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Integrin Beta1 Over-Expression Associates With Resistance to Tyrosine Kinase Inhibitor Gefitinib in Non-Small Cell Lung Cancer

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ABSTRACT
The epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) such as gefitinib and erlotinib have been widely used in treating patients with advanced non-small cell lung cancer (NSCLC). However, acquired resistance to EGFR TKI almost occurs in every patient eventually. To identify its potential mechanism, we established a human NSCLC cell line PC9/AB2 which was 576-fold decrease in gefitinib sensitivity compared with its parental PC9 cell lines. No EGFR-T790M mutation or abnormal expression of c-Met protein was found in PC9/AB2 cells. Over-expression of integrin β1 was found, accompanied with increase of the cells' adhesion and migration. To further confirm the role of integrin β1 in gefitinib acquired resistance, we transferred its siRNA-expressing plasmid and its whole cDNA expressing plasmid into PC9/AB2 and into PC9 cells, respectively. The sensitivity of NSCLC cells to gefitinib was negatively correlated with integrin β1 expression levels. All these data suggest that up-regulation of integrin β1 might be an important factor for gefitinib resistance in NSCLC cell line PC9/AB2. J. Cell. Biochem. 111: 1565–1574, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: INTEGRIN Β1; GEFITINIB; RESISTANCE; NON-SMALL CELL LUNG CANCER

Epidermal growth factor receptor (EGFR) is involved in many important processes of carcinogenesis of many solid cancers, including cell proliferation, invasion, angiogenesis and resistance of chemotherapy and radiotherapy [Salmon et al., 1995; Nicholson et al., 2001]. It is over-expressed in the cancer tissue compared with its surrounding normal tissue. Thus, it has become an attractive target for cancer therapy. Small molecular inhibitors and monoclonal antibodies targeting EGFR signaling pathway have been developed over the past few decades.

Small molecular inhibitors such as gefitinib and erlotinib inhibit downstream signal transduction of EGFR via inhibition of phosphorylation of EGFR, thereby inducing the apoptosis of cancer cells [Herbst et al., 2004; Sharma et al., 2007]. A series of clinical studies proved their anti-tumor activity in the treatment of advanced non-small cell lung cancer (NSCLC) [Paez et al., 2004; Bell et al., 2005]. Erlotinib significantly improves overall survival in the second or third line treatment of advanced NSCLC compared with placebo [Mehić et al., 2008]. Gefitinib produces similar efficacy to that of docetaxel in the second line setting [Cufer et al., 2006]. So, the agents have been approved in many countries as second or third-line therapy for advanced NSCLC. About 10–20% of advanced NSCLC patients could get dramatic clinical response to EGFR TKIs [Fukuoka et al., 2003; Kris et al., 2003]. These cases often harbor activating mutations of EGFR. Deletion mutations in exon 19 and the substitution of leucine with arginine in exon 21 account for 85–90% of all the mutations [Rodenhuis et al., 1988; Rodenhuis and Slebos, 1990; Suzuki et al., 1990; Gazdar et al., 2004]. These mutations strongly sensitize the cancer cells to EGFR TKIs, thus being clear-cut predictors of major responses to this class of drugs [Yun et al., 2007].

Although the patients with mutant EGFR display dramatic response to EGFR TKIs, duration of response is typically 9–10 months and then most patients eventually acquire resistance to the agents [Inoue et al., 2006; Sutani et al., 2006], leading to treatment failure. Mechanisms for acquired resistance to EGFR TKIs have been widely studied. A secondary mutation of EGFR gene in exon 20 (T790M) and c-Met gene amplification have been found to be related with acquired resistance to EGFR TKIs in NSCLC [Kobayashi et al., 2005; Kwak et al., 2005; Onitsuka et al., 2005].

The secondary mutation in EGFR, T790M, can abrogate the binding of gefitinib or erlotinib to ATP-binding pocket of the EGFR kinase domain [Engelman et al., 2006], thereby leading to the drug resistance. The incidence of primary T790M mutation is quite rare before EGFR-TKI treatment, but it increases remarkably after the treatment. Gefitinib-sensitive NSCLC cell lines could be rendered

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resistant when introduced with T790M mutant EGFR [Engelman et al., 2006]. Many studies have confirmed the fact that T790M mutation accounts for about 50% of acquired resistance to EGFR TKIs [Jackman et al., 2006; Rosell et al., 2007; Sharma et al., 2007; Yoshida et al., 2007].

Besides T790M mutation, c-Met gene amplification is also an important reason for the acquired resistance to gefitinib. c-Met, a kind of transmembrane protein with activity of receptor tyrosine kinase, is often over-expressed in NSCLC and correlates with cell proliferation, motility, and invasion. Application of c-Met inhibitors can significantly reverse EGFR TKI resistance and has some efficacy in some lung cancer patients. It has reported that c-Met gene amplification is present in about 21% of patients with gefitinib resistance [Bean et al., 2007].

Except for above two mechanisms, others that account for the remaining about 30% of acquired resistance are still unclear. In recent years, some studies have shown that integrin-mediated adhesion of epithelial cells to extracellular matrix (ECM) induces prolonged tyrosine phosphorylation and partial activation of EGFR [Bill et al., 2004]. Others studies also found that ECM was associated with chemotherapy resistance [Rintoul and Sethi, 2002; Helleman et al., 2010] and can induce recovery of HCC cells from Gefitinib-induced apoptosis [Giannelli et al., 2004]. Thus it was supposed that integrins, the receptor of ECM, might play some role in the acquired resistance to EGFR TKIs.

We established a gefitinib-resistant NSCLC cell line PC9/AB2 from PC9 cells according to the method of the related literature [Koizumi et al., 2005], which is about 576-fold more resistant. We found that the adhesion and migration of PC9/AB2 was enhanced significantly compared with its parental cells, associated with over-expression of integrin β1. Introduction of siRNA against integrin β1 into the PC9/AB2 cell line could partially recover sensitivity of the cells to gefitinib while introduction of integrin β1 cDNA into PC9 cell line was associated with gefitinib resistance. These data suggested integrin β1 might play some role in the acquired resistance to EGFR TKIs in the cell line.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

Human NSCLC cell line PC9 was provided by Cancer Institute of Medical School, Tongji University, China. The gefitinib-resistant NSCLC subline PC9/AB2 was induced from PC9 cells according to the method in the literature [Koizumi et al., 2005] and was continuously subcultured with 2 μmol/L of gefitinib for an additional 6 months. The resistance of PC9/AB2 cells to gefitinib has been proved to maintain for at least 1 year in the medium without gefitinib. All cells were kept at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin.

CELL PROLIFERATION ASSAY

The cells (5×10^3/well) were seeded into 96-well plate in quadruplicate and were exposed to various concentrations of gefitinib. After 72 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and incubated. After 4 h, crystalline formation was dissolved with Dimethyl sulfoxide (DMSO) and the absorbance at 530 nm was read using the microplate-reader for ELISA MK-2 Labsystems Dragon. The IC_{50} was defined as the concentration needed for a 50% reduction of the absorbance based on the survival curves. Percent survival was calculated as: (mean absorbance of the replicate wells containing drugs – mean absorbance of the replicate background wells)/(mean absorbance of the replicate drug – free wells – mean absorbance of the replicate background wells). The test was performed independently three times. All results were derived from quadruplicate experiments yielding almost similar results.

CELL ADHERENT AND MIGRATION ASSAY

The 96-well plates were pre-coated with 100 μl of solution containing fibronectin (FN) (Millipore) (20 mg/L) per well at 4°C overnight, followed by blocking with 100 μl of bovine serum albumin (BSA) solution (10 mg/ml) for 1 h at room temperature, washed three times with PBS, and then kept in 4°C till used. The cells were trypsinized, and resuspended in DMEM with 0.5% serum. The 100 μl of cells (2×10^4/well) were seeded into the coated 96-well plates and incubated at 37°C. After 30 min the non-adherent cells were removed by washing with PBS and then 200 μl of serum-free DMEM and 20 μl MTT solution were added to each well and incubated for 4 h. Absorbance was read at 530 nm using a Delta Soft ELISA analysis program.

A wound-scratch assay was performed to examine the cellular migration. In this study, a uniform cell-free area at six-well plate was created by scratching the confluent monolayer of cells with a plastic micropipette tip as described by Lu et al. [2004]. The plate was washed with PBS, added into each well with serum-free DMEM and incubated at 37°C for 24 h. The wound area was inspected at different time intervals (0, 24 h) to determine the distance migrated by the cells. The test was performed independently three times.

APOPTOSIS ASSAY

Flow cytometry and transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) Kit (Promega, USA) were used for apoptosis assay. In flow cytometry assay, PC9 cells and PC9/AB2 cells were plated in six-well plates. Twenty-four hours later, gefitinib (1 μmol/L) was added into experimental wells and incubated for another 48 h. The cells were harvested, washed with PBS and resuspended in 500 μl binding buffer. The Cell were stained with 5 μl of Annexin V-PE and incubated for 5 min at room temperature in the dark. Quantification of apoptosis was determined by flow cytometry.

In TUNEL assay, cells were seeded in 24-well plates and exposed to gefitinib (5 or 15 μmol/L) for another 48 h. Apoptosis was assessed by the TUNEL assay kit (GENMED, China) following the manufacturer's protocol. Apoptotic index (AI) (%) was calculated by the formula: positive staining cells/tumor cells number × 100%.

DNA SEQUENCING

Total DNA was extracted from tumor cells according to the protocol of Shanghai Chaoshi Bio Technologies Co., Ltd (China). Genotypes of the exons 19–21 of EGFR were determined by PCR-based DNA sequencing. The primers for EGFR (exons 19–21) were used as

published [Willmore-Payne et al., 2006]. All sequencing reactions were performed by Ying Jun Company of Invitrogen (USA).

WESTERN BLOT ASSAY

Cells were washed twice with ice-cold PBS and lysed in 0.1 ml of lysis buffer on ice for 30 min. Insoluble debris was removed by centrifuging at 13,000 rpm for 15 min at 4°C. Electrophoresis and blotting procedures were done according to methods described previously. Primary antibodies against human integrin beta1/CD29 (R&D, USA), human MET/HGFR (ABGENT, USA), phosphorylated EGFR, Akt, Erk1/2, phosphorylated Akt and phosphorylated Erk1/2 (CST, USA) were used according to the manufacturer's instructions. Blotting quantification was done with an Odyssey® Infrared Imaging system (LI-COR, USA).

PLASMID TRANSFECTION

The plasmid expressing siRNA against integrin β1 mRNA (5′-GGATTCTGACAGCTTTAA A-3′) and the control scrambled siRNA (5′-TTCTCCGAACGTGTCACGT-3′) were purchased from Kang Cheng Technology Co., Ltd (China). Both the plasmids carried GFP gene. PC9/AB2 cells were transfected with integrin β1-siRNA plasmid or scrambled siRNA plasmid by the Lipofectamine 2000 reagent according to the manufacturer's protocol. In brief, the cells were treated with siRNA plasmid-Lipofectamine 2000 complexes and incubated at 37°C in a CO_2 incubator. After 24 h, G418 was added to select the transfected cells. Fluorescence microscope, real-time RT-PCR and Western blot were used to determine efficiency of the transfection. When the cells were grown to 30% confluence, a single-cell cloning was done in 96-well plate by dilution method. The cells stably transfected with integrin β1-siRNA were named as AB2/17-2 and the cells with scrambled siRNA were named AB2/N as a control.

Full-length integrin β1 cDNA was cloned into an expression vector (pcDNA3.1). The integrin β1 cDNA plasmid and the vacant vector were provided by Professor Xiliang Zha (Fudan University, China). PC9 cells were transfected with integrin β1 cDNA plasmid also by the Lipofectamine 2000. After 24 h, G418 was added to select the transfected cells. The cells stably transfected with integrin β1 cDNA plasmid were named as PC9/D6 and with vacant vector as PC9/PCD.

QUANTITATIVE REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QRT-PCR)

Total RNAs were extracted from cells (1×10^6 cells) using Trizol plus kit (TaKaRa, Japan). First-strand cDNA synthesis was done using Promega kit. Synthesized cDNA was used for qRT-PCR analysis using Lightcylcer (Roche, Switzerland) following the manufacturer's instructions. Integrin β1 primers were specifically designed by BoShang Bio-Technology Co., Ltd (China). β-Actin was used as the internal control. The Nucleotide sequences for the primers are listed in Table I. Amplifications were carried out in the 20 μl reaction mixtures in the following conditions 95°C for 2 min and followed by 40 cycles of 95°C for 20 s, 55°C for 20 s and 72°C for 35 s; and then 72°C for 3 min. The copy number of integrin β1 gene was determined by: target gene copy number $2^{-\Delta\Delta Ct}$ = (CT target gene − CT reference gene) experimental group − (CT target gene − CT reference gene) control group.

TABLE I. Primer Sequences of Integrin β1 Gene and β-Actin Gene for qRT-PCR

Integrinβ1(IGT B1)	
Forward	5′-CGGATGGTGTGTTACGATGAC-3′
Reverse	5′-CAGGATTCAGGGTTTCTCAGATG-3′
β-actin	
Forward	5′-CACTCTTCCAGCCTTCCTTCC-3′
Reverse	5′-AGGTCTTTGCGGATGTCCAC-3′

CELL CYCLE ANALYSIS

The cells were incubated in six-well plates for 24 h, and cell culture medium was replaced by fresh medium containing 10% FBS with or without gefitinib, and incubated for another 48 h. The cells were trypsinized, fixed in ice-cold 70% ethanol overnight, and stained with propidium iodide containing 1 mg/ml RNase (Sigma, USA), according to the instructions of the Cell Cycle Phase Determination Kit (Cayman Chemical Company, USA). Samples were analyzed on a flow cytometry (Becton Dickinson, USA). Cell cycle parameters from 10,000 events were analyzed using multi-cycle software.

STATISTICAL ANALYSES

Values were expressed as mean ± SD. Statistical analysis was done by independent-samples *t*-test. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

PC9/AB2 CELLS SHOW SIGNIFICANT RESISTANCE TO GEFITINIB

Gefitinib had little effect on cell proliferation in PC9/AB2 cell line. Value of IC_{50} for gefitinib in PC9/AB2 cells were about 24.2 ± 5.45 μmol/L, 576 times higher than in PC9 cells (0.042 ± 0.01 μmol/L, $P < 0.001$) (Fig. 1A). The resistance in PC9/AB2 cells was maintained for 1 year when they were kept in gefitinib-free medium. Morphological changes were also observed in the two cells lines when exposed to gefitinib (1 μmol/L). After 72 h, PC9 cells became rounded, detached from the bottom of the plate and collapsed into debris. However, no morphology change was observed in PC9/AB2 cells. Before gefitinib treatment, apoptotic cells were 0.92% and 0.48% for PC9 and PC9/AB2 cells, respectively. After 48 h of 1 μmol/L gefitinib treatment, apoptosis was significantly increased in PC9 cells but not in PC9/AB2 cells (38.48% vs. 2.22%) (data not shown).

ADHESION AND MIGRATION INCREASE IN PC9/AB2 CELLS

To further investigate the biological behavior changes, we detect the adhesion and migration in the two cell lines. We found the adhesion ability was dramatically increased in PC9/AB2 in both FN-pre-coated and non-coated conditions compared with PC9 cells ($P < 0.05$). Number of PC9/AB2 cells adhered to pre-coated plates was much higher than to non-coated plates ($P < 0.05$) (Fig. 1C). Similarly, PC9/AB2 showed stronger migration ability. They migrated to cover the entire wound area after 24 h of scratching (Fig. 1D). These data demonstrated that the adhesion and migration capability of gefitinib-resistant cell line PC9/AB2 were significantly increased compared with PC9 cells.

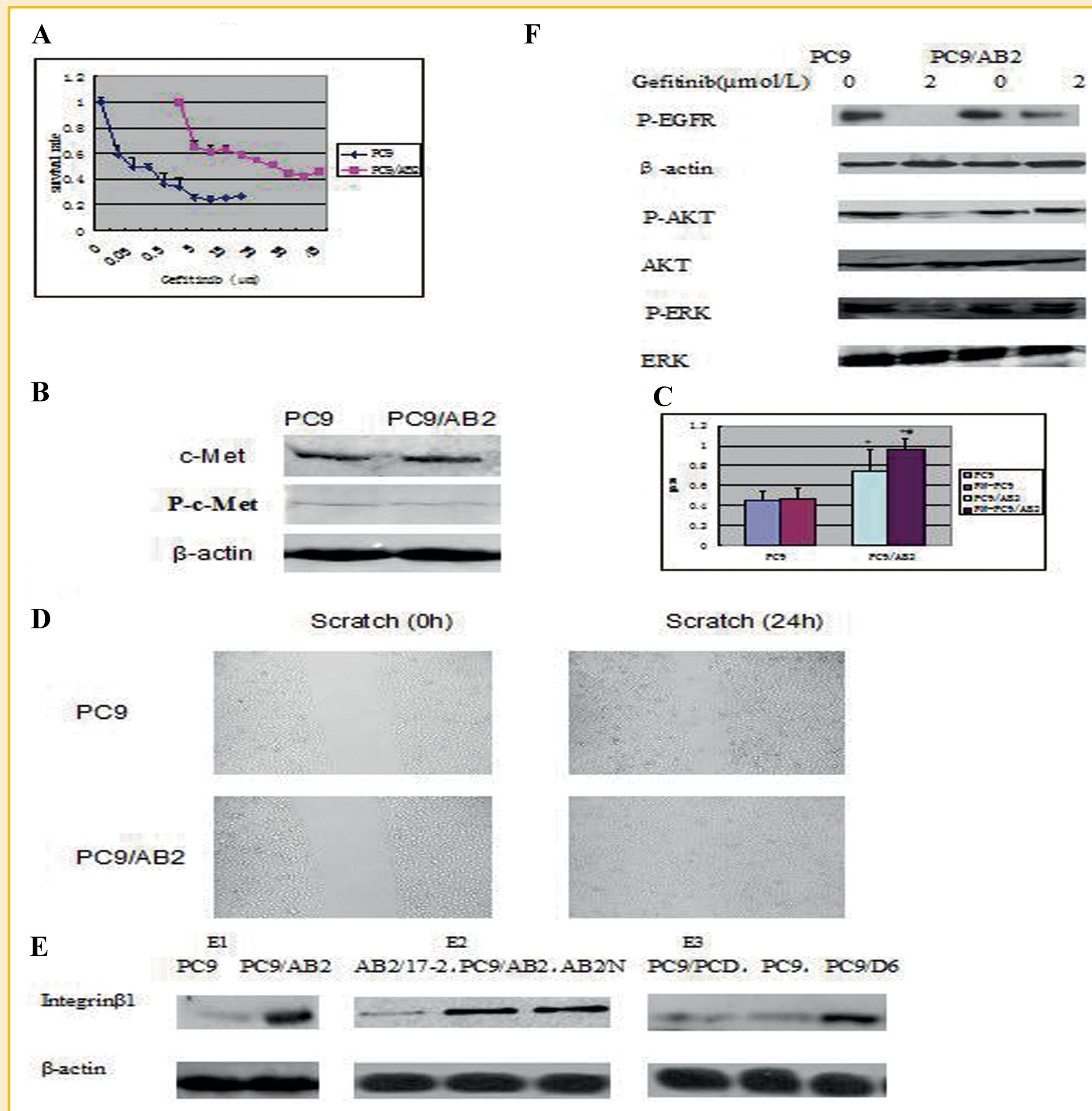

Fig. 1. Characterization of gefitinib-resistant cell line PC9/AB2. A: Cells (~5,000 per well) were seeded on 96-well plates in quadruplicate, and grew in the indicated concentrations of gefitinib. After 72 h, they were subjected to MTT assay. B: Western blot analysis of c-Met and phosphorylated c-Met in PC9 and PC9/AB2 cells. C: Cell adhesion capability of the cell lines to both FN-coated and non-coated cell plates were measured with a Delta Soft ELISA analysis program. FN, fibronectin; $^{*}P < 0.05$ AB2 versus PC9; $^{\#}P < 0.05$ FN-coated versus non-coated. D: Migration assays of PC9 and PC9/AB2 cells. The wound area was inspected at 0 and 24 h after scratching. E: Western blot analysis of integrinβ1 in cell lines. F: Western blot analysis of changes of downstream of EGFR signaling pathway in PC9 and PC9/AB2 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

NO T790M MUTATION AND NO C-MET OVER-EXPRESSION IN PC9/AB2

T790M mutation of EGFR is reported to be associated with acquired resistance to gefitinib [Kobayashi et al., 2005; Kwak et al., 2005; Onitsuka et al., 2005]. So, genotypes of EGFR were determined. Both PC9 and PC9/AB2 harbored exon 19 deletions (15 bp deletion) but no other mutations in EGFR (data not shown). We also detected protein expression of c-Met which is related with resistance to gefitinib. There was no significant difference in c-Met protein expression between PC9 and PC9/AB2 cell lines (Fig. 1B).

EXPRESSION OF INTEGRIN β1 INCREASES IN PC9/AB2 CELLS

Integrins are a family of heterodimeric transmembrane proteins as receptors for ECM proteins such as fibronectin (FN), laminin (LN), and collagens. The coordinated cellular response to matrix attachment through integrins has been shown to induce a panoply of changes in cellular behaviors, including cell survival, proliferation, migration, gene transcription, and differentiation [Bill et al., 2004]. So we analyzed the expression of integrin β1 in both mRNA and protein levels in both cell lines. Interestingly, the mRNA of integrin β1 was about 2.75 times higher in PC9/AB2 cells than in

PC9 cells by real-time PCR. Furthermore, the integrin β1 protein was increased in PC9/AB2 (Fig. 1E1). So, over-expression of integrin β1 might play some role in acquired resistance to gefitinib in PC9/AB2 cells.

REDUCTION OF INTEGRIN β1 GENE EXPRESSION BY siRNA RESTORES SENSITIVITY OF NSCLC CELLS TO GEFITINIB

We supposed that integrin β1 might induce acquired resistance to gefitinib in PC9/AB2 cells. PC9/AB2 cells were stably transfected with the plasmid expressing siRNA against integrin β1 or scramble siRNA (Fig. 2A). Integrinβ1 siRNA significantly decreased protein expression of the integrin by 36% compared with PC9/AB2 cells, while scramble siRNA had no influence on its expression (Figs. 1E2 and 3E, $P < 0.05$). After knockdown of integrin β1, PC9/AB2 partially recovered sensitivity to gefitinib. The Values of IC_{50} in AB2/17-2, PC9/AB2, and AB2/N were (1.9 ± 1.28) μmol/L, (24.2 ± 5.45) μmol/L, and (25.45 ± 7.58) μmol/L respectively. The difference of IC_{50} among the cell lines were highly significant (Fig. 2B). Accordingly, apoptotic cells were increased remarkably in AB2/17-2 compared with that in PC9/AB2

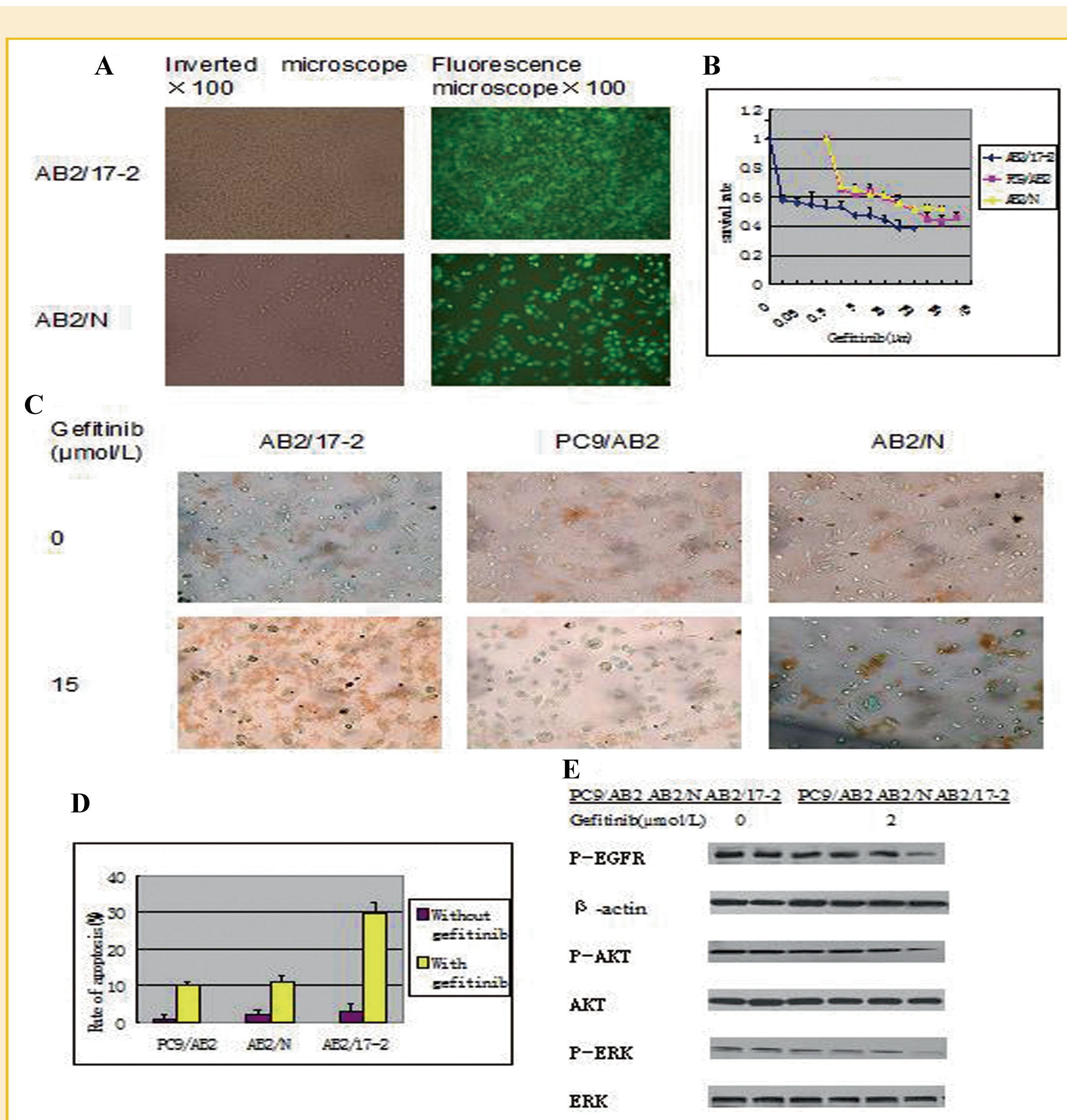

Fig. 2. Characterization of integrin β1-inhibit cell line AB2/17-2. A: Fluorescence microscope analysis of GFP gene expression in AB2/17-2 and AB2/N. B: Cells (~5,000 per well) were seeded on 96-well plates in quadruplicate, and grew in the indicated concentrations of gefitinib. After 72 h, they were subjected to MTT assay. C: TUNEL assay of apoptotic cells in three cell lines (PC9/AB2, AB2/N, and AB2/17-2) treated with or without 15 μmol/L of gefitinib for 48 h. Apoptotic cells are stained with dark brown. D: Apoptosis rate of the three cell lines (PC9/AB2, AB2/N, and AB2/17-2), Bars. E: Western blot analysis of changes of downstream of EGFR signaling pathway in AB2/17-2, AB2/N, and PC9/AB2 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

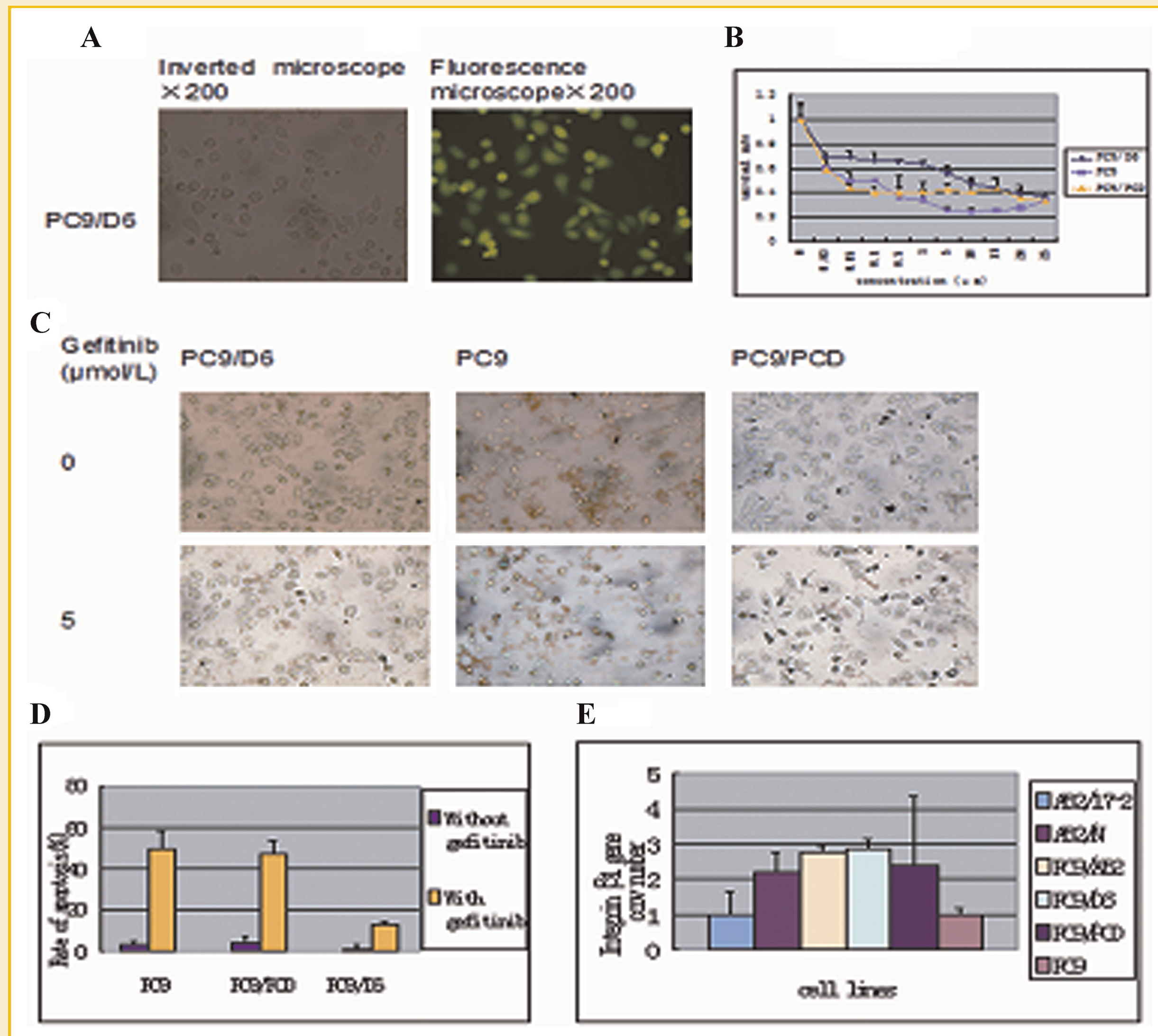

Fig. 3. Characterization of integrin β1-overexpress cell line PC9/D6. A: Fluorescence microscope analysis of GFP gene expression in PC9/D6. B: Cells (~5,000 per well) were seeded on 96-well plates in quadruplicate, and grew in the indicated concentrations of gefitinib. After 72 h, they were subjected to MTT assay. C: TUNEL assay of apoptotic cells in three cell lines (PC9/D6, PC9, PC9/PCD) treated with or without 5 μmol/L of gefitinib. Apoptotic cells are stained with dark brown. D: Apoptosis rate of the three cell lines (PC9/D6, PC9, PC9/PCD), Bars. E: qRT-PCR analysis of integrinβ1 gene in all the six cell lines (AB2/17-2, AB2/N, PC9/AB2, PC9/D6, PC9/PCD, PC9). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and AB2/N after gefitinib treatment (15 μmol/L) (Fig. 2C,D). The number of apoptotic cells was 30% in AB2/17-2 cell lines and 10% in PC9/AB2 and AB2/N cell lines ($P < 0.05$). Gefitinib treatment caused G1 arrest in the 3 cell lines (Table II).

UP-REGULATED INTEGRIN β1 EXPRESSION IN PC9 CELL LINE LEADS TO ACQUIRED RESISTANCE TO GEFITINIB

To further confirm the role of integrin β1 in acquired resistance to gefitinib, we stably transfected PC9 cells with integrin β1 cDNA plasmid (Fig. 3A) or empty plasmid. The integrin β1 mRNA expression in PC9/D6 was 4.45-fold higher than that in its parental cell line (Figs. 1E3 and 3E).

After transfection of integrin cDNA plasmid, the cells acquired resistance to gefitinib. Values of IC_{50} for gefitinib was (9.26 ± 1.20) μmol/L in PC9/D6 cells, which was a 220-fold higher than in its parental PC9 cells [(0.042 ± 0.01) μmol/L] (Fig. 3B). Forty-eight hours after gefitinib treatment (5 μmol/L), both PC9 and PC9/PCD cells developed a considerably higher number of apoptotic cells (about 50%), while the apoptosis in the integrinβ1 over-expressed PC9/D6 cells was significantly inhibited (Fig. 3C,D). Cell cycle analysis revealed that gefitinib treatment resulted in G1 arrest in all the three cell lines. The decrease of S cell cycle in cell line PC9/D6 was significant ($P < 0.05$), which was not found in cell line PC9 and PC9/PCD (Table III).

CHANGE OF DOWNSTREAM OF EGFR SIGNALING PATHWAY

We compared expression of phosphorylated EGFR, phosphorylated Akt and phosphorylated Erk1/2 using Western blot in PC9 and PC9/AB2 cell lines (Fig. 1F). Gefitinib significantly decreased levels of phosphorylated EGFR, phosphorylated Akt and phosphorylated

TABLE II. Cell Cycle of Integrinβ1-Inhibit Cell Lines ($\bar{x} \pm s$, %)

Cells	Before treatment			15 μmol/L gefitinib		
	G0/G1	S	G2/M	G0/G1	S	G2/M
PC9/AB2	68.03 ± 9.90	17.2 ± 2.78	15.77 ± 6.43	86.97 ± 11.75	6.07 ± 5.26	5.3 ± 7.13
AB2/N	57.67 ± 7.10	24.03 ± 2.55	18.3 ± 9.59	84.93 ± 5.17	9.67 ± 2.78	5.37 ± 2.65
AB2/17-2	60.8 ± 13.72	17.85 ± 3.6	19.7 ± 7.78	77.45 ± 0.21*	12.5 ± 3.39	10.05 ± 3.46*

Compared with before treatment, *$P < 0.01$.

Erk1/2 in PC9 cells, but not in PC9/AB2 cells. Then we also detected expression of phosphorylated EGFR, phosphorylated Akt and phosphorylated Erk1/2 in PC9/AB2 and AB2/N, AB2/17-2 cell lines in presence and in absence of gefitinib (Fig. 2E). Gefitinib markedly decreased levels of phosphorylated EGFR, phosphorylated Akt and phosphorylated Erk1/2 in AB2/17-2 cells, but not in PC9/AB2 and AB2/N cells. These data suggested that EGFR signaling pathway could be suppressed with 2 μmol/L gefitinib and down-regulation of integrin β1 by siRNA can inhibit downstream signaling of EGFR and improve the sensitivity of gefitinib in NSCLC (Fig. 4).

DISCUSSION

Comparison in difference between the acquired resistant cell line and its parental sensitive cell line in gene expression or signaling pathway is a useful approach to elucidate the mechanism of acquired gefitinib resistance. Several studies have induced gefitinib-resistant NSCLC cell lines, such as PC9/ZD, H3255, HCC827, and RPC9 cells, etc. [Koizumi et al., 2005; Engelman et al., 2007; Ogino et al., 2007; Su et al., 2008]. In current study, we induced a high gefitinib-resistant cell line PC9/AB2 whose sensitivity to gefitinib was decreased by 576 times. As to acquired resistant mechanisms to gefitinib in NSCLC, T790M mutation in exon 20 of EGFR and c-met amplification have been proposed and confirmed in preclinical and clinical studies. So, we detected genotypes of exons 19–21 of EGFR and c-met protein expression in the PC9 and PC9/AB2 cells. No other mutations were found in the two cell lines except for exon 19 deletion mutation. Expression level of c-met protein was comparable between the two cell lines. So, we could deduce that T790M mutation and c-met amplification might not be the main reasons for acquired resistance in PC9/AB2 cell line.

Jida et al. reported that they established 5 clones of erlotinib resistant PC-9 cell line (harboring EGFR exon 19 deletion) (designated as PC-9ER1-5) by exposing PC-9 to low-dose erlotinib. The IC50 values for the parental PC-9 cells and PC-9ER1-5 cells were 0.02 and 33 μmol/L respectively. No T790M mutation or MET amplification was found in all the 5 resistant cell lines. Although phospho-EGFR was suppressed in both PC-9 and PC-9ER1-5 with 2-μmol/L erlotinib, phospho-Akt was not suppressed in PC-9ER1-5. The combination of erlotinib and PHA-665752, MET tyrosine kinase inhibitor, did not suppress phospho-Akt or cell proliferation in PC-9ER1-5. All together, their results indicated that other mechanisms leading to Akt activation caused resistance to EGFR-TKIs [Jida et al., 2009].

Integrin beta1, that associates with the adhesion and migration capability of tumor cells and have a key role in the growth and metastasis of tumors, is an important molecular of the adhesion-mediated drug resistance. It is also the receptor of ECM that is involved in drug resistance including EGFR TKI. Integrin abnormal adhesion and migration was found in PC9/AB2 cells accompanied with over-expression of integrin β1. Moreover, EGFR and integrin β1 can activate Akt through different signaling pathways. Akt, also called PKB, is a serine/threonine kinase that plays a major role in cell survival [Velling et al., 2008]. So, integrin pathway may play some role of in the acquired resistance of NSCLC to EGFR TKIs. To confirm our hypothesis, we established the cell lines with stable down- and up-expression of integrin β1 by siRNA or integrin β1 cDNA plasmid into PC9/AB2 cells and PC9 cells, respectively. After reduction of integrin β1, PC9/AB2 cells partially restored sensitivity to gefitinib while over-expression of integrin β1 led to resistance of PC9 cells to gefitinib. Expression level of integrin β1 was negatively correlated with gefitinib sensitivity in these two cell lines.

Koizumi et al. [2005] had established a gefitinib-resistant PC-9 subline (termed PC-9/ZD) using same way. There was also no T790M mutation in the PC-9/ZD subline. However they found that the downregulation of activated AKT was closely correlated with the cellular sensitivity to gefitinib that is similar with our result. So all these data suggest that integrin β1-AKT/ERK alerted the cellular sensitivity to gefitinib.

TABLE III. Cell Cycle of Integrinβ1-Overexpress Cell Lines ($\bar{x} \pm s$, %)

Cells	Before treatment			5 μmol/L gefitinib		
	G0/G1	S	G2/M	G0/G1	S	G2/M
PC9	57.5 ± 21.11	25.57 ± 10.98	15.93 ± 9.84	77.83 ± 8.18	11.2 ± 7.83	10.97 ± 1.48
PC9/PCD	68.8 ± 7.19	19.3 ± 1.35	11.93 ± 7.73	70.43 ± 3.55	17.33 ± 8.4	9.23 ± 2.2
PC9/D6	46.43 ± 7.76	36.57 ± 16.48*	17.13 ± 8.87	82.27 ± 5.96	8.73 ± 0.9*	8.93 ± 5.06

Compared with before treatment, *$P < 0.01$.

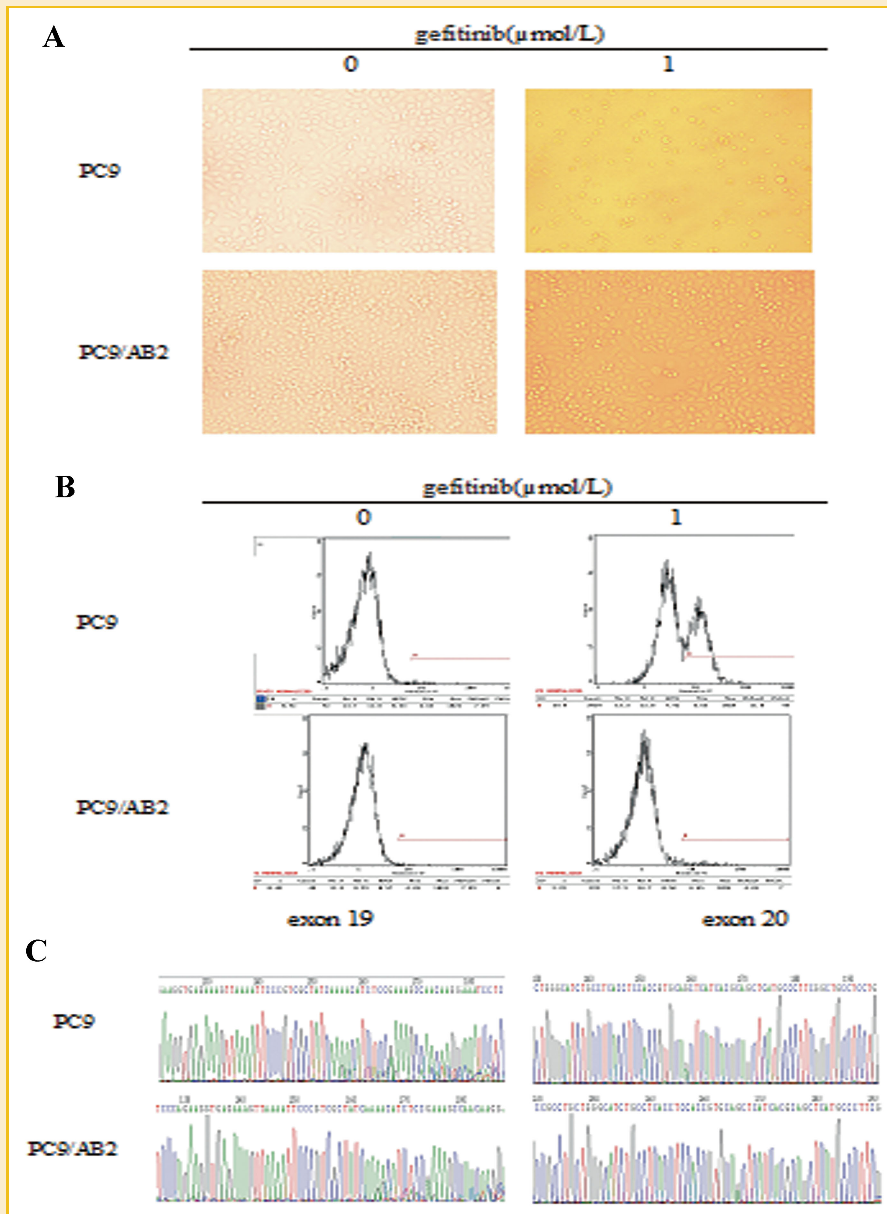

Fig. 4. Characterization of gefitinib-resistant cell line PC9/AB2. A: Light microscopic analysis of PC9 and PC9/AB2 cells treated with or without 1 μmol/L of gefitinib for 72 h. B: Apoptosis analysis of PC9 and PC9/AB2 cells with or without 1 μmol/L of gefitinib by flow cytometry and Annexin V-PE assay. C: Direct sequencing of EGFR exons 19–21 in PC9 and PC9/AB2 cells. The original 15 bp deletion in exon 19 was identified. No T790M or other mutations were found. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In summary, we have established a gefitinib-resistant subline, PC9/AB2, from the highly sensitive NSCLC cell line PC9. The occurrence of overexpression of integrin β1 is most likely the predominant mechanism for the resistance, which could be reversed by the interference of siRNA of integrin β1. We suggest that integrin β1 may play a role in regulating gefitinib sensitivity.

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