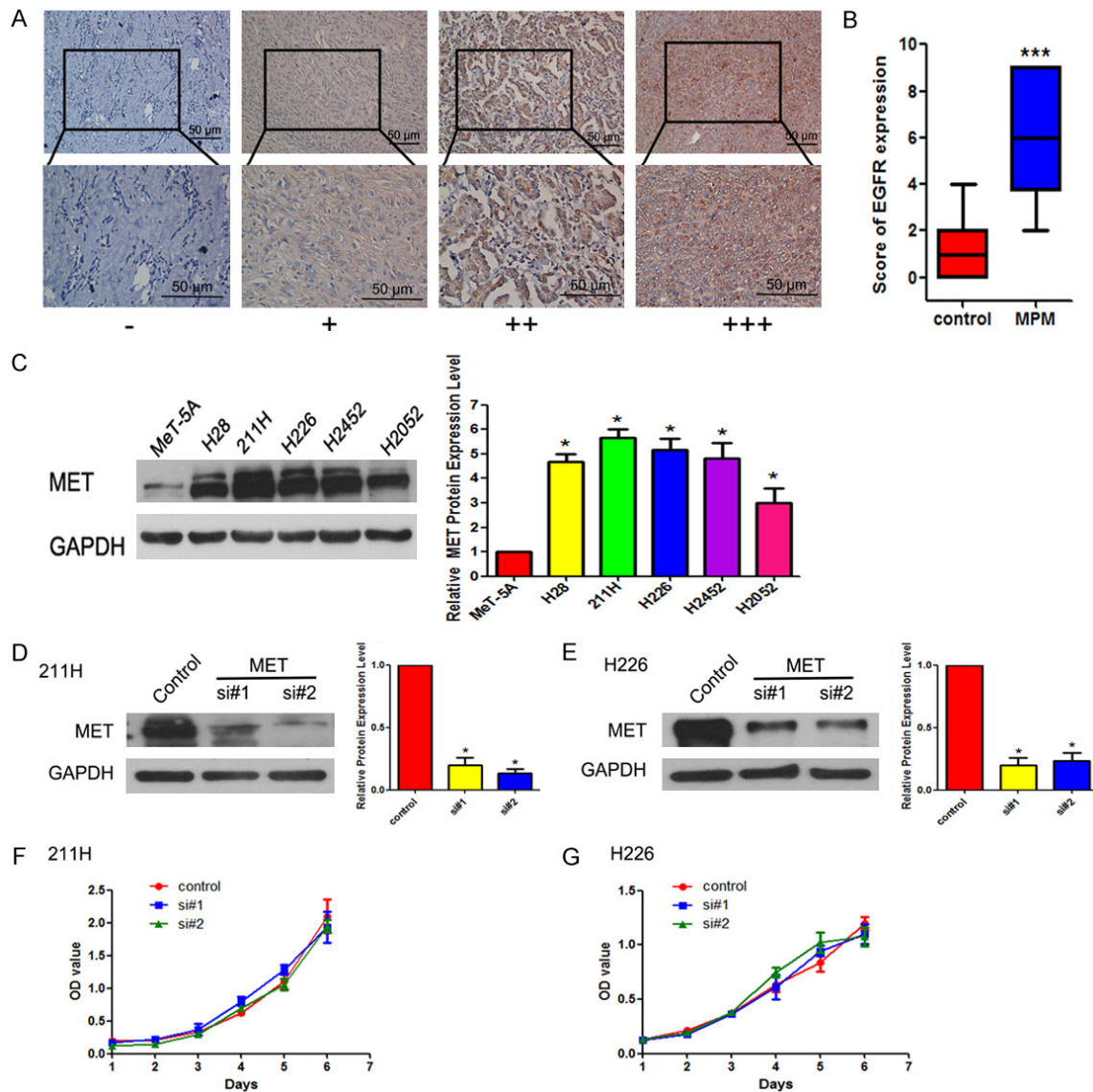
Figure 1. MET was over-expression in MPM specimens and cell lines and down-regulation of MET has little effect on MPM cell proliferation. A. Representative graphs showing typical examples of the four intensity grades of MET staining in tumor samples of the cohort of 24 MPM patients. B. Box plots was presented for comparing the expression of MET between normal pleura tissues and MPM specimens (***, P<0.001). C. Western Blotting was used to determine MET expression in different MPM cell lines and MeT-5A. GAPDH was used as loading control. The data were analyzed using the Gel-Pro analyzer. The data represented mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to MeT-5A group (One-Way ANOVA). D and E. MET protein levels were detected by Western Blotting. Data from Western Blot analysis showed that transfection of MET-siRNA inhibited the expression of MET. GAPDH was used as a loading control. The data were analyzed using the Gel-Pro analyzer. The data represent mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to control group (One-Way ANOVA). F and G. Cell proliferation assay of 211H and H226 cells was performed after transfection of MET-siRNA. Down-regulation of MET did not significantly inhibit cell proliferation of 211H and H226 cells.

excised and weighed. The ratio of growth inhibition (IR) was estimated according to the following formula:

IR (%) = 1- (Mean tumor weight of experimental group)/(Mean tumor weight of control group) × 100%.

Statistical analysis

Data was expressed as mean ± SD and all experiments were performed in triplicate and representative results were presented. All statistical analyses were carried out using the Statistical Package for the Social Science
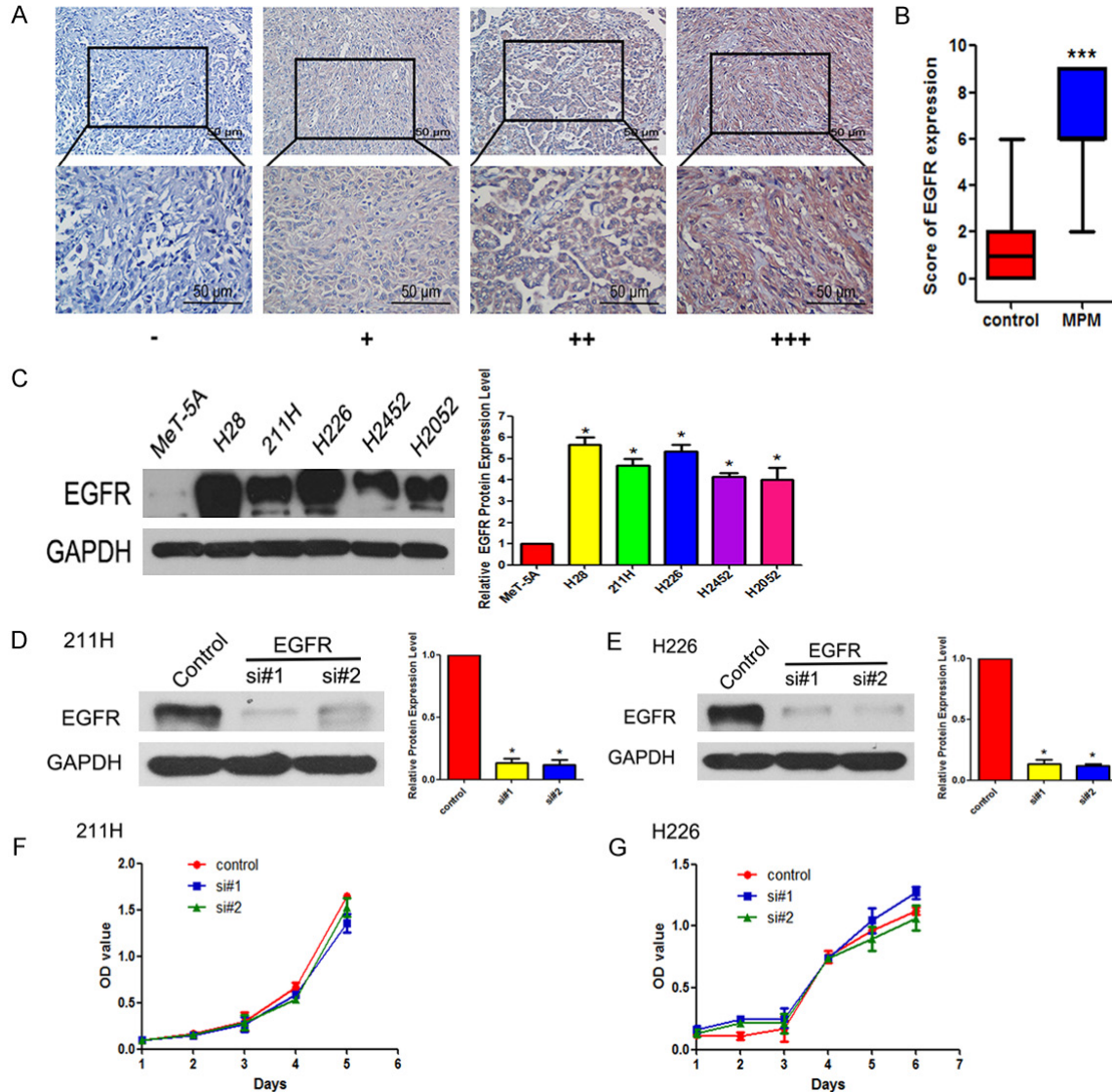
Figure 2. EGFR was over-expression in MPM specimens and cell lines and down-regulation of EGFR has little effect on MPM cell proliferation. A. Representative graphs showing typical examples of the four intensity grades of EGFR staining in tumor samples of the cohort of 24 MPM patients. B. Box plots was presented for comparing the expression of EGFR between normal pleura tissues and MPM specimens (***, P<0.001). C. Western Blotting was used to determine EGFR expression in different MPM cell lines and MeT-5A. GAPDH was used as loading control. The data were analyzed using the Gel-Pro analyzer. The data represent mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to control group (One-Way ANOVA). D and E. EGFR protein level was detected by Western Blotting. Data from Western Blot analysis showed that transfection of EGFR-siRNA inhibited the expression of EGFR. GAPDH was used as a loading control. The data were analyzed using the Gel-Pro analyzer. The data represent mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to control group (One-Way ANOVA). F and G. Cell proliferation assay of 211H and H226 cells was performed after transfection of EGFR-siRNA. Down-regulation of EGFR did not significantly inhibit cell proliferation of 211H and H226 cells.

(SPSS, Chicago, IL), Version 19.0. Student's *t* test was used to compare the difference between groups. Wilcoxon matched-samples rank sum test was used to compare the difference of IHC score of MPM and para-carcinoma. Fisher's extra test was used to analyze the correlation between gene expression and the clinicopathological characteristics. Survival curves were plotted using the Kaplan–Meier method and log-rank test to compare their differences. P<0.05 in all cases was considered statistically significant.

Results

Frequent over-expression of MET in MPM specimens and cell lines

To confirm the relationship between MET deregulation and MPM development, MET expression was examined by IHC in 24 cases of MPM specimens and 24 cases of relevant normal pleura tissues. The categorization of IHC grade of MET expression in the total 24 MPM tissues was as follows: - (n = 1; 4.2%), + (n = 5; 20.8%), ++ (n = 8; 33.3%) and +++ (n = 10; 41.7%), exhibits were the representative pictures (**Figure 1A**). MET was rarely detectable in normal pleura tissues whereas higher expression of MET was observed in MPM samples. There was obviously statistical difference between MPM tissues and relevant para-carcinoma tissues (P<0.001) (**Figure 1B**). Using a panel of five MPM cell lines (H28, 211H, H226, H2452, H2052) and immortalized mesothelial cell (MeT-5A), we determined the protein expression of MET by Western Blot analysis. All of mesothelioma cell lines expressed relatively high levels of MET compared with MeT-5A. GAPDH was used as loading protein (**Figure 1C**).

Down-regulation of MET has little effect on MPM cell proliferation

MET signaling is known to play an important and essential role in cell survival. To understand whether suppression of MET was associated with the inhibition of MPM cell growth, MTT assay was conducted after down-regulation of MET by si-RNA. The Western Blotting result indicated that MET/si-RNA down-regulated MET protein after 48 h treatment (**Figure 1D**, **1E**). However, we found that down-regulation of MET seemed to have no significant effect for 211H cells and H226 cells growth (**Figure 1F**, **1G**), which may due to co-activatiion of EGFR in mesothelioma cells which may have compensated for the cancer growth-inhibitory effect mediated by MET down-regulation.

EGFR was over-expressed in MPM specimens and cell lines

In order to confirm above hypothesis, we evaluate the EGFR expression level in MPM tumor samples. As expected, EGFR was over-expression in 87% MPM specimens. There was signifi-

cant statistical difference between MPM tissues and relevant normal pleura tissues (P<0.001) (**Figure 2B**). Exhibits were the representative pictures of the categorization grade of EGFR expression in the total 24 MPM tissues (**Figure 2A**). We further found that EGFR was highly expressed in mesothelioma cell lines compared with negative control, MeT-5A cell (**Figure 2C**).

Down-regulation of EGFR has little effect on MPM cell proliferation

EGFR signaling is also known to play an important and essential role in cell survival. To understand whether suppression of EGFR was associated with the inhibition of MPM cell growth, MTT assay was conducted after down-regulation of EGFR by si-RNA. The Western Blotting result indicated that EGFR/si-RNA down-regulated EGFR protein after 48 h treatment (**Figure 2D**, **2E**) in two different cells. However, we found that down-regulation of EGFR also has no obvious effect for 211H cells and H226 cells survival (**Figure 2F**, **2G**), suggesting inhibiting EGFR alone is not enough to suppress MPM tumor growth.

Simultaneously suppress MET and EGFR can remarkably inhibit MPM cells proliferation

As independent down-regulation of MET or EGFR has little effect for MPM inhibition, we further detected the effect of simultaneous down-regulation MET and EGFR. The Western Blotting result indicated that MET and EGFR proteins were down-regulated by MET/si-RNA and EGFR/si-RNA after 48 h treatment in 211H and H226 cells (**Figure 3A**, **3C**). Colony formation assay in these cells showed that inhibition of MET or EGFR alone did not significantly affect proliferation of MPM, consistent with the MTT assays we have done. In contrast, simultaneous suppression of MET and EGFR caused a remarkable inhibition of proliferation in MPM cells (P<0.001) (**Figure 3B**, **3D**).

Downstream of MET and EGFR was active in MPM specimens and cell lines

As MET and EGFR were co-expression in more than 70% MPM specimens and most cell lines, we further detected whether downstream of MET and EGFR was active in MPM specimens
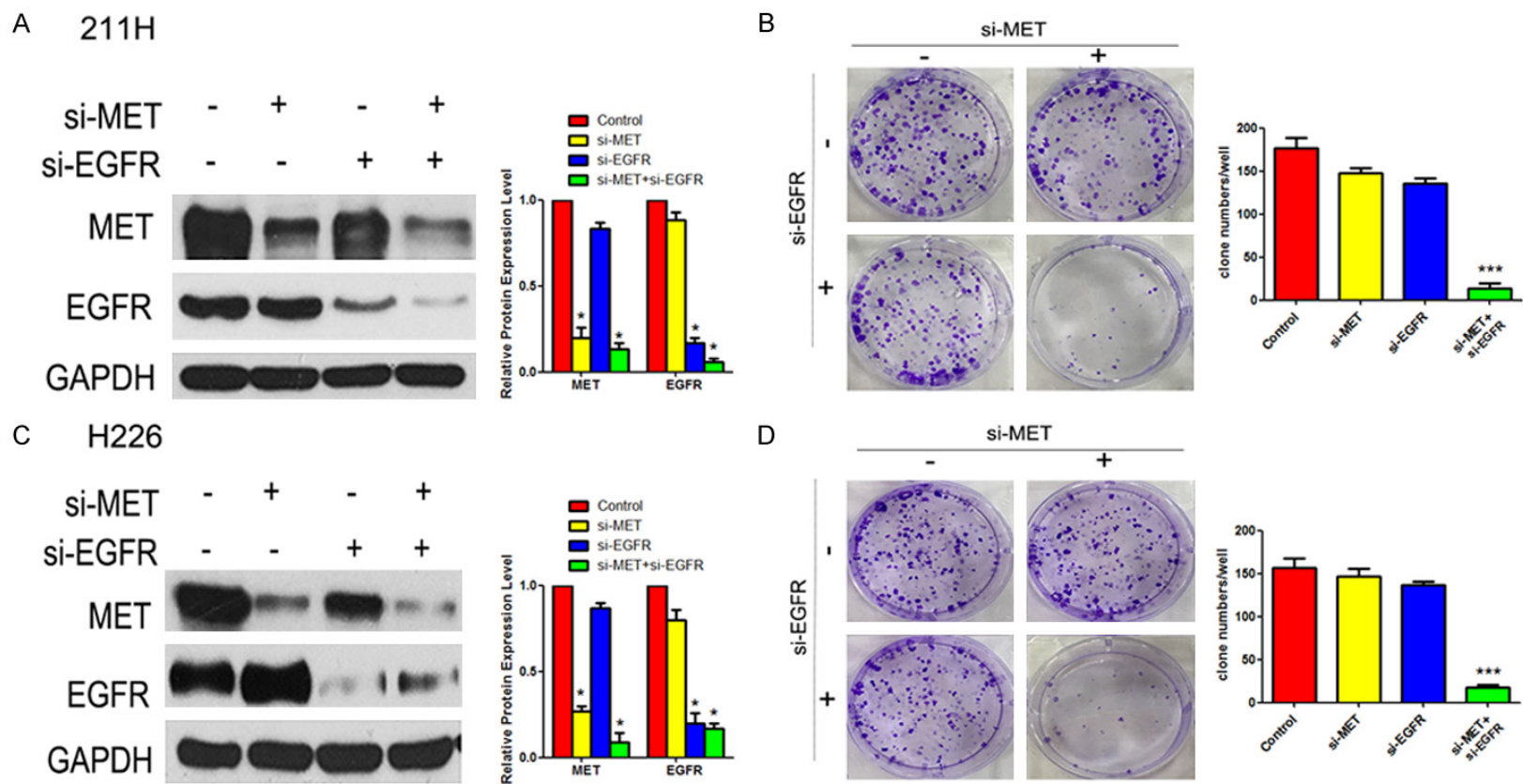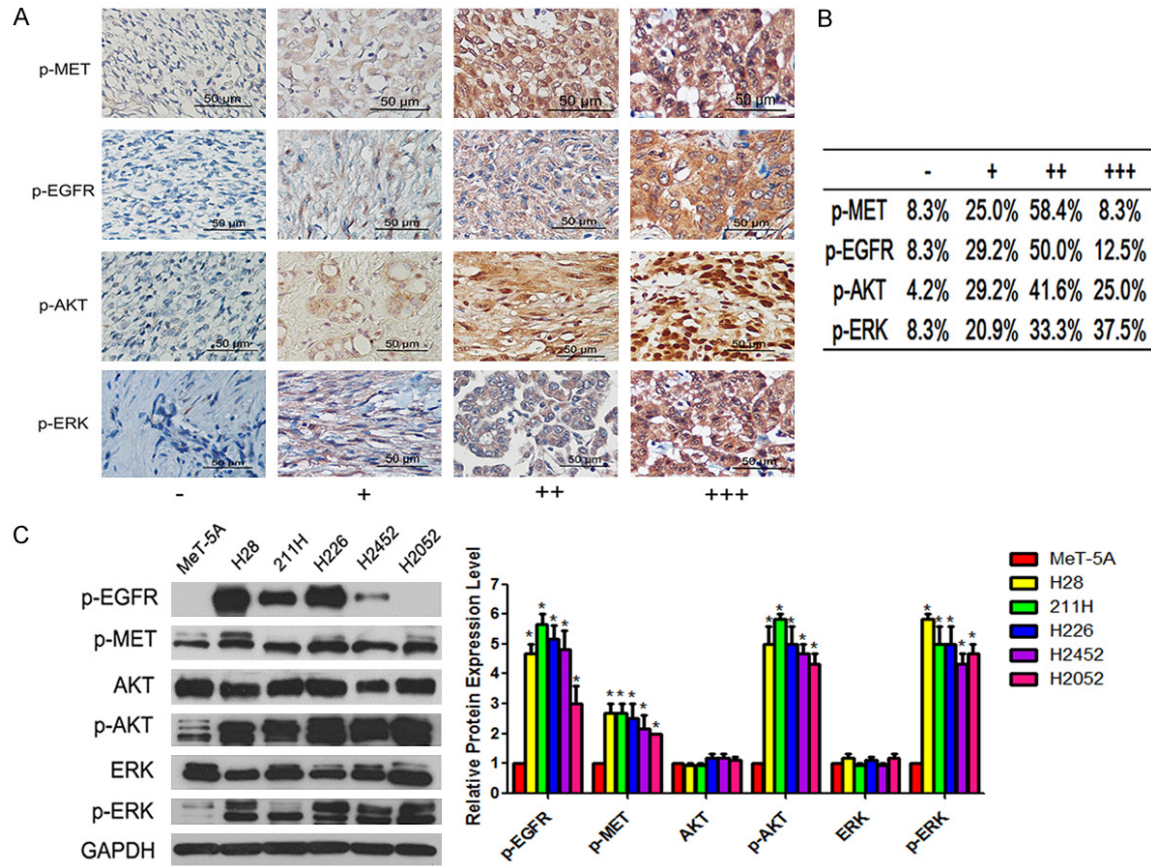
Figure 3. Simultaneously suppress MET and EGFR can remarkly inhibit MPM cells proliferation. A and C. MET and EGFR protein levels were detected by Western Blotting after transfection of MET-siRNA and EGFR-siRNA. GAPDH was used as a loading control. The data represent mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to control group (One-Way ANOVA). B and D. Clone formation assay of 211H and H226 cells was performed after transfection. Down-regulation of MET or EGFR alone did not significantly inhibit cell proliferation of 211H and H226 cells. Suppression of MET in combination with EGFR caused a marked inhibition of proliferation. Quantitative analysis of clone numbers of **Figure 4B** and **4D**, values represented mean ± SD of three independent experiments (***, P<0.001).

Figure 4. Downstream of MET and EGFR was active in MPM cells and specimens. A. Representative graphs showing typical examples of the four intensity grades of p-MET, p-EGFR, p-AKT and p-ERK staining in tumor samples of the cohort of 24 MPM patients. B. Statistics of four intensity grades of p-MET, p-EGFR, p-AKT and p-ERK in tumor samples of 24 MPM patients. C. Western Blot analysis showed the levels of p-MET, p-EGFR and their downstream signaling pathway proteins. GAPDH was used as a loading control. The data were analyzed using the Gel-Pro analyzer. The data represent mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to control group (One-Way ANOVA).

and cell lines. Results indicated that p-MET, p-EGFR, p-AKT and p-ERK were high expression in 66.7%, 62.5%, 66.6% and 70.8% MPM tissues (**Figure 4A**, **4B**). Furthermore, with the stimulation of EGF and HGF, MET, EGFR and their downstream proteins AKT and ERK were actived in most mesothelioma cell lines (**Figure 4C**), demonstrating that AKT and ERK pathways were active in MPM specimens and cell lines at the same time.

Simultaneous treatment with MET and EGFR inhibitors induced remarkably inhibitory effect on MPM cell proliferation in vitro

As we found MET and EGFR were co-high-expressed in most MPM cells and 70% specimens, we hypothesis that concurrent inhibition of MET and EGFR could have effect on MPM cells inhibition. As all of our 5 MPM cell lines

express wild-type EGFR (**Figure 5A**), we choose afatinib, the wild-type EGFR inhibitor, combined with MET inhibitor crizotinib to test the treatment efficacy of MPM. Cell viability was examined in 211H and H226 cells after treatment with crizotinib, afatinib alone or their combination for 72 h (**Figure 5B**, **5C**). According to the IC$_{50}$ of crizotinib and afatinib alone (**Table 1**), we designed 6 combination treatment groups. Combination effect was analyzed using combination index plots by the Compusyn software. The combination of crizotinib and afatinib exhibited significant synergistic cytotoxic effect on the cell lines tested (**Table 2**). In addition, long-term colony formation assay in MPM (211H and H226) cells also showed strong synergy of combination of crizotinib and afatinib whereas crizotinib or afatinib alone could only cause limited growth inhibition effect (P<0.001) (**Figure 5D**, **5E**).
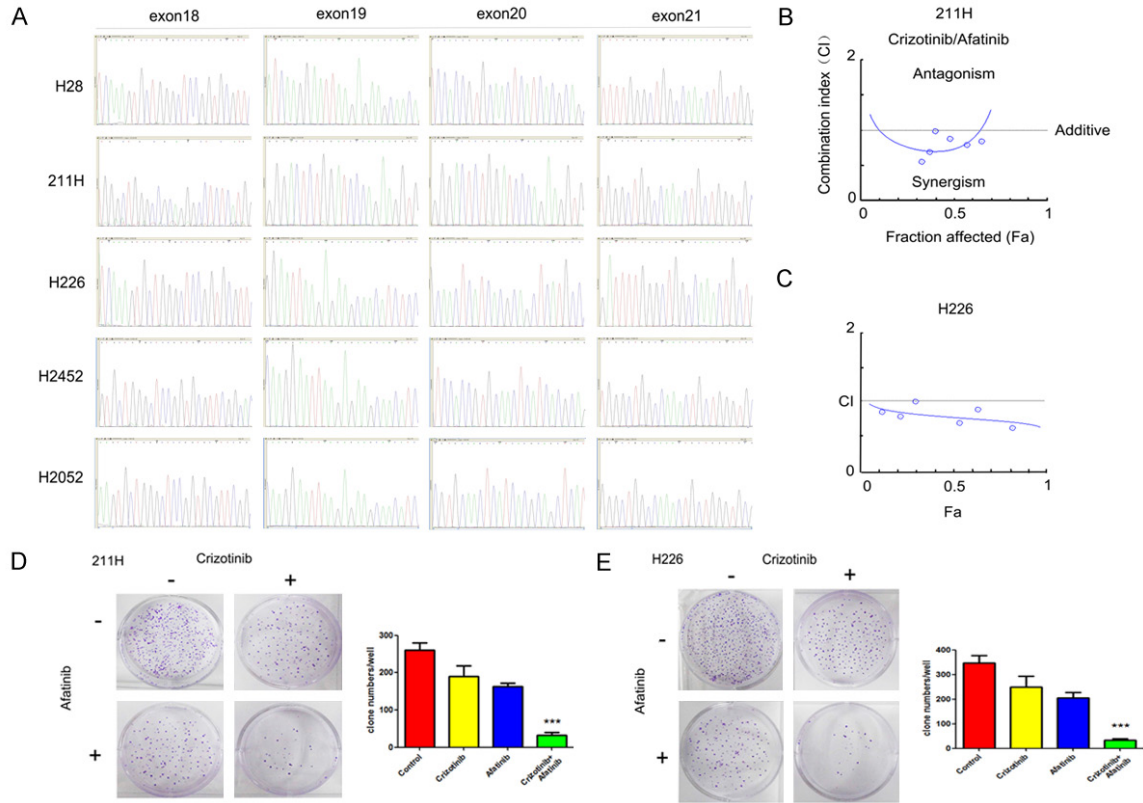
Figure 5. Simultaneous treatment with MET and EGFR inhibitors induced significantly anticancer effect on MPM cell proliferation *in vitro*. A. Five MPM cell lines express wild-type EGFR validated by DNA sequencing. B, C. Cells were treated with crizotinib or afatinib alone or their combination at serial concentrations for 72 h. Cell viability was measured by MTT assay. Combination index (CI) plot analysis of crizotinib and afatinib combinations showed that they interacted synergistically in 211H and H226 cells. CI = 1 shows additive effect, CI<1 is synergism and CI>1 is antagonism. Each experiment was carried out independently and repeated at least three times. D and E. Long-term colony formation assay of 211H and H226 cells with the combination of crizotinib and afatinib Cells were cultured in crizotinib (1 µM) alone, afatinib (1 µM) alone, or their combinations. The cells were fixed, stained and photographed after 14 days. Quantitative analysis of clone numbers of **Figure 4D** and **4F**, values represented mean ± SD of three independent experiments (***, P<0.001).

Table 1. IC_{50} of MET or EGFR inhibitor for a series of MPM cells

Cell Line	Crizotinib (µM)	Afatinib (µM)
H28	5.770 ± 0.126	2.420 ± 0.379
211H	1.870 ± 0.056	1.849 ± 0.004
H226	5.173 ± 0.038	4.660 ± 0.123
H2452	4.478 ± 0.072	3.328 ± 0.275
H2052	15.156 ± 0.009	16.473 ± 0.119

Simultaneous treatment with MET and EGFR inhibitors induced significant anticancer activity on MPM cells in vivo

To verify the synergistic anticancer activity observed *in vitro*, the antitumor activity of crizo-tinib and afatinib alone or their combination was further investigated in a mouse xenograft model derived from 211H cells. Mice were treated daily by oral gavage with saline, crizo-tinib, afatinib or their combination as described in "Methods and Materials". While the treatment with crizotinib or afatinib alone did not appreciably inhibit tumor growth, the drug combination was found to exhibit remarkable anti-cancer activity on the tumor xenografts inhibition (P<0.01) (**Figure 6A**, **6B**). The mean weights of tumors excised from mice were 1.08 ± 0.14, 0.98 ± 0.08, 0.96 ± 0.18, 0.50 ± 0.10 g for saline, crizotinib, afatinib and combination groups, respectively (P<0.01) (**Figure 6C**). The tumor growth inhibition rate (IR) of the combination group was calculated to be 53.67%.

Table 2. Inhibition rates of single and combination of crizotinib and afatinib

| | Crizotinib | | Afatinib | | Crizotinib+Afatinib | | |
	Concentration (μM)	Inhibitor rate (%)	Concentration (μM)	Inhibitor rate (%)	Concentration (μM)	Inhibitor rate (%)	CI value
211H	3.00	55 ± 2.3	3.00	58 ± 1.8	3+3	65 ± 9.2	0.84
	1.50	29 ± 1.8	1.50	49 ± 4.3	1.5+1.5	57 ± 3.6	0.79
	0.75	6 ± 0.3	0.75	44 ± 5.3	0.75+0.75	48 ± 5.7	0.88
	0.38	9 ± 0.1	0.38	40 ± 2.5	0.38+0.38	40 ± 3.6	0.99
	0.19	1 ± 0.6	0.19	33 ± 0.8	0.19+0.19	37 ± 1.9	0.69
	0.09	1 ± 0.4	0.09	28 ± 1.7	0.09+0.09	33 ± 3.3	0.55
H226	5.00	45 ± 4.6	5.00	63 ± 3.8	5+5	83 ± 7.5	0.6
	2.50	36 ± 2.7	2.50	35 ± 3.2	2.5+2.5	64 ± 2.9	0.87
	1.25	20 ± 0.5	1.25	27 ± 1.9	1.25+1.25	54 ± 4.6	0.67
	0.63	27 ± 5.9	0.63	20 ± 0.9	0.63+0.63	30 ± 2.1	0.99
	0.32	16 ± 0.2	0.32	11 ± 0.4	0.32+0.32	22 ± 0.9	0.77
	0.16	6 ± 0.3	0.16	3 ± 0.8	0.16+0.16	12 ± 1.1	0.84
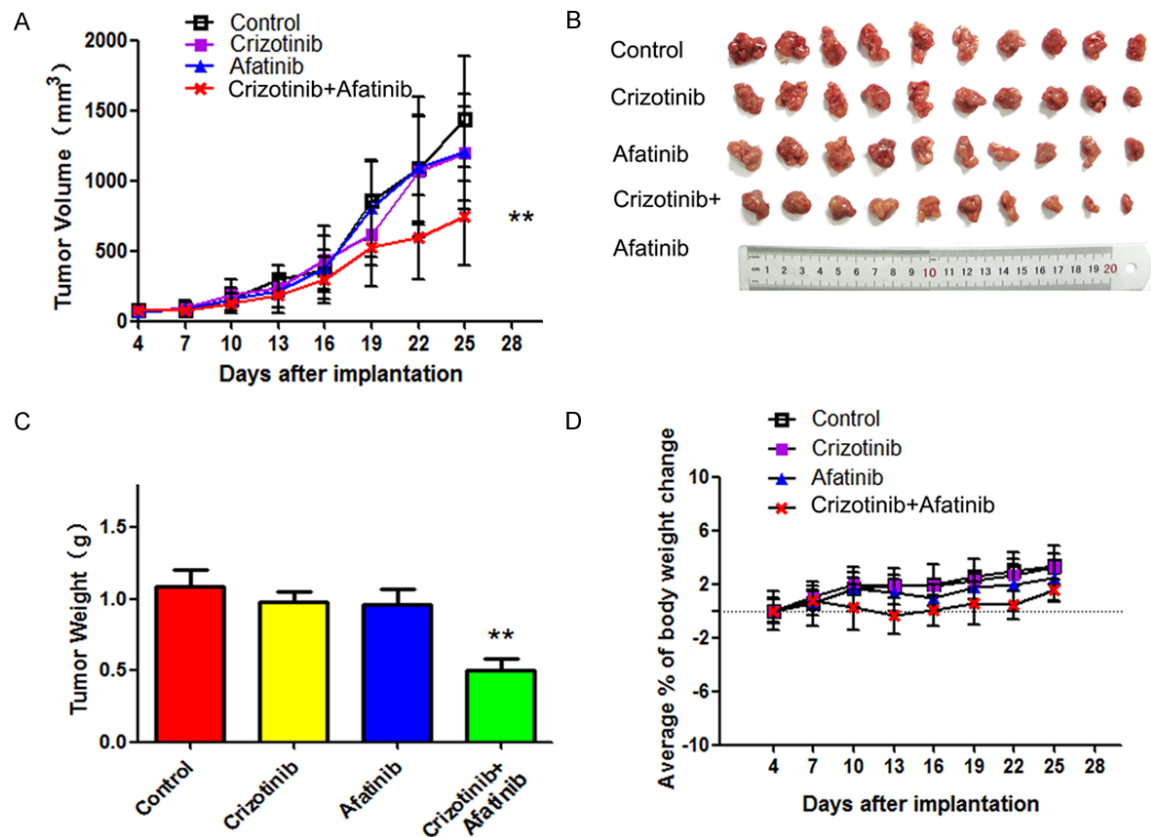
Figure 6. Simultaneous treatment with MET and EGFR inhibitors induced remarkably greater anticancer effect on MPM cell xenografts *in vivo*. A. The changes of tumor volume over time following the implantation. Data points represented the mean ± SD of tumor volumes of each group. n = 10. B. Image of excised xenograft tumors from four groups. C. Mean tumor weight (n = 10) of excised xenograft tumors. Error bars indicate SD. D. The changes in body weight Each point represents the mean ± SD of body weight from each group. (**, P<0.01).

Importantly, the drug combination was also well tolerated because the experimental mice in the combination group did not suffer notable body weight reduction (**Figure 6D**).
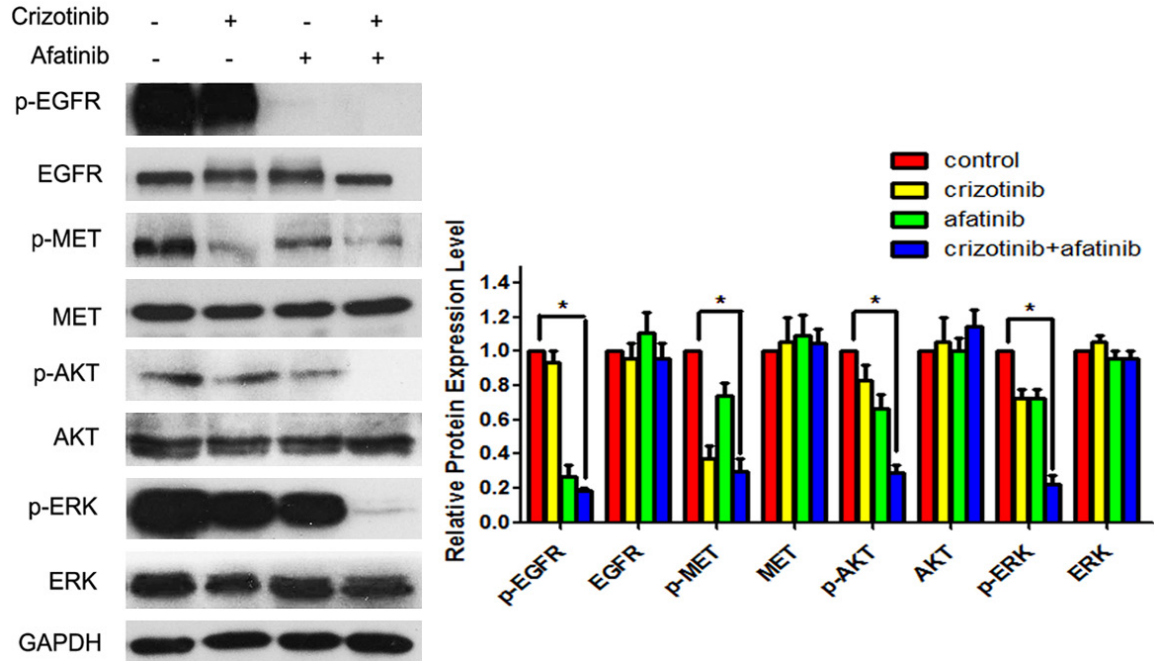
Figure 7. Effect of combination of crizotinib and afatinib on downstream signaling pathways. 211H cells were treated with crizotinib (2 µM) and afatinib (2 µM) alone or in combination for 24 h. HGF (40 ng/ml) and EGF (50 ng/ml) were added to culture medium before collected the cell lysates. Equal amount of protein was loaded for Western Blot analysis as described in "Materials and Methods". GAPDH was used as a loading control. The data were analyzed using the Gel-Pro analyzer. The data represent mean ± SD derived from three independent experiments. Significant changes are indicated as follows: *, P<0.05, compared to control group (One-Way ANOVA).

Effect of combination of crizotinib and afatinib on downstream cell survival signaling pathways

MET and EGFR can active signals through the AKT and MAPK pathways, so we examined the effect of crizotinib and afatinib on MET, EGFR, AKT, and ERK in MPM cells. 211H cells were treated with crizotinib (2 µM) and afatinib (2 µM) alone or their combination for 24 h. HGF (40 ng/ml) and EGF (50 ng/ml) were added to culture medium before collection of cell lysates. Both p-MET and p-EGFR were found to be increased after the stimulation by HGF and EGF. As expected, they were suppressed by their specific inhibitor (crizotinib and afatinib). Importantly, compared with crizotinib or afatinib alone treatment, the drug combination was found to cause more dramatic inhibition of p-AKT and p-ERK1/2. Total AKT and ERK protein levels were unchanged (**Figure 7**). These results indicated that only by combining the MET and EGFR inhibitors (crizotinib and afatinib), MPM survival signaling pathways could be significantly blocked.

Kaplan-Meier curve for overall survival of MPM patients with co-expression of MET and EGFR

To further elucidate the clinical role of MET and EGFR expression in MPM patients, we examined the relationship between single or co-expression of MET and EGFR, and patient therapeutic effect. A difference in overall survival rate (OS) was observed between high MET expression group (median survival time = 14.5 months) and low MET expression group (median survival time = 21.7 months) (P = 0.006) (**Figure 8A**). On the other hand, no relationship in overall survival rate between high EGFR expression group and low EGFR expression group (P = 0.197) (**Figure 8B**). However, a striking difference in overall survival was observed between the MET and EGFR co-high-expression group (median survival time = 13.5 months) and non-co-high-expression group (median survival time = 20.5 months) (P<0.001) (**Figure 8C**). These results indicated that co-high-expression of MET and EGFR in MPM may be used as a useful prognostic marker for MPM treatment.
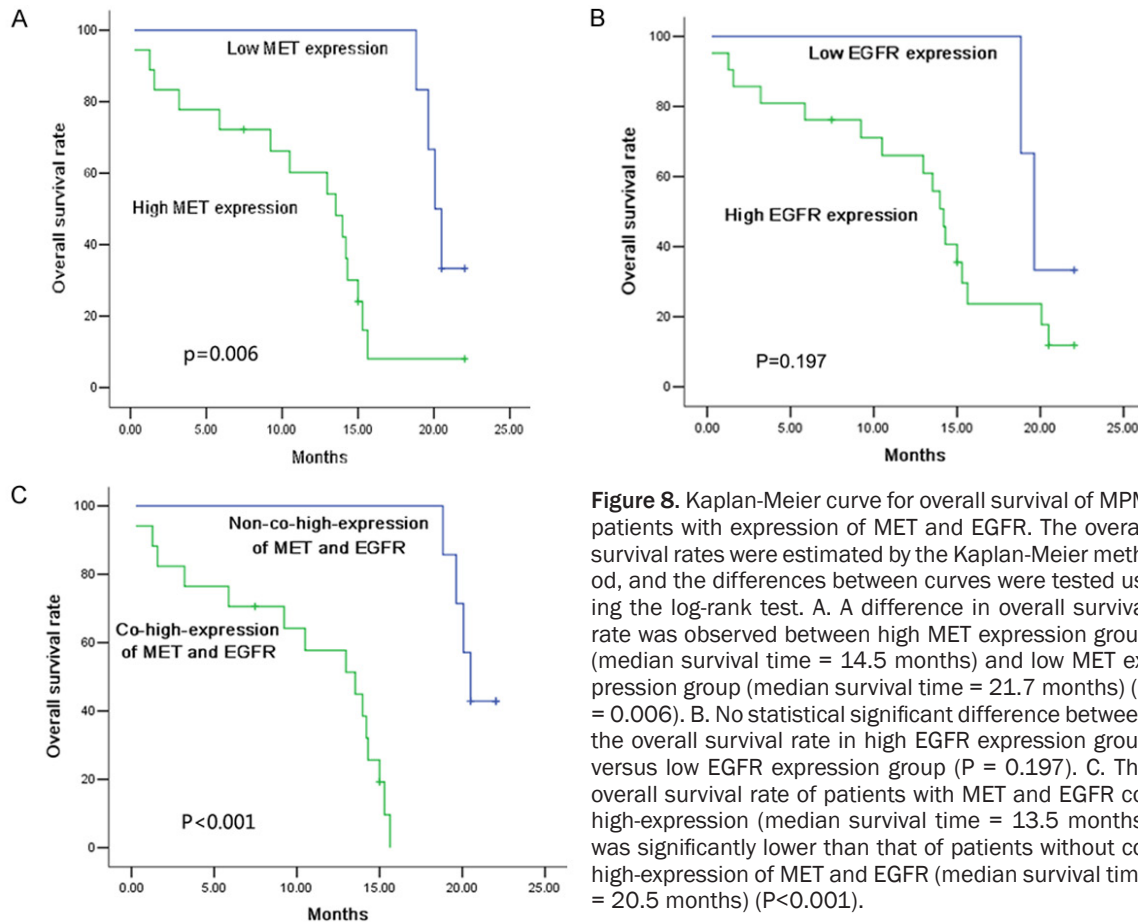
Figure 8. Kaplan-Meier curve for overall survival of MPM patients with expression of MET and EGFR. The overall survival rates were estimated by the Kaplan-Meier method, and the differences between curves were tested using the log-rank test. A. A difference in overall survival rate was observed between high MET expression group (median survival time = 14.5 months) and low MET expression group (median survival time = 21.7 months) (P = 0.006). B. No statistical significant difference between the overall survival rate in high EGFR expression group versus low EGFR expression group (P = 0.197). C. The overall survival rate of patients with MET and EGFR co-high-expression (median survival time = 13.5 months) was significantly lower than that of patients without co-high-expression of MET and EGFR (median survival time = 20.5 months) (P<0.001).

Discussion

MPM is a rare and invasive cancer, which is generally associated with prior asbestos exposure. Due to the relatively long latency period of the disease, the incidence of mesothelioma is still rising. Unfortunately, chemotherapy and surgery therapy are ineffective, and the prognosis is always very poor. Hence, there is a pressing need for effective therapies for MPM.

Targeted therapy for MPM is widely studied due to targeted drugs in the list have gained encouraging results on anti-tumor therapy. In our study, we analyzed the relationship of MET and EGFR co-expression pattern and patient's overall survival rate. A striking difference in overall survival rate was observed between the MET and EGFR co-high-expression group (median survival time = 13.5 months) and non-co-high-expression group (median survival time = 20.5 months) (P<0.001). MET is related to signals that promote cell survival, proliferation, movement, invasiveness, branching morphogenesis

and angiogenesis following binding of its ligand HGF [16]. EGFR is associated with a cascade of intracellular signal transduction pathways that could result in cell proliferation, invasion, anti-apoptosis and metastasis, when it is binding with ligands such as EGF or transforming growth factor alpha (TGF-α) [17, 18]. Among various intracellular signal pathways stimulated by MET and EGFR, the major ones are the PI3K/AKT/mTOR pathway which could lead to apoptosis inhibition, and the RAS/RAF/MAPK pathway which caused cell proliferation, metastasis and invasion [19]. These findings underscore the importance of MET and EGFR co-high-expression in the prognosis of MPM patients, and provided the basis for early MPM targeted therapy.

We further tried to search a targeted strategy to treat MPM. In our study, we confirmed MET and EGFR were remarkably over-expressed in MPM tissues and cell lines. Moreover, we found single inhibiting MET or EGFR by si-RNA caused little growth inhibition in MPM cell lines. In con-
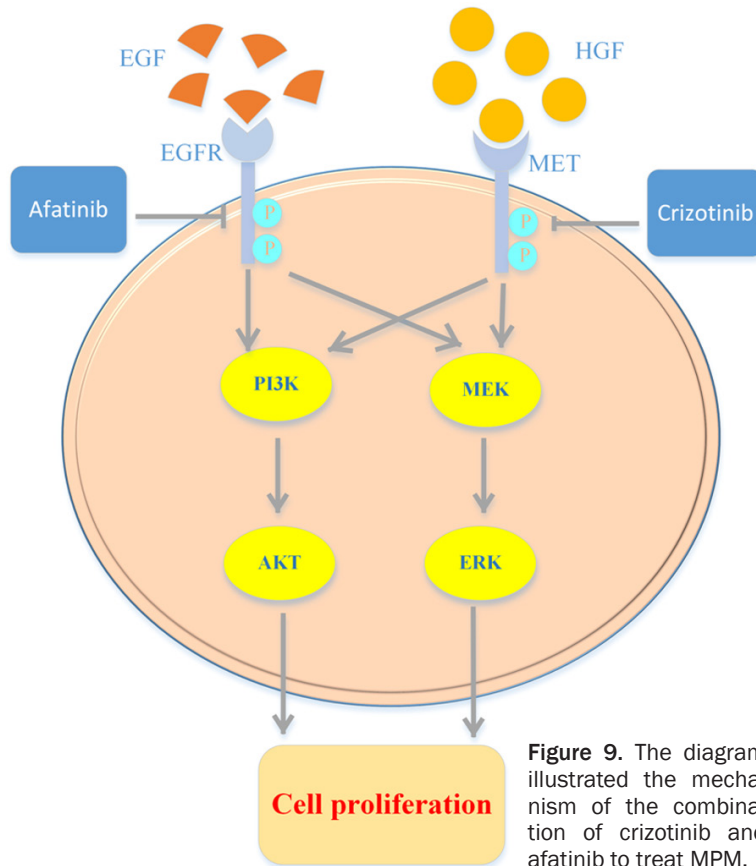
Figure 9. The diagram illustrated the mechanism of the combination of crizotinib and afatinib to treat MPM.

gy effect when crizotinib was combined with afatinib compared with the scarcely inhibition effect by the single drug alone. Furthermore, in the 211H xenograft, crizotinib or afatinib alone group had little anticancer effect compared with the control group. However, the combination of crizotinib and afatinib induced pronounced anticancer effect than either crizotinib or afatinib treatment alone. These results indicated that the combination of crizotinib and afatinib had potential antitumor effect to MPM in vitro and in vivo.

It is necessary to understand detailed mechanisms of the synergistic effect of crizotinib and afatinib. In MPM, MET and EGFR pathways were active at the same time which improved the proliferation of tumor. AKT and ERK were co-downstream signal pathways of MET and EGFR. Because of the cross-talk, the inhibition of the survival pathways by a single RTK inhibitor can be overridden by activation of another RTK. AKT and ERK signal pathways could not be inhibited completely when single inhibited MET or EGFR, so MPM could not be inhibited. Compared with crizotinib or afatinib alone treatment, p-AKT and p-ERK1/2 were more dramatically inhibited by the drug combination. Therefore, MPM survival signaling pathway can be blocked only by combination of crizotinib and afatinib (**Figure 9**).

Taken together, our results advocate the use of combination of crizotinib and afatinib to treat MPM. Our research was extension of the previous study and provided potential strategy for clinical trial of MPM tumor therapy.

Acknowledgements

trast, suppression of both MET and EGFR caused significantly inhibition of proliferation in MPM cells. These results illustrated that the necessary to inhibit MET and EGFR at the same time to treat MPM. As we found that 5 MPM cell lines express wild-type EGFR through Sanger sequencing, we choose afatinib, the both mutated and wild-type EGFR inhibitor, combined with MET inhibitor crizotinib to test the treatment efficacy for MPM. In previous studies, afatinib not only inhibited the models with harboring "activating" mutations in exons 19 and 21 of the EGFR gene, but was also active in lung cancer models harboring wild-type EGFR or the EGFR L858R/T790M double mutant [20]. Crizotinib was originally developed as a MET inhibitor which could inhibit MET phosphorylation and signal transduction, tumor cell proliferation and induce apoptosis, and was later shown to also be a potent inhibitor of ALK. In our study, the combination of crizotinib and afatinib was found to exhibit significant synergistic cytotoxic effect in 211H and H226 cells by MTT assay. Long-term colony formation assays of MPM cells also showed strong syner-

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Disclosure of conflict of interest

None.

Abbreviations

MPM, Malignant pleural mesothelioma; IHC, Immunohistochemistry; TKIs, tyrosine kinase inhibitors; c-MET/MET, cellular-mesenchymal to epithelial transition factor; RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; PI3-K, phosphoinositide 3-kinase; EGFR, Epidermal growth factor receptor; NSCLC, non-small cell lung cancer; HGF, hepatocyte growth factor; EGF, epidermal growth factor; FDA, Food and Drug Administration; HER2, human epidermal receptor 2; PBS, phosphate-buffered saline; DAB, diaminobenzidine; DMSO, dimethylsulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; CI, combinational index.

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