

Downregulation of c-Met expression does not enhance the sensitivity of gastric cancer cell line MKN-45 to gefitinib

JIN-AN MA, CHUNHONG HU, WENJUAN LI, JING REN, FANGWEN ZOU,
DONGAI ZHOU, WEN ZOU, YAJUN WEI and YING ZHOU

Department of Oncology, The Second Xiangya Hospital, Central South University,
Changsha, Hunan 410011, P.R. China

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Abstract. The aim of the present study was to investigate the effect of downregulation of the c-Met gene on signal transduction and apoptosis in gastric cancer MKN-45 cells; furthermore, the study aimed to determine whether altered c-Met gene expression affected MKN-45 sensitivity to gefitinib. Three c-Met-specific small interfering RNAs (siRNAs) were synthesized and transfected into MKN-45 cells. Messenger RNA (mRNA) and protein levels of c-Met and its downstream signaling molecules [phosphoinositide 3-kinase (PI3K) and AKT] were examined using reverse transcription polymerase chain reaction and western blot analysis 48 h following transfection. Cell apoptosis was evaluated using Annexin-V/propidium iodide double staining and fluorescence-activated cell sorting analysis. An MTT assay was performed in order to measure the 50% inhibitory concentration (IC_{50}) of gefitinib on MKN-45 cells. The results of the present study demonstrated that 48 h post-transfection with c-Met siRNA, MKN-45 cells showed significantly downregulated expression of c-Met mRNA and protein as well as an increased rate of apoptosis ($P<0.05$). In addition, following c-Met siRNA transfection mRNA and protein levels of PI3K and AKT were not significantly altered in MKN-45 cells ($P>0.05$); however, a marked decrease in the expression levels of phosphorylated (p)-PI3K and p-AKT was observed ($P<0.05$). Furthermore, the IC_{50} of gefitinib in MKN-45 cells was not significantly decreased. In conclusion, knockdown of the c-Met gene promoted gastric cancer cell apoptosis and inhibited downstream p-PI3K and p-AKT; however, the sensitivity of MKN-45 cells to gefitinib was not increased.

Introduction

At present, surgical resection is the primary treatment method for gastric cancer. However, for gastric cancer in the advanced or metastasized stages, surgery may not be an option and therefore, other therapies, including adjuvant therapy, salvage chemotherapy and cytotoxic treatment, are used (1); however, the effect of these therapies is often limited.

Trastuzumab, in combination with chemotherapy, has been reported to have a significant impact on the treatment of advanced human epidermal growth factor receptor 2 (HER2)-overexpressing gastric cancer. The results of this study demonstrated an increase in the long-term survival of patients, therefore suggesting its potential as a targeted therapy for gastric cancer (2). The identification of effective drug targets for novel therapies is an increasingly important field of drug research.

Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is an important transmembrane receptor, with its protein-tyrosine kinase activity residing in the intracellular domain. Activation of EGFR via growth factor ligand-binding triggers an intracellular signal transduction pathway, which further initiates intracellular responses by regulating downstream molecules. EGFR was reported to be highly associated with the incidence and development of gastric cancer (3). Furthermore, it was reported that patients with EGFR-overexpression had worse prognoses compared with those of EGFR-negative patients (4).

c-Met is a high-affinity hepatocyte growth factor (HGF) receptor, which also possesses tyrosine kinase activity. Studies have revealed that c-Met was frequently overexpressed in 46.1-77.3% of patients with gastric cancer (5-7); in addition, increased c-Met expression was reported to be highly associated with gastric cancer staging and poor prognosis as well as tumor cell migration, invasion and metastasis (8). Studies have shown that the HGF fragment NK4 acted as a HGF antagonist, improving the sensitivity of gastric cancer cells to the orally active EGFR tyrosine kinase inhibitor (EGFR-TKI), gefitinib (9). Phase II clinical studies of metastatic gastric and gastroesophageal junction adenocarcinoma have shown that following gefitinib administration to 75 late-stage patients, one patient showed a partial response (PR) and in 13 patients increase disease control was achieved (10). Another phase II

Correspondence to: Dr Chunhong Hu, Department of Oncology, The Second Xiangya Hospital, Central South University, 86 Middle Renmin Road, Changsha, Hunan 410011, P.R. China
E-mail: chunhonghu66@163.com

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clinical study examined the effect of an EGFR-TKI, erlotinib, on gastrointestinal and gastric adenocarcinoma. Out of 43 patients with gastroesophageal junction adenocarcinoma, one demonstrated a complete response (CR) and four showed a PR; however, no significant results were observed in any of the 25 gastric adenocarcinoma patients (11).

These clinical studies provided evidence for the minimal sensitivity of gastric cancers to EGFR-TKI. Therefore, it has been hypothesized that this may be due to drug resistance; however, the mechanism of sensitivity of certain gastroesophageal junction carcinoma to EGFR-TKI remains to be elucidated.

Numerous tyrosine kinase receptors are located on the surface of tumor cells, and activation of these receptors triggers signal transduction networks, which are able to crosstalk with each other (12,13). In theory, these pathways may have a synergistic role in cancer signaling and therefore, targeting these pathways may be an effective novel strategy for disease management. EGFR mutations in non-small-cell lung cancer (NSCLC) increased the sensitivity of cells to EGFR-TKI treatment (14); in addition, the continuous overexpression of c-Met was reported to be functionally relevant to EGFR-TKI resistance (15-17). Non-mutated EGFR and c-Met have been shown to be overexpressed in the gastric cancer cell line MKN-45 (18,19). The aim of the present study was to investigate whether altering c-Met gene expression by using small interfering RNAs (siRNAs) affected the sensitivity and resistance of MKN-45 cells to gefitinib.

Materials and methods

Cell line and culture. The human gastric cancer cell line MKN-45 was purchased from American Type Culture Collection (Manassas, VA, USA). MKN-45 was grown and passaged routinely at 37°C in a humidified 5% CO₂ atmosphere in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL).

siRNA transfection. Three pairs of siRNAs for c-Met and one pair of control siRNAs were designed and synthesized (Shanghai GeneChem Co., Ltd., Shanghai, China). siRNA sequences were as follows: c-Met-siRNA1 sense, 5'-GUGCCACUACUACAUUUATT-3' and anti-sense, 5'-UAAAUGUAGUUAGUGGCACTT-3'; c-Met-siRNA2 sense, 5'-GUCCCGAGAAUGGUCAUAATT-3' and anti-sense, 5'-UUAUGACCAUUCUCGGGACTT-3'; c-Met-siRNA3 sense, 5'-GCCUGAAUGAUGACAUCUUTT-3' and anti-sense, 5'-AGAAUGUCAUCAUUCAGGCTT-3'; control siRNA sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'. The transfection efficiency was analyzed using fluorescence microscopy (Axioskop40; Carl Zeiss AG, Jena, Germany) using the methods described previously (20).

Reagents. LipofectamineTM 2000 transfection kits were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Polyclonal goat anti-human-c-Met, -PI3K, -phosphorylated (p)-PI3K, -AKT and -p-AKT (1:300) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit

immunoglobulin G (IgG) was purchased from Proteintech (Chicago, IL, USA). Primers for polymerase chain reaction (PCR) and the reverse-transcription PCR (RT-PCR) kit were obtained from Takara Bio, Inc. (Dalian, China).

Transfection. siRNA was transfected with the LipofectamineTM 2000 kit according to the manufacturer's instructions. In brief, cells were seeded into six-well plates until they reached 70-90% confluence. Cells were divided into five groups: Control cells; cells transfected with Lipofectamine only; cells transfected with 200 pmol siRNA1, siRNA2 or siRNA3; and cells transfected with control siRNA. A total of 5 μ l LipofectamineTM 2000 was added to 250 μ l serum-free medium and mixed for 5 min at room temperature. An appropriate amount of siRNA (final concentration of 200 pmol) was then added and incubated for 20 min. The mixture was then added to phosphate-buffered saline (PBS)-washed cells and incubated at 37°C for 4 h. 10% FBS/DMEM medium was then added to achieve a final volume of 2 ml.

Western blot analysis. Following transfection, cells were lysed for 48 h and then separated using 8% SDS-PAGE. Prior to incubation, the membrane was blocked with primary antibodies. Rabbit-anti-human-c-Met, -PI3K, -p-PI3K, -AKT, -p-AKT or β -actin (internal control) antibodies (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) were incubated with the membranes for 2 h at room temperature, washed using 1X Tris-buffered saline with Tween 20 and incubated with a HRP-labeled secondary antibody (goat-anti-rabbit IgG; 1:500; Proteintech) for 90 min. An enhanced chemiluminescence kit from Perkin-Elmer (Waltham, MA, USA) was used to detect the signal. Western blot analyses were quantified by densitometry and analyzed using the Quantity One image analysis system (Bio-Rad, Hercules, CA, USA).

RT-PCR. Total RNA was isolated using TRIzol[®] (Invitrogen Life Technologies) 48 h following transfection. RNA purity was measured using a spectrometer, and 2 μ g RNA was reverse-transcribed in a 20- μ l reaction system. The specific primers used were as follows: c-Met forward, 5'-CCTCACCATAGCTAATCTTGGGACA-3' and reverse, 5'-CACAATCACTTCTGGAGACACTGGA-3'; PI3K forward 5'-AGGCTGTGATTGGGCGTA-3' and reverse, 5'-AAGCAACCTCAAAGGGAAA-3'; AKT forward, 5'-ATGGCACCTTCATTGGCTAC-3' and reverse, 5'-CAGTCTGGATGGCGGTTG-3'. The housekeeping gene GAPDH was used as the internal control (forward, 5'-CAAGGTCATCCATGACAACCTTTG-3' and reverse, 5'-GTCCACCACCCTGTTGCTGTAG-3'). The cycling conditions were 95°C for 30 sec, 40 cycles of 95°C for 5 sec, and then 60°C for 30 sec.

MTT assay. In brief, cells were seeded into 96-well plates at a density of 6,000 cells/well 24 h following transfection. A series of concentrations of gefitinib were then added and incubated for 48 h. MTT was added with the final concentration of 5 mg/ml for 4 h. Medium was replaced with 150 μ l dimethyl sulfoxide and incubated for 10 min. Optical density was measured at 490 nm using a Wellsan MK3 ELISA reader (LabSystems, Dragon, Finland) in order to determine

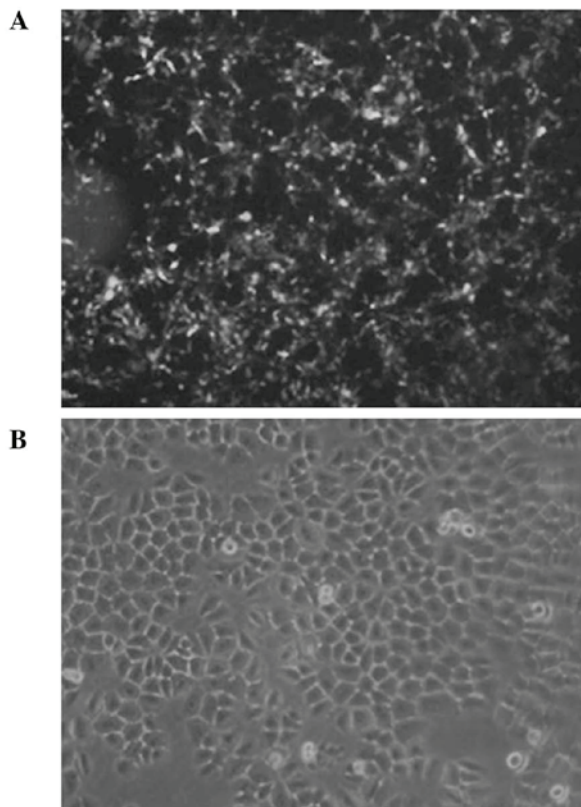


Figure 1. Fluorescence microscopy of transfected and untransfected gastric cancer MKN-45 cells. (A) c-Met small interfering RNA transfected MKN-45 cells 6 h post-transfection. (B) Untransfected MKN-45 cells. Images were captured using a bright-field fluorescence microscope (magnification, x10).

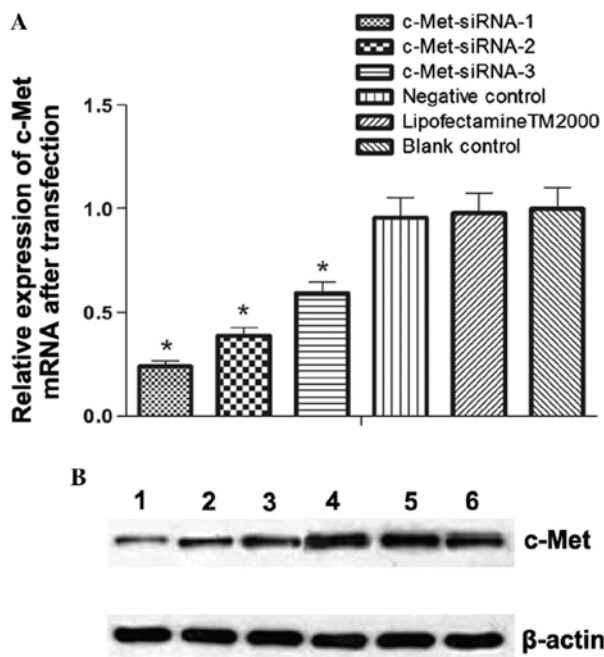


Figure 2. Relative expression of c-Met in gastric cancer MKN-45 cells following transfection of c-Met siRNAs. (A) c-Met mRNA expression levels following transfection of c-Met siRNAs and controls. * $P < 0.05$ compared with negative control. (B) c-Met protein expression following transfection with: 1, c-Met-siRNA-1; 2, c-Met-siRNA-2; 3, c-Met-siRNA-3; 4, negative control siRNA; and 5, LipofectamineTM 2000, as well as 6, un-transfected blank control. Expression was measured relative to β -actin. siRNA, small interfering RNA; mRNA, messenger RNA.

Table I. Apoptotic rates of MKN-45 cells following transfection.

Group	Apoptotic rate (%)	P-value
c-Met-siRNA-1	35.43±4.6	<0.05
Lipofectamine TM 2000	11.82±2.30	>0.05
Normal control	7.02±2.24	

Mean \pm standard deviation. P-values are relative to the normal control (n=3). siRNA, small interfering RNA.

the IC_{50} of gefitinib. $IgIC_{50}$ served as a standard control, $IgIC_{50} = X_m - I [P - (3 - P_m - P_n) / 4] m$ where X_m is the numerical value of the maximum designed concentration; I is the numerical value of the maximum dose/adjacent doses, is the sum of positive reaction rates, P_m is the maximum positive reaction rate and P_n is the smallest positive reaction rate.

Fluorescence-activated cell sorting (FACS). Cells were dissociated into a single-cell suspension and the apoptotic rate was assayed using flow cytometry (Becton-Dickinson FACSCalibur flow cytometer; BD Biosciences, Franklin Lakes, NJ, USA with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining according to the manufacturer's instructions (Hong Kong Jiamei Century Biotechnology, Ltd., Hong Kong, Japan).

Statistical analysis. Values are expressed as the mean \pm standard deviation. Differences between groups were assessed by one-way analysis of variance using SPSS 19.0 statistical software package (International Business Machines Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

Transfection efficiency. Fluorescent-labeled negative control siRNAs were transfected into MKN-45 cells in order to monitor siRNA uptake. Six hours post-transfection, transfection efficiency was analyzed using fluorescence microscopy. As shown in Fig. 1, 80% transfection was achieved using an siRNA: LipofectamineTM 2000 ratio of 40 pmol:1 μ l, which was adopted throughout the study.

c-Met mRNA levels following transfection. The expression of c-Met was calculated by normalizing values relative to GAPDH. The results demonstrated that all c-Met siRNA constructs significantly downregulated c-Met expression ($P < 0.05$); however, siRNA-c-Met-1 had the most obvious effect (Fig. 2A).

c-Met protein expression following transfection. c-Met protein expression was normalized to β -actin and compared following transfection. The relative expression levels of c-Met in siRNA groups 1, 2 and 3 were 0.258 ± 0.021 , 0.379 ± 0.018 and 0.485 ± 0.040 , respectively; each siRNA group showed significantly decreased c-Met protein expression compared

Table II. PI3K/AKT mRNA levels following c-Met knockdown.

Group	PI3K mRNA	P-value	AKT mRNA	P-value
c-Met-siRNA-1	0.450±0.017	>0.05	0.215±0.018	>0.05
Negative control	0.455±0.030	>0.05	0.225±0.016	>0.05
Lipofectamine™ 2000	0.453±0.021	>0.05	0.219±0.025	>0.05
Blank control	0.465±0.025	-	0.229±0.024	-

Mean ± standard deviation. P-values are relative to the blank control (n=3). PI3K, phosphoinositide 3-kinase; mRNA, messenger RNA; siRNA, small interfering RNA.

to that of the control ($P<0.05$). The strongest suppression of c-Met expression was observed following transfection of c-Met-siRNA-1 (Fig. 2B). Accordingly, c-Met-siRNA-1 was used in the subsequent functional experiment.

Apoptosis of MKN-45 cells prior to and following c-Met gene silencing. Annexin V-FITC/PI double staining and FACS analysis was used to evaluate the apoptotic rate of MKN-45 cells (Fig. 3). Early apoptotic cells are Annexin-positive and PI-negative, and are therefore represented in the lower-right quadrant of the photomicrographs; Annexin- and PI- positive cells in the upper-right quadrant are late apoptotic or necrotic cells. The total apoptotic rate was obtained by calculating the sum of these two quadrants. The apoptotic rate of MKN-45 cells (Table I) following c-Met-siRNA transfection was significantly higher than that in control siRNA-transfected or Lipofectamine™ 2000 only-transfected cells ($35.43\pm4.6\%$ vs. 7.02 ± 2.24 and $11.82\pm2.30\%$, respectively; $P<0.05$); this therefore indicated that c-Met was involved in MKN-45 apoptosis.

Impact of c-Met knockdown on PI3K and AKT signaling. PI3K and AKT are important downstream genes of c-Met (21). Following transfection, the relative mRNA expression of PI3K and AKT was examined using quantitative PCR. As shown in Table II, expression levels of PI3K and AKT showed no significant difference to those of the groups transfected with control siRNA ($P>0.05$). In addition, protein expression levels of PI3K and AKT were not altered by c-Met knockdown ($P>0.05$) (Table III; Fig. 4). By contrast, protein levels of p-PI3K and p-AKT were significantly downregulated compared to those of the group transfected with control siRNA (Table IV; Fig. 4). These results therefore indicated that c-Met signaling was attenuated by the downregulation of c-Met transcription.

IC₅₀ of gefitinib in MKN-45 cells. The IC₅₀ values of gefitinib on MKN-45 cells were determined following transfection using an MTT assay (Table V), with the IgIC₅₀ values in the un-transfected and transfected cells being 2.595 ± 0.010 and 2.566 ± 0.206 , respectively. Un-transfected and transfected cells demonstrated comparable responses to gefitinib (Fig. 5), indicating that drug-sensitivity was independent of c-Met expression. siRNA transfection did not affect the inhibition rate of MKN-45 cells following treatment with different concentrations of gefitinib.

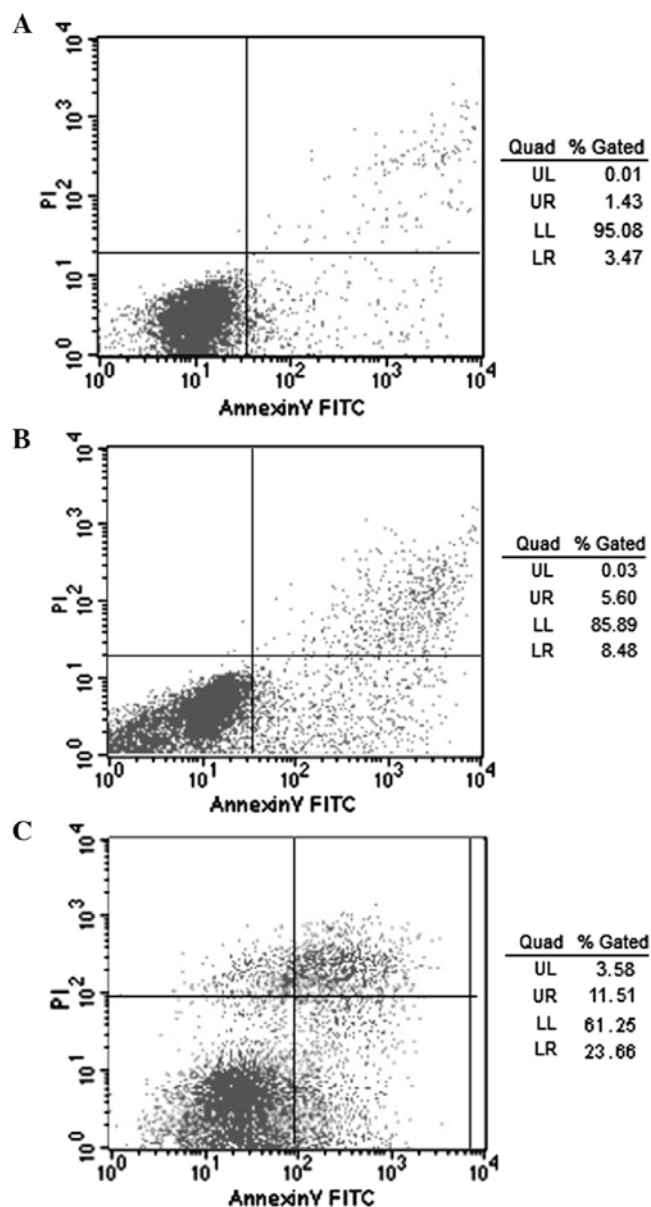


Figure 3. Fluorescence-activated cell sorting and Annexin V-FITC/PI double-labeled staining was used to determine the apoptotic rate of MKN-45 cells. Sorting of MKN-45 cells following transfection with (A) Normal siRNA control; (B) Lipofectamine™ 2000; and (C) c-Met-siRNA-1. Early apoptotic cells are Annexin-positive and PI-negative, lower-right quadrant of the photomicrographs; late apoptotic or necrotic cells are Annexin- and PI- positive cells, upper-right quadrant. FITC, fluorescein isothiocyanate; PI, propidium iodide; siRNA, small interfering RNA; Quad, quadrant; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

Table III. PI3K/AKT protein levels following c-Met knockdown.

Group	PI3K protein	P-value	AKT protein	P-value
c-Met-siRNA-1	0.466±0.050	>0.05	0.200±0.030	>0.05
Negative control	0.475±0.020	>0.05	0.218±0.050	>0.05
Lipofectamine™ 2000	0.470±0.030	>0.05	0.215±0.010	>0.05
Blank control	0.477±0.010	-	0.229±0.020	-

Mean ± standard deviation. P-values are relative to the blank control (n=3). PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA.

Table IV. p-PI3K and p-AKT levels following c-Met knockdown.

Group	p-PI3K protein	P-value	p-AKT protein	P-value
c-Met-siRNA-1	0.190±0.020	<0.05	0.125±0.040	<0.05
Negative control	0.380±0.020	>0.05	0.195±0.020	>0.05
Lipofectamine™ 2000	0.388±0.035	>0.05	0.188±0.020	>0.05
Blank control	0.395±0.030	-	0.198±0.030	-

Mean ± standard deviation. P-values are relative to the blank control (n=3). p-, phosphorylated; PI3K, phosphorylated-phosphoinositide 3-kinase; siRNA, small interfering RNA.

Table V. IC₅₀ of gefitinib in MNK-45 cells.

Group	IC ₅₀ (μmol/l)	IgIC ₅₀	P-value
Untransfected	393.650±8.594	2.595±0.010	0.136
Transfected	368.648±17.368	2.566±0.206	

Mean ± standard deviation (n=3). Optical density was measured at 490 nm. P-value compares transfected and un-transfected cell values. IC₅₀, 50% inhibitory concentration; IgIC₅₀, IC₅₀ for immunoglobulin synthesis.

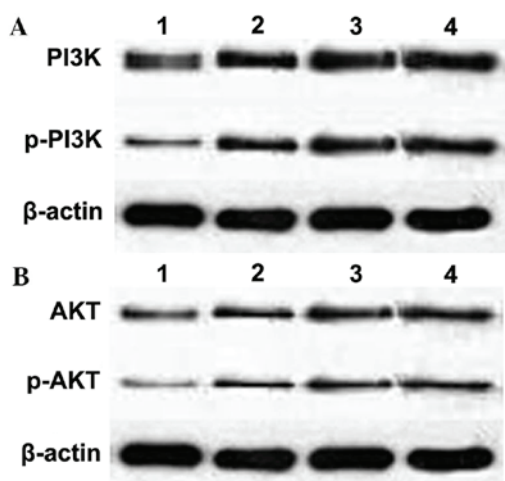


Figure 4. Protein expression of c-Met downstream signaling genes PI3K and AKT following c-Met knockdown. Western blot analysis revealed protein expression levels of (A) PI3K and (B) AKT following transfection of MKN-45 cells. Lanes: Cells transfected with 1, c-Met-siRNA-1; 2, negative siRNA control; and 3, Lipofectamine™ 2000 as well as 4, untransfected blank control. β-actin was used as an internal control. PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA.

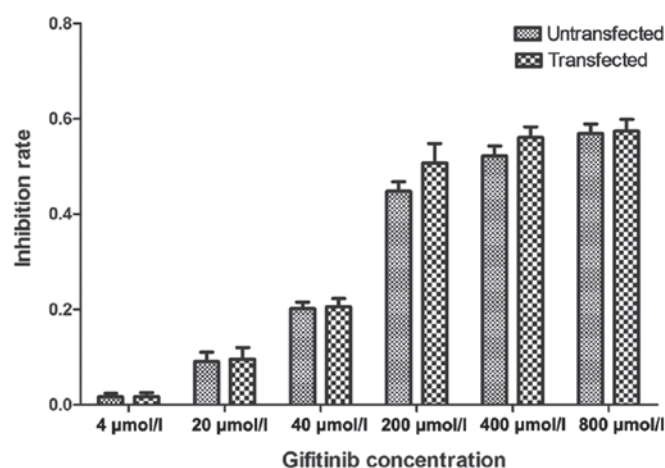


Figure 5. MTT assay determines the effect of gefitinib on growth inhibition in MKN-45. MKN-45 untransfected cells and cells transfected with c-Met small interfering RNA 1 were incubated with different concentrations of gefitinib for 48 h and growth inhibition was measured using an MTT assay for cell viability.

Discussion

EGFR and c-Met are tyrosine kinase receptors that share downstream signaling transduction pathways, including the mitogen-activated protein kinase and PI3K/AKT pathways (22). Gefitinib has been reported to inhibit the intracellular tyrosine kinase domain of EGFR and be effective in the treatment of NSCLC, which harbors mutations in EGFR at exons 19 and 21. Gastric cancers highly express EGFR; however, studies have shown that gastric cancers exhibited minimal sensitivity or were unresponsive to gefitinib. Certain studies have indicated that high c-Met expression may be responsible for acquired

resistance to EGFR-TKI in lung cancer (23-26) and c-Met amplification may activate the receptor tyrosine-protein kinase, human epidermal growth factor receptor 3 (ErbB3; HER3)-dependent PI3K/AKT signaling and therefore result in gefitinib resistance (27).

A previous study (8) reported that the HGF inhibitor NK4 enhanced the sensitivity of peritoneally spread gastric cancer to gefitinib *in vivo* (8). HGF is a ligand for the c-Met receptor, therefore suggesting that abnormal c-Met expression may alter the sensitivity of gefitinib to EGFR-TKI. The aim of the present study was to explore whether downregulation of c-Met enhanced the sensitivity of gastric cancer to gefitinib.

Numerous studies have demonstrated that blocking c-Met signaling inhibited cell proliferation and invasion as well as induced apoptosis (28-31). In addition, the use of siRNAs to knockdown c-Met in gastric cancer cells was reported to trigger apoptosis (31). The results of the present study revealed that the apoptotic rate of MKN-45 cells transfected with c-Met siRNA was significantly higher than that of the control groups; this therefore confirmed that c-Met mediated apoptosis in gastric cancer.

In the present study, three siRNA oligos for c-Met were transfected into MKN-45 cells, which resulted in high rates of c-Met inhibition ($P < 0.05$), indicating successful c-Met downregulation. c-Met-siRNA-1 showed the most effective results and therefore was used for the subsequent experiments.

Previous studies have shown that the IC_{50} value of gefitinib on gastric cancer cells was 400 $\mu\text{mol/l}$; therefore, throughout the present study, this value was used as a guideline to test the inhibition rate of different doses of gefitinib on MKN-45 cells (18). The results revealed that the IC_{50} values of gefitinib were unchanged, despite c-Met knockdown. It was speculated that MKN-45 cell resistance to gefitinib was not dependent on c-Met expression and the molecular mechanism of EGFR-TKI-resistance in MKN-45 cells remains to be elucidated. In c-Met-addicted gastric cancer, the inhibition or gene silencing of c-Met may be an effective approach for reversing drug resistance. However, EGFR and HER activation were reported to induce drug resistance (32), suggesting that there is complex cross-talk between EGFR and c-Met (33).

Phase II clinical studies on NSCLC patients demonstrated that the combination of the c-Met inhibitor tivantinib (ARQ197) and erlotinib did not increase the overall survival rate; however, in patients with KRAS mutations, tivantinib was shown to increase the beneficial effect of erlotinib (34). In the present study, the hypothesized increase of gefitinib sensitivity in c-Met-silenced MKN-45 cells was not observed. In addition, mRNA and protein levels of the downstream targets of c-Met, PI3K and AKT were not significantly altered following c-Met knockdown; by contrast, siRNA transfection was shown to attenuate p-PI3K and p-AKT levels, which may explain the unaltered sensitivity to gefitinib in MKN-45 cells. However, the precise molecular mechanism of gastric cancer insensitivity to gefitinib remains to be elucidated.

In NSCLC, c-Met was markedly associated with high-grade amplification that conferred acquired resistance to EGFR-TKIs in EGFR-mutant cancers. In gastric cancer, EGFR and HER3 activation was reported to result in acquired

resistance to c-Met inhibitors. The results of the present study indicated that c-Met downregulation promoted MKN-45 cell apoptosis; however, it did not increase the sensitivity of gastric cancer cells to gefitinib. In conclusion, these results suggested that c-Met expression was not associated with or had little effect on the resistance of gastric cancer cells to gefitinib. Further studies are required in order to determine whether KRAS gene mutations or other signaling molecules downstream of EGFR and c-Met are involved in mediating gefitinib resistance.

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