Blockade of DNA methylation enhances the therapeutic effect of gefitinib in non-small cell lung cancer cells

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Abstract. The sensitivity of lung cancer to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) has been found to be associated with mutations in the tyrosine kinase domain of EGFR. However, not all mutations are sensitive to gefitinib. While CpG island methylation in the promoter region of the EGFR gene and transcriptional silencing are common in solid tumors, the role of the EGFR gene promoter methylation in affecting resistance to TKIs in non-small cell lung cancer (NSCLC) remains unknown. In this study, we examined the correlation between EGFR gene promoter methylation and the therapeutic effect of gefitinib in NSCLC cells. Three NSCLC cell lines with different EGFR mutation statuses and levels of sensitivity to EGFR-TKIs were used in this study: H1650 (del E746-A750), H1299 (wild-type EGFR) and PC-9 (del E746-A750). Cells were treated with gefitinib or 5-aza-2'-deoxy cytidine (5-aza-CdR), a methylation inhibitor, alone or in combination. Subsequently, the methylation status of the EGFR gene promoter was examined by methylation-specific PCR (MSP). Cell survival and apoptosis assays were performed using the Cell Counting Kit-8 (CCK-8) and flow cytometry. In addition, western blot analysis and quantitative real-time PCR were used to examine the expression levels of EGFR protein and mRNA. Our study showed that the promoter region of the EGFR gene in PC-9 cells was unmethylated, and that the cells were sensitive to gefitinib. By contrast, the promoter region of the EGFR gene in the H1650 and H1299 cells was methylated, and the cells were resistant to gefitinib. Of note, the combination treatment with 5-aza-CdR and gefitinib further enhanced the growth inhibitory effects and led to the induction of apoptosis, while a significant reduction in the expression of EGFR protein and mRNA was observed in the H1650 and H1299 cells. These results suggest that blockade of DNA methylation may enhance the antitumor effects of EGFR-TKIs and gefitinib in NSCLC cells. Thus, EGFR gene promoter methylation may be a potential mechanism for acquired resistance to gefitinib.

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Introduction

Lung cancer is the most lethal malignancy worldwide. Treatment options remain limited for some patients with advanced lung cancer. Approximately half of limited stage lung cancer patients relapse despite curative intent surgery/radiation and adjuvant chemotherapy (1). Thus, there is an urgent need to develop novel or modified therapeutic approaches to enhance the efficacy of treatment for this malignancy.

Epidermal growth factor receptor (EGFR), a 170 kDa membrane-bound protein encoded by 28 exons on chromosome 7p12, is a typical member of the tyrosine kinase (TK) family and belongs to a subfamily that consists of four closely related members: EGFR (ErbB1), HER-2/neu (ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4). All members have an extracellular ligand-binding domain, a single membrane spanning domain and an intracellular domain (2,3); they are also known to have intrinsic TK activity apart from ErbB3 (4). EGFR has been shown to play a central role in the occurrence and progression of multiple solid tumors, including lung cancer (5). Several anti-EGFR molecules have been reported to induce neoplastic growth inhibition. Among these, gefitinib (Iressa), an orally available synthetic anilinoquinazoline agent, selectively binds to the TK region of the intracellular domain of EGFR, prevents ATP binding, and blocks EGFR signaling transduction pathways, thereby inhibiting cancer cell growth (6). Over the years, non-small cell lung cancer (NSCLC) containing EGFR mutations has been shown to be sensitive to gefitinib, and this agent has been successfully used in the treatment of these patients (7,8). Unfortunately, some patients with EGFR gene mutations develop resistance to TK inhibitor (TKI) treatment after a certain period of time, and the mechanisms behind this resistance remain unknown.

Aberrant hypermethylation in the gene promoter has become one of the major mechanisms for silencing tumor suppressor or other cancer-associated genes in many types of human cancer, and it is an epigenetic modification that plays an important role in the control of gene expression in mammalian cells (9). Recently, the role it plays in carcinogenesis has raised considerable interest (10). In many tumor types, CpG island hypermethylation in the promoter region of several tumor suppressor genes has been observed and has been shown to

correlate closely with the loss of mRNA and protein expression. While hypermethylation typically affects tumor suppressor genes, it also silences oncogenes, such as cyclooxygenase-2 (COX-2) (11) and telomerase reverse transcriptase (TERT) (12). Since the promoter region of the EGFR gene contains a CpG island that extends into exon 1, we hypothesized that EGFR gene promoter methylation may influence the antitumor effect of gefitinib on NSCLC cells.

Materials and methods

Cell culture and reagents. Three NSCLC cell lines with different EGFR mutation statuses and levels of sensitivity to EGFR-TKIs, obtained from the Shanghai Cell Station, Chinese Academy of Sciences, Shanghai, China were used: H1650 (del E746-A750), H1299 (wild-type EGFR) and PC-9 (del E746-A750). These cells were maintained in RPMI-1640 culture medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin, and they were incubated in humidified air and 5% CO_2 at 37˚C. Gefitinib was a generous gift from AstraZeneca (Cheshire, UK). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20˚C. Gefitinib solution was prepared in fresh medium prior to each experiment and the control cells were treated with medium containing an equal concentration of DMSO. 5-Aza-deoxycytidine (5-aza-CdR, decitabine), a methylation inhibitor, was purchased from Sigma (St. Louis, MO, USA) and prepared as described above.

Methylation-specific PCR (MSP). For the demethylation experiments, NSCLC cells (H1650 and H1299) were treated with varying concentrations (1-10 μM) of 5-aza-CdR for up to 72 h. The PC-9 cells were not treated with 5-aza-CdR due to their unmethylated status. Genomic DNA was extracted by proteinase K digestion followed by purification with a series of phenol/chloroform and isopropyl alcohol precipitations. The extracted DNA samples were stored in TE buffer at -20˚C until use. Bisulfite-based DNA modification, which converted all unmethylated cytosines to uracil, was performed by using the Methylcode Bisulfite Conversion kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The modified DNA was used as a template for MSP with primers specific for either the modified-methylated or unmethylated EGFR gene promoter sequences. PCR amplification was performed with the following primer sets that included the CpG island of EGFR: forward primer, 5'-GGTTGGGTTTGTAAGTTCGC-3' and reverse primer, 5'-ATAAACAACGATAACCCCCG-3' for the methylated EGFR sequence (150 bp); and forward primer, 5'-GGTTGGGTTTGTAAGTTTGT-3' and reverse primer, 5'-ATAAACAACAATAACCCCCA-3' for the unmethylated EGFR sequence (150 bp). The PCR amplification program consisted of 10 min at 95˚C, followed by 40 cycles of 30 sec denaturation at 95˚C, 30 sec of annealing at 56˚C, 30 sec of extension at 72˚C, with a final extension at 72˚C for 10 min for both primers using HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA). PCR products were separated on 3% agarose gels with ethidium bromide and visualized under UV illumination. As the positive control, M.*Sss*I methylase (New England BioLabs, Ipswich, MA, USA) was used to methylate normal human peripheral blood.

Cell proliferation assays. Cell proliferation assays were performed using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Briefly, the H1650, H1299 and PC-9 cells were seeded at a density of 3,000-5,000 cells/well in 96-well microtiter plates for 24 h. While the H1650 and H1299 cells were treated with increasing concentrations of gefitinib (0.01-100 μM), 5-aza-CdR (0.01-100 μM), or a combination of gefitinib and 5-aza-CdR for up to 72 h, the PC-9 cells were treated with increasing concentrations of gefitinib (0.01-100 μM) alone for 72 h. Subsequently, 10 μl of the CCK-8 solution were added to each well in the plate. The absorbance (A) at 450 nm was measured using a microplate reader, and a calibration curve was prepared using the data obtained from the wells that contained known numbers of viable cells. Each experiment was carried out in five replicate wells at each drug concentration and repeated at least three times.

Apoptosis measurements. The apoptosis assay was conducted using Annexin V staining and flow cytometry assays. The H1650 and H1299 cells seeded in 6-well plates were treated with gefitinib (1 μM), 5-aza-CdR (1 μM), or a combination of gefitinib and 5-aza-CdR based on their sensitivity to various drugs for 72 h. PC-9 cells were treated with various concentrations of gefitinib (0.01-100 μM) for 72 h. Subsequently, the treated and untreated cells were collected and washed with PBS. Annexin V staining was performed following the manufacturer's instructions (Trevigen, Inc., Gaithersburg, MD, USA). Briefly, the cells were incubated for 15 min at room temperature in the presence of 1 μl of Annexin V-FITC, 1 μl of propidium iodide, and 98 μl of 1X binding buffer (all reagents were provided by the manufacturer). Following incubation, 400 μl of 1X binding buffer were added to each tube, and the cells were analyzed by flow cytometry.

Western blot analysis. Cells were lysed in a buffer containing 1 mM protease inhibitor [phenylmethylsulfonyl fluoride (PMSF)] and were cleared by centrifugation at 12,000 rpm for 10 min. Protein concentration was determined using the BCA assay (Bio-Rad, Hercules, CA, USA). Protein (80 μg) was dissolved in loading buffer, denatured by heating at 100˚C for 5 min, and subsequently separated on 8% polyacrylamide gels by SDS-gel electrophoresis. After separation, the proteins were transferred onto an immunoblot polyvinylidene difluoride membrane (Bio-Rad). Overall protein loading was confirmed by Ponceau S staining. Membranes were blocked with 5% non-fat milk for 2 h at 37˚C and then incubated with anti-EGFR antibody (Millipore, Billerica, MA, USA) overnight at 4˚C. The membranes were washed three times with 1X PBST and incubated with secondary antibody conjugated with peroxidase (Dako, Carpinteria, CA, USA) for 1 h at 37˚C. After a final wash with PBST, the membranes were developed using chemiluminescence and exposed to X-ray film. The expression of EGFR was normalized to β-actin.

RT-PCR and quantitative real-time PCR. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was qualitatively assessed using a Nanodrop UV spectrophotometer. cDNA was generated using an OmniScript RT kit (Qiagen) according to the manufacturer's instructions. Quantitative gene

Table I. The IC_{50} values of gefitinib and 5-aza-CdR in the NSCLC cell lines.

NSCLC cell line	Histology	EGFR mutation status	EGFR mutation status	5-aza-CdR IC_{50} (μM)[a]	Gefitinib IC_{50} (μM)	
					No 5-aza-CdR	5-aza-CdR
H1650	AD	mut	Methylated	15.91±1.42	14.53±1.13	2.93±0.95
H1299	LC	wt	Methylated	16.69±1.64	18.64±1.98	3.41±1.01
PC-9	AD	mut	Unmethylated		1.42±0.73	

[a]The IC_{50} value was defined as the concentration required for a 50% reduction in the absorbance calculated based on the cell viability curves. EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; AD, adenocarcinoma; LC, large cell; mut, mutant; wt, wild-type; IC_{50}, half maximal inhibitory concentration.

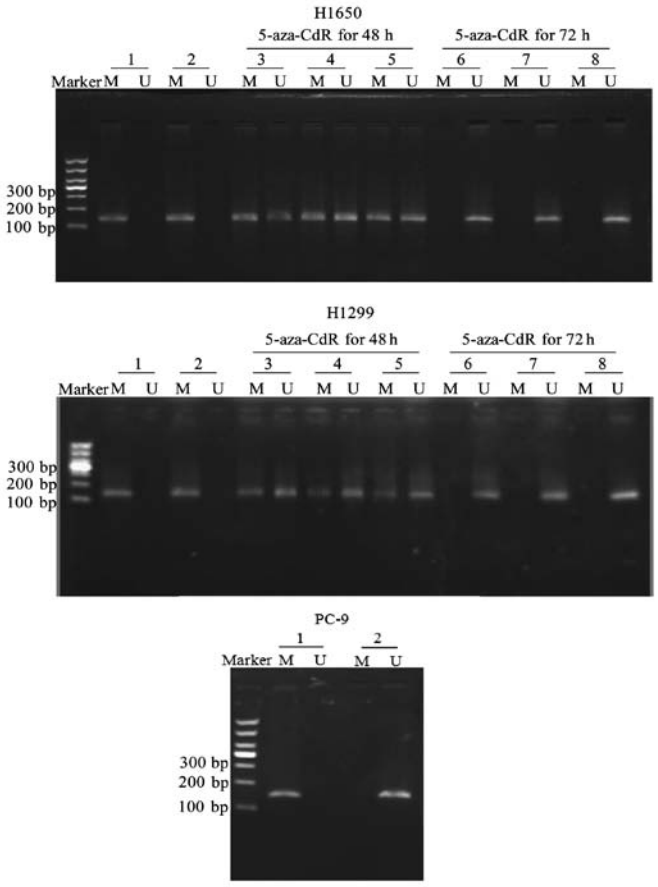

Figure 1. The CpG island methylation status in the promoter of the EGFR gene in NSCLC cells. NSCLC cells were treated with various concentrations (1-10 μM) of 5-aza-CdR, a methylation inhibitor, for up to 72 h. Subsequently, the cells were harvested. Genomic DNA was extracted by proteinase K digestion followed by purification with a series of phenol/chloroform and isopropyl alcohol precipitations. The modified DNA was used as a template for MSP with primers specific for either the modified-methylated or the modified-unmethylated EGFR promoter sequences. The methylation status of the EGFR gene was determined by MSP. PCR products were separated on 3% agarose gels with ethidium bromide and visualized under UV illumination. As the positive control, M.*Sss*I methylase was used to methylate normal human peripheral blood. Marker, DL1,000 DNA Marker; lane 1, positive control; lane 2, vehicle control; lane 3, 1 μM 5-aza-CdR; lane 4, 5 μM 5-aza-CdR; lane 5, 10 μM 5-aza-CdR; lane 6, 1 μM 5-aza-CdR; lane 7, 5 μM 5-aza-CdR; lane 8, 10 μM 5-aza-CdR; M, product of methylated specific primer (150 bp); U, product of unmethylated specific primer (150 bp).

expression analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the Universal TaqMan PCR protocol (qPCR) and the ABI 7300 sequence detection system. Briefly, 2 μl of cDNA were used for each RT reaction. The 20 μl PCR reaction mixture contained 1X primers and probe mixture (Applied Biosystems, Foster City, CA, USA). The assay IDs were as follows: Hs01076092-m1 (EGFR) and Hs99999905-m1 (GAPDH); 1X ABsolute qPCR mix (Roche, USA). The PCR conditions were 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, and 60˚C for 1 min. Each sample was assayed in triplicate with commercial RNA as the positive control and RNase-free water as the negative control. Relative gene expression quantifications were calculated according to the comparative Ct method using GAPDH as the internal control and commercial RNA control as calibrators on each plate. The final results were determined by the formula, $2^{-\Delta\Delta Ct}$ (13).

Statistical analyses. Data are expressed as the means ± SD. Statistical analysis was performed using SPSS software version 13.0. Data were analyzed by one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

CpG island methylation status determination in the promoter of the EGFR gene in NSCLC cells. Three NSCLC cell lines with different EGFR gene mutation statuses were chosen in this study, including a cell line containing wild-type EGFR (H1299), a cell line containing mutant EGFR resistant to TKIs (H1650) and a cell line sensitive to TKIs (PC-9). The histological data of these NSCLC cell lines are shown in Table I. The methylation status of the EGFR gene was determined by MSP in the H1650, H1299 and PC-9 cells. As shown in Fig. 1, no methylation was observed in the PC-9 cells, while hypermethylation was detected in the H1650 and H1299 cells. We found that the EGFR gene promoter became unmethylated when the cells were treated with 1-10 μM 5-aza-CdR for 48 and 72 h (Fig. 1).

Induction of growth inhibition by 5-aza-CdR and gefitinib. We then examined the effects of 5-aza-CdR and gefitinib on cell growth. The half maximal inhibitory concentration (IC_{50}) values of gefitinib in the H1650, H1299 and PC-9 cells were 14.53±1.13,

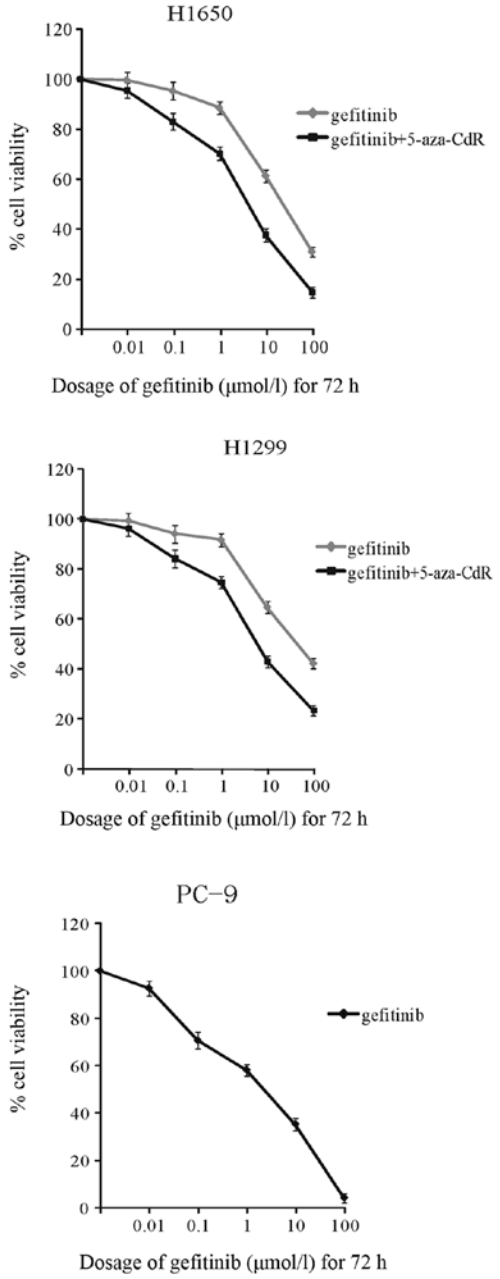

Figure 2. Induction of growth inhibition by 5-aza-CdR and gefitinib. H1650 and H1299 cells were treated with increasing concentrations of gefitinib (0.01-100 μM) or a combination of gefitinib (0.01-100 μM) and 5-aza-CdR (1 μM) for up to 72 h. PC-9 cells were treated with increasing concentrations of gefitinib (0.01-100 μM) for up to 72 h. Subsequently, 10 μl of the CCK-8 solution was added to each well of the plate. The absorbance (A) at 450 nm was measured using a microplate reader. A calibration curve was prepared using the data obtained from the wells that contained known numbers of viable cells. Each experiment was carried out in five replicate wells for each drug concentration and repeated at least three times.

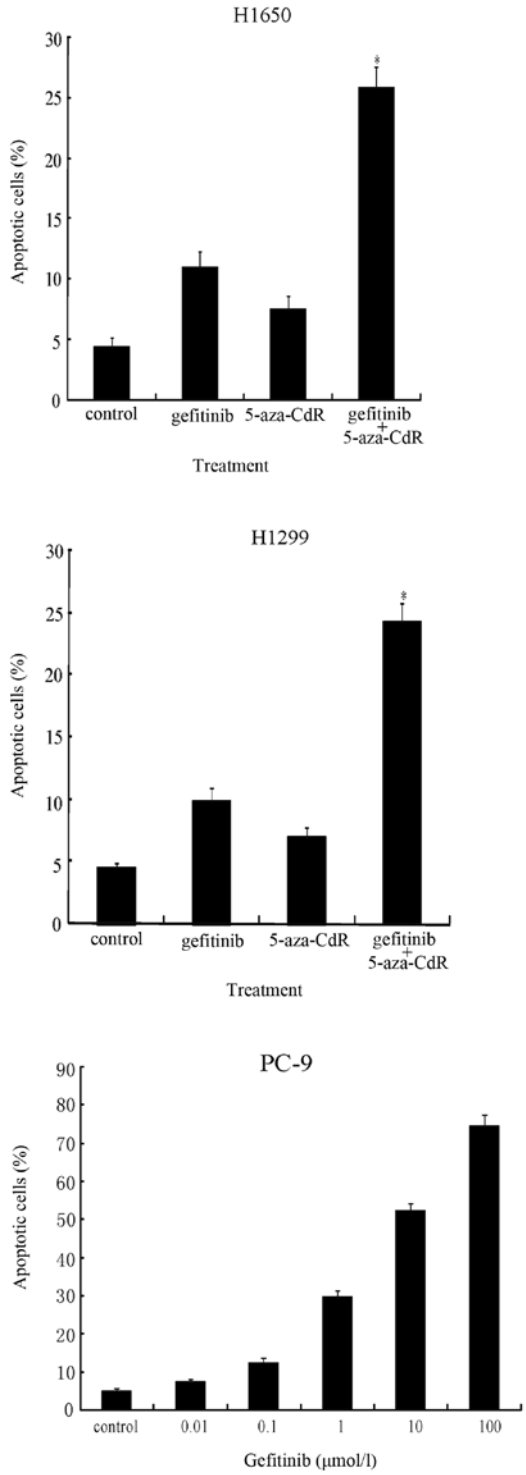

Figure 3. Induction of apoptosis by gefitinib and 5-aza-CdR. H1650 and H1299 cells seeded in 6-well plates were treated with gefitinib (1 μM), 5-aza-CdR (1 μM), or a combination of gefitinib and 5-aza-CdR based on their levels of sensitivity to various drugs for 72 h. PC-9 cells were treated with increasing concentrations of gefitinib, as indicated, for up to 72 h. Subsequently, the treated and untreated cells were collected and the apoptosis assay was conducted using Annexin V staining, followed by flow cytometry analysis according to the manufacturer's instructions. The percentage of apoptotic cells was calculated based on the instructions on the Annexin V-FITC/PI staining kit. Results are expressed as the means ± SD. *P<0.05, compared to the control group.

18.64±1.98 and 1.42±0.73 μM, respectively. Of note, we found that in the presence of 1 μM 5-aza-CdR, the IC_{50} values of gefitinib decreased to 2.93±0.95 and 3.41±1.01 μM in the H1650 and H1299 cells, respectively. For the combined treatment, H1650 and H1299 cells were treated with varying concentrations (0.01-100 μM) of gefitinib in the presence of 1 μM 5-aza-CdR; an enhanced inhibition of cell growth was observed (Fig. 2). These results suggested that 5-aza-CdR increased the cellular sensitivity of gefitinib as a cell growth inhibitor. The PC-9 cells, which are unmethylated in the EGFR gene promoter, were more sensitive to gefitinib than the H1650 and H1299 cells (Fig. 2).

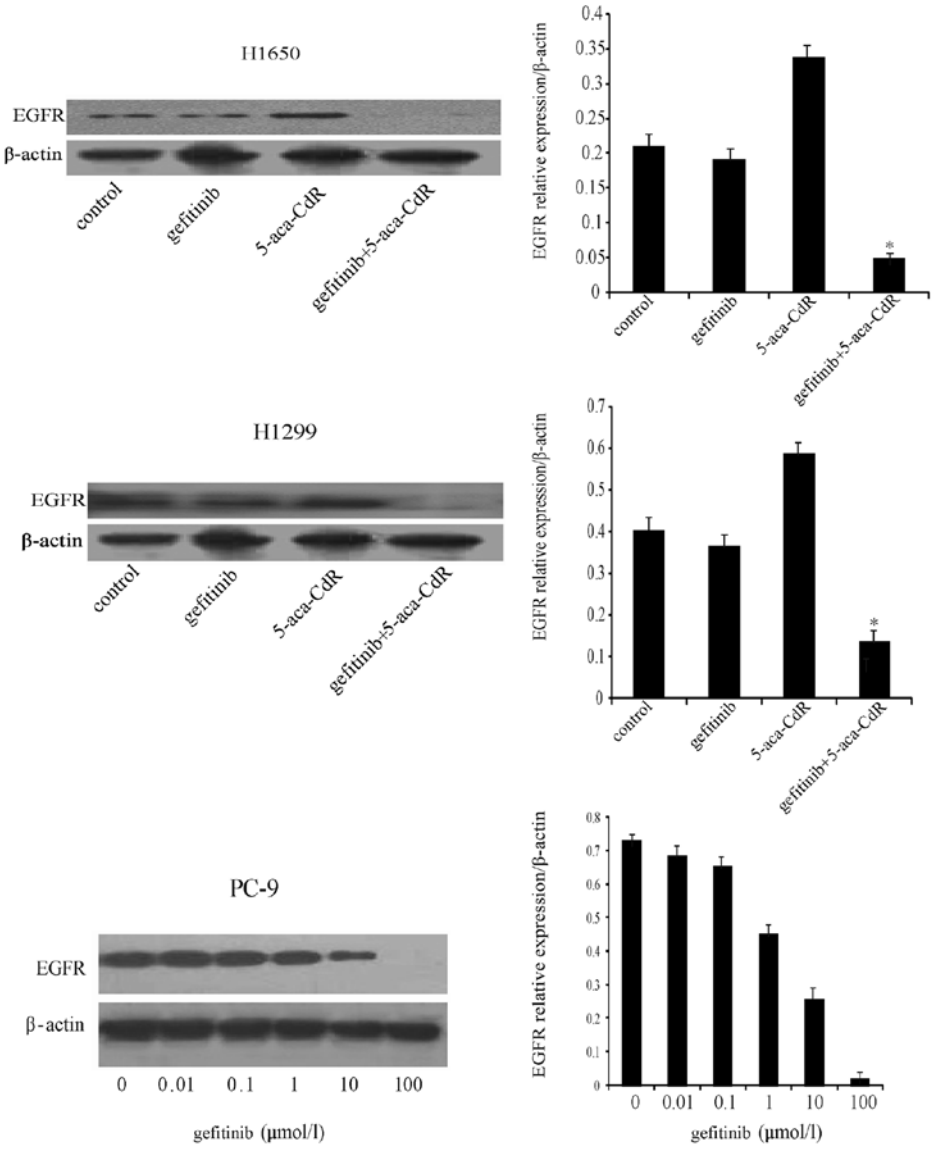

Figure 4. Effects of gefitinib and 5-aza-CdR on EGFR protein expression. H1650 and H1299 cells were treated with gefitinib (1 μM), 5-aza-CdR (1 μM), or a combination of gefitinib and 5-aza-CdR for up to 72 h. In a separate experiment, PC-9 cells were treated with increasing concentrations of gefitinib, as indicated, for up to 72 h. Subsequently, EGFR protein expression levels were determined by western blot analysis. The bar graphs indicate the densitometry results from western blot analysis. *P<0.05, compared to the control group.

Induction of apoptosis by gefitinib and 5-aza-CdR. We hypothesized that a greater sensitivity to gefitinib could be achieved by EGFR demethylation in the H1650 and H1299 cells. We found that the H1650 and H1299 cell lines were relatively resistant to gefitinib with IC_{50} values of 14.53±1.13 μM and 18.64±1.98 μM, respectively. Compared to either 5-aza-CdR or gefitinib treatment alone, a significant additional increase in apoptosis was observed in the H1650 cells (25.73%, P<0.05) and H1299 cells (24.27%, P<0.05) treated with a combination of 5-aza-CdR (1 μM), which showed maximal demethylation, and gefitinib (1 μM) for 72 h as determined by Annexin V staining (Fig. 3). The treatment of the unmethylated PC-9 cells with gefitinib also increased apoptosis in a dose-dependent manner (Fig. 3).

Effects of gefitinib and 5-aza-CdR on EGFR protein expression. In order to determine whether gene methylation affects protein expression, the H1650 and H1299 cells were treated with gefitinib (1 μM), 5-aza-CdR (1 μM), or both. As shown in Fig. 4, EGFR protein expression was low in the two cell lines. To confirm that the low expression of EGFR was due to hypermethylation, the H1650 and H1299 cells were treated with 5-aza-CdR, which resulted in CpG island demethylation in the EGFR gene promoter (Fig. 1). We found that EGFR protein expression was increased following treatment with 5-aza-CdR. A significant decrease in EGFR protein expression was observed in the H1650 and H1299 cells after treatment with a combination of gefitinib and 5-aza-CdR compared to treatment with either agent alone. A dose-dependent reduction in EGFR protein expression was observed in the PC-9 cells treated with gefitinib (Fig. 4).

Effect of gefitinib and 5-aza-CdR on EGFR mRNA expression. We also evaluated EGFR mRNA expression by real-time PCR (qPCR) in the H1650, H1299 and PC-9 cells. Cells exposed to 5-aza-CdR for 72 h demonstrated an induction of EGFR

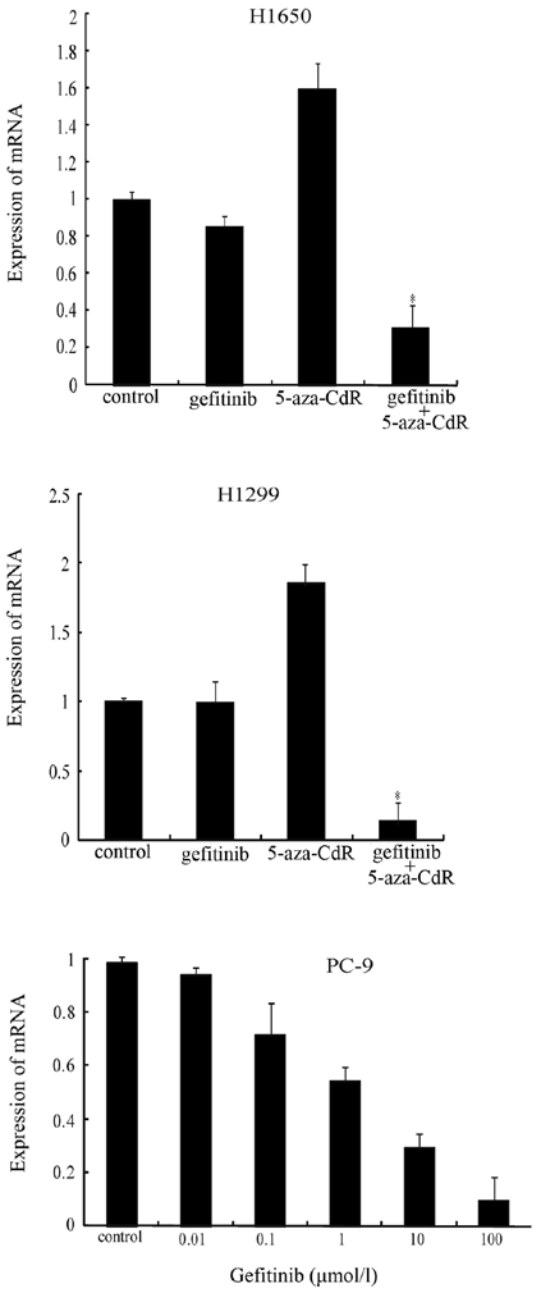

Figure 5. Effect of gefitinib and 5-aza-CdR on EGFR mRNA expression. H1650 and H1299 cells were treated with gefitinib (1 μM), 5-aza-CdR (1 μM), or a combination of gefitinib and 5-aza-CdR for up to 72 h. In a separate experiment, PC-9 cells were treated with increasing concentrations of gefitinib, as indicated, for up to 72 h. Subsequently, EGFR mRNA expression levels were determined by RT-PCR analysis. Results are presented as the means ± SD. *P<0.05, compared to the control group.

mRNA expression (Fig. 5). The effect of the simultaneous exposure of the NSCLC cells to gefitinib in combination with 5-aza-CdR at concentrations below the IC_{50} value was examined. Similar to the protein expression, further inhibition of EGFR mRNA expression was observed with the combination treatment (Fig. 5); these results indicated that the cells became more sensitive to gefitinib in the presence of 5-aza-CdR and suggested a clear synergistic effect. As expected, a dose-dependent reduction in EGFR mRNA expression was observed in the PC-9 cells treated with gefitinib (Fig. 5).

Discussion

The majority of patients with metastatic NSCLC have a poor outcome, with a median survival time of less than ten months (14). The development of EGFR-TKIs has significantly affected the treatment of NSCLC. EGFR-TKIs, such as gefitinib and erlotinib, have been found to be efficient in the treatment of certain NSCLC patients with EGFR mutations (7,8). However, resistance to TKIs has been found in some NSCLC patients with EGFR mutations. Furthermore, most primary NSCLC cell lines, including cell lines containing wild-type EGFR, are resistant to EGFR-TKI treatment. The exact reasons for this remain unknown. Further investigation in this area would unveil new information and may enhance the therapeutic efficacy and survival of patients with NSCLC. Our study showed that the NSCLC cell line, H1650, which contains a EGFR mutation (del E746-A750), was less sensitive to EGFR-TKIs (15). Since lung cancer cells rapidly develop drug resistance to single TKI therapy, studies on other potential agents that can complement and enhance the anti-neoplastic activity of TKIs are required. Previously, promoter hypermethylation has been recognized as a potential mechanism by which genes regulating cellular proliferation are silenced during cancer development (16,17). Promoter hypermethylation involves DNA methylation of CpG islands in or near the promoter region of certain genes, rendering them transcriptionally silent. In this study, we demonstrate that the expression level of the EGFR gene is low in the H1650 and H1299 cells. To elucidate the mechanism of the downregulation of EGFR gene expression, we focused on examining the promoter methylation of the EGFR gene in lung cancer cells.

In this study, we demonstrate that the promoter of the EGFR gene is methylated in the H1650 and H1299 cells, which correlates with the downregulation of EGFR gene expression. Of note, 5-aza-CdR, a demethylating agent, increased EGFR gene transcription. This suggests that epigenetic regulation may be responsible for controlling EGF-R gene expression. The downregulation of expression of important cellular growth control genes, such as Ras-association domain family member 1 (RASSF1A) and hypoxia-inducible factor-1 (HIF-1), has been shown to play an important role in cancer progression and metastasis, resulting in a poor outcome that is associated with the promoter hypermethylation of these genes (18,19).

DNA methylation and the associated silencing have been shown to be involved in the development of drug resistance (20), which prompted investigations for the use of a hypomethylation approach to re-sensitize malignant cells to classical cytotoxic agents. 5-Aza-CdR, a potent DNA methylation inhibitor, has exhibited potent anti-neoplastic activity in animal models and has shown promising anticancer activity in hematological malignancies (21), as well as lung cancer (22). Since 5-aza-CdR reactivates genes through blockade of DNA methylation, it has an important therapeutic potential in inhibiting tumorigenesis. This led us to investigate the potential mechanisms of action of the therapeutic agents in malignant tumors, including lung cancer. Montero *et al* (23) showed that 5-aza-CdR treatment increased the sensitivity to gefitinib, an EGFR-TKI, in EGFR-methylated breast cancer cells. Other published data has suggested that folic acid inhibits the constitutive and induced the activity of the EGFR promoter in colon cancer cells through methylation, as this effect was reversed by 5-aza-CdR (24,25).

In a study by Momparler and Ayoub (26), it was observed that patients with stage IV NSCLC who received five cycles of 5-aza-CdR treatment survived 81 months. Consistent with these data, in this study, by evaluating the anti-neoplastic effects of 5-aza-CdR and gefitinib, we showed that a combination of lower doses (<IC_{50} values) of 5-aza-CdR and gefitinib significantly inhibited NSCLC cell growth. Similarly, 5-aza-CdR further increased the induction of apoptosis by gefitinib in NSCLC cells. These results suggest that NSCLC cells with EGFR methylation are resistant to gefitinib, and that 5-aza-CdR, a hypomethylating drug, may increase the cellular sensitivity to gefitinib in controlling NSCLC cell growth and apoptosis. The PC-9 cells, which did not harbor a CpG island methylation within the EGFR promoter, were more sensitive to gefitinib than the H1650 and H1299 cells. Thus, 5-aza-CdR had no further effect on PC-9 cell proliferation.

Promoter methylation of the EGFR gene is one of the key mechanisms that affects cancer cell sensitivity to TKIs. Previous studies have shown that certain somatic mutations within the TK and ATP-binding domain of the EGFR gene are associated with a response to EGFR-TKIs in NSCLC (27,28). The positive association between EGFR mutations and response to erlotinib or gefitinib in NSCLC patients has been shown in several clinical studies, with a significant enhancement of patient survival (29). However, in larger randomized studies, such as the BR.21 trial, a similar survival advantage was observed for patients treated with erlotinib, independent of EGFR mutations or wild-type EGFR gene, indicating that EGFR mutations were not the only biomarker for predicting NSCLC survival with small-molecule EGFR-TKI treatment (30). Our results suggested that the CpG island methylation status in the EGFR promoter influenced the sensitivity to gefitinib in NSCLC cells.

Our results demonstrated the enhanced effects of 5-aza-CdR on EGFR-TKI-induced cell growth inhibition and the induction of apoptosis. The reduced expression of EGFR may be due to hypermethylation. In addition, 5-aza-CdR treatment resulted in CpG island demethylation in the promoter of the EGFR gene. These results suggest that demethylation potentially induces EGFR gene expression. Whether this could affect the sensitivity and therapeutic efficacy of gefitinib remains unknown. In general, methylation causes gene silencing and demethylation induces gene expression. EGFR expression is not considered as a significant predictive factor for a response to gefitinib (31). There is no clear correlation between EGFR expression and gefitinib sensitivity. We reasoned that the demethylation effect of 5-aza-CdR caused the induction of EGFR gene expression. Thus, the balance between methylation and demethylation changes may contribute to the synergistic effects of this combination treatment. On the basis of EGFR demethylation, this would enhance the therapeutic response of gefitinib in NSCLC cells. Further mechanistic studies are required to confirm these results.

Two major EGFR-TKI-resistance mechanisms have been revealed. Gefitinib is an ATP competitive inhibitor of EGFR-TKI. It has been found that the T790M secondary mutation increases the affinity of the oncogenic mutant EGFR for ATP, and this leads to the reduced efficacy of EGFR-TKIs (31). Almost half of the patients with acquired resistance appear to have this mutation (32,33). Met proto-oncogene (MET) amplification is another mechanism that escapes the antitumor effect of EGFR-TKIs, which allows cancer cell survival by persistent enhancement of Akt signaling and MET amplification when the EGFR signal is blocked in the presence of EGFR-TKIs. MET amplification has been found in approximately 20% of patients with acquired resistance (34,35). The link between MET amplification and promoter methylation of the EGFR gene involving EGFR-TKI resistance has not been reported; whether 5-aza-CdR has any direct or indirect effect on ATP or MET amplification needs to be determined. A recent study showed that 5-aza-CdR decreased c-Met protein expression in NSCLC cells (36), suggesting a potentially novel mechanism of this agent in controlling cancer cell growth.

In conclusion, the data presented in this study show that NSCLC cells with EGFR methylation are more resistant to gefitinib, and that the combination treatment with 5-aza-CdR, a demethylating agent, increases the sensitivity to gefitinib. These results suggest that the combination of a demethylating agent with an EGFR-TKI may have a synergistic anti-lung cancer effect and that the blockade of DNA methylation of the EGFR gene promoter region should be considered as one of the potential mechanisms for reversing resistance to EGFR-TKIs in NSCLC cells. Further studies to better define the role of 5-aza-CdR in controlling cancer cell growth and the potential synergistic antitumor effects of TKIs and 5-aza-CdR in NSCLC are warranted.

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