

Isolation and characterization of erlotinib-resistant human non-small cell lung cancer A549 cells

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Abstract. Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, is an effective therapy for non-small cell lung cancer (NSCLC). However, resistance to erlotinib reduces its efficacy. To investigate the basis of erlotinib resistance, we isolated erlotinib-resistant human NSCLC A549 cells, termed A549/ER cells. The A549/ER cells were found to be resistant to erlotinib, as well as paclitaxel and gemcitabine. We then performed a PCR array to investigate the resistance to erlotinib in A549/ER cells. *EGFR* expression in A549/ER cells was decreased compared to A549 cells. The expression of *fibroblast growth factor 2* (*FGF2*) and *p21* in A549/ER was increased when compared to A549 cells. Our results suggest that the down-regulation of EGFR and up-regulation of FGF2 is related to resistance to erlotinib in A549/ER cells.

Introduction

Non-small cell lung cancer (NSCLC) is a significant cause of cancer-related mortality in the US and worldwide (1). Epidermal growth factor receptor (EGFR) is overexpressed in various epithelial types of cancer, including NSCLC, and its inhibitors have been investigated as first-line or subsequent therapy options for patients with advanced metastatic NSCLC (2). Two classes of EGFR inhibitor, monoclonal antibodies, including cetuximab, and small-molecule tyrosine kinase inhibitors (TKIs), including erlotinib and gefitinib, have been

studied in phase III trials and are used clinically to treat NSCLC (3-8). Although erlotinib is an effective therapy for NSCLC, resistance to erlotinib reduces its efficacy. Erlotinib resistance is associated with changes in EGFR itself or in the expression of other genes (9). In particular, the T790M mutation has been reported in 50% of EGFR TKI-resistant tumors (10,11). To investigate the basis of erlotinib resistance, erlotinib-resistant human NSCLC A549 cells, termed A549/ER cells, were isolated. A PCR array was performed to identify erlotinib resistance in A549/ER cells.

Materials and methods

Reagents. Paclitaxel, ethyl methanesulfonate and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The gemcitabine hydrochloride used was from LKT Laboratories, Inc. (MN, USA). Erlotinib was from Chemie Tek (Indianapolis, IN, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution (10,000 U/ml penicillin and 10,000 µg/ml streptomycin) were from Hyclone (UT, USA). RT-2 Profiler PCR Array Human Cancer Drug Resistance and Metabolism (PAHS-004A-2) was from SA Biosciences (Frederick, MD, USA).

Cell culture. The A549 cell line, derived from NSCLC, was maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

Cell proliferation by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay. Cell proliferation *in vitro* was measured using a MTT colorimetric assay in 96-well plates. The cells (5x10³) were inoculated into each well. Following overnight incubation (37°C in 5% CO₂), anti-cancer agents were added to the cultured and incubated for 3 days. A total of 50 µl of MTT (1 mg/ml) was added to each well and the plates were incubated for an additional 4 h. Following aspiration of the culture medium, the resulting formazan was dissolved with

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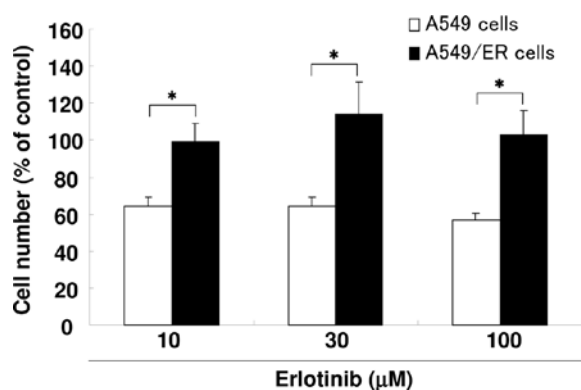


Figure 1. Sensitivity of erlotinib in A549 and A549/ER cells. A549 and A549/ER cells were treated with various concentrations of erlotinib for 72 h and cell viability was determined using MTT assay in each cell line. The columns and bars are the mean \pm SD. * $P < 0.05$.

100 μ l of dimethylsulfoxide. The plates were read at 570 nm using a microplate reader.

Chronic erlotinib exposure. Erlotinib-resistant A549/ER cells were isolated by the A549 cells with increasing concentrations of erlotinib following ethyl methanesulfonate-induced mutagenesis, and then incubated in a selection medium with erlotinib (1-100 μ M).

Reverse transcription-polymerase chain reaction (RT-PCR) method. Total cellular RNA was extracted by the RNeasy Mini kit (Qiagen Sciences, MD, USA). RNA quality and concentration were confirmed in NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For RT-PCR, 1 μ g of total RNA was used for cDNA synthesis using an iScript cDNA synthesis kit (Bio-Rad, CA, USA), according to the manufacturer's instructions. The conditions for the RT-PCR were: 5 min at 95°C and then 28 cycles of amplification in PCR master mix (Promega, WI, USA) at 95°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min. The primers used for this analysis were: p21, forward 5'-ctcttcggccagtgacagc-3' and reverse 5'-agagtctcaggtccacctgg-3'; fibroblast growth factor 2 (FGF2), forward 5'-ccttgcccaggatggcgca-3' and reverse 5'-ttgaccgtaagtattgtagt-3'; EGFR, forward 5'-gccacaggccaggtctgcat-3' and reverse 5'-ccggcgtctgcgtacttcag-3'; and GAPDH, forward 5'-gtcttcaccaccatggagaagg-3' and reverse 5'-ggcaggtcaggtccaccactga-3'.

Genomic DNA extraction and single nucleotide polymorphism genotyping. Genomic DNA from A549 and A549/ER cells were extracted with a QIAamp DNA Mini kit (Qiagen Sciences). The conditions for the RT-PCR were: 5 min at 95°C and then 55 cycles of amplification in PCR master mix (Promega) at 95°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min. The mutations were genotyped using PSQ96MA (Qiagen, Germantown, MD, USA).

The primers used for this analysis were: T790M, forward 5'-tgggcatctgcctcacct-3', reverse 5'-ctttgtgtcccgacat-3' and sequence primer 5'-cctcacctccaccgt-3'; L861Q, forward 5'-agcaggaacgtactggtgaa-3', reverse 5'-gcctcctctgcagtgattc-3' and sequence primer 5'-tcacagatttgggc-3'.

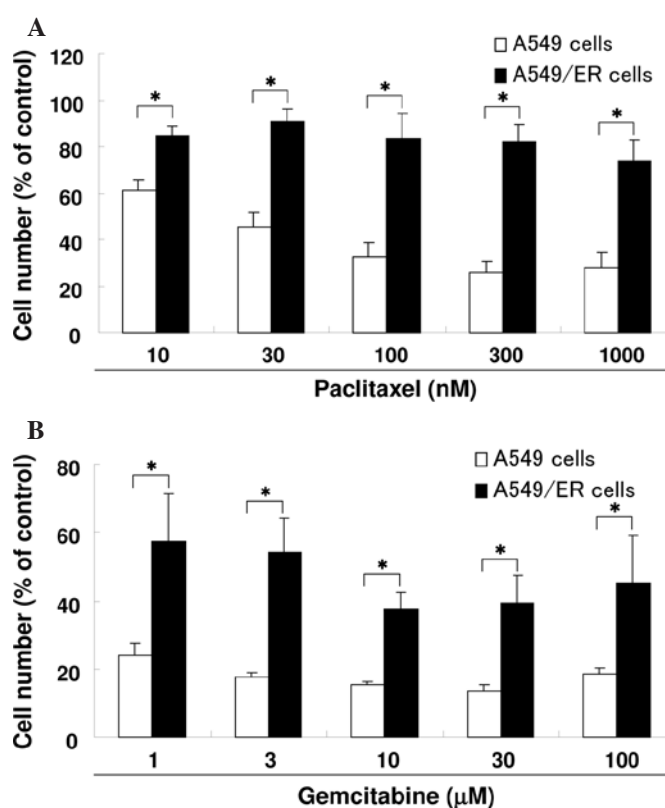


Figure 2. Sensitivity of A549 and A549/ER cells to paclitaxel and gemcitabine. A549 and A549/ER cells were treated with various concentrations of (A) paclitaxel and (B) gemcitabine for 72 h and cell viability was determined using MTT assay in each cell line. The columns and bars are the mean \pm SD. * $P < 0.05$.

PCR array. Total cellular RNA was extracted by the RNeasy Mini kit (Qiagen Sciences). RNA quality and concentration were confirmed in the NanoDrop ND-100 Spectrophotometer (Thermo Scientific). For RT-PCR, 1 μ g of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. After cDNAs were mixed with SYBR-Green Supermix, the mixtures were added to the plates of RT-2 Profiler PCR Array Human Cancer Drug Resistance and Metabolism. The conditions for real-time PCR were: 10 min at 95°C and then 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The data from PCR array were normalized according to the manufacturer's guidelines using software from SA Bioscience.

Statistical analysis. Data are presented as the mean \pm SD. Statistical analysis was performed using StatView 5.0. (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P < 0.05$.

Results

Establishment of erlotinib-resistant non-small cell lung cancer A549 cells. To isolate erlotinib-resistant A549/ER cells, A549 cells were cultured in stepwise selection containing an increasing erlotinib concentration, from 10 to 100 μ M. The sensitivity to erlotinib was examined in each cell line. Results showed that the A549/ER cells were more resistant than the parental A549 cells (Fig. 1).

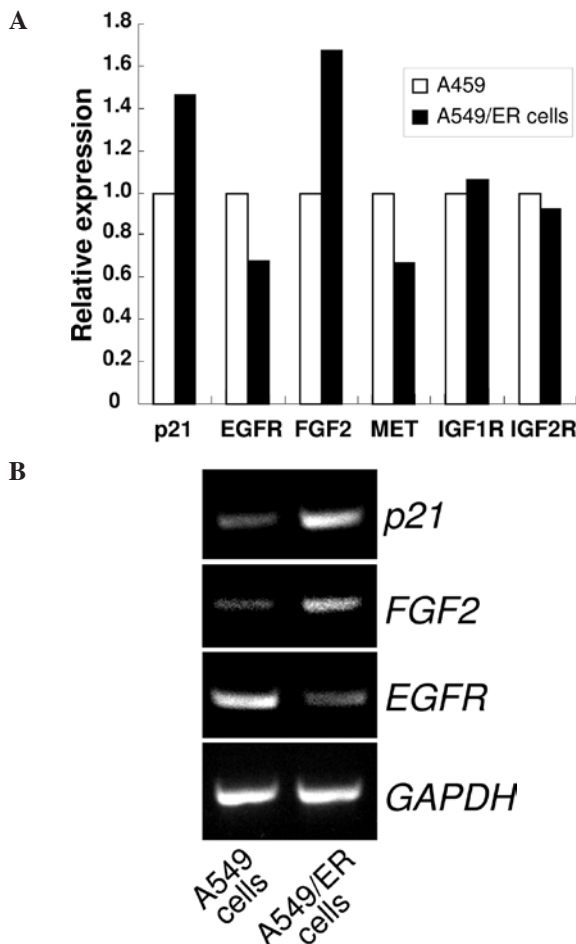


Figure 3. Expression of *EGFR*, *MET*, *p21* and *FGF2* in A549 and A549/ER cells. (A) Expression of *p21*, *EGFR*, *MET*, *FGF2*, *IGF1R* and *IGF2R* in A549 and A549/ER cells was detected by PCR array. Following preparation of the total RNA, 1 μ g was used for cDNA synthesis. The expression of genes was determined by PCR array, as described in Materials and methods. Data are normalized to the expression of each gene in parental A549 cells. (B) Expression of *EGFR*, *FGF2* and *p21* in A549 and A549/ER cells. The expression of *EGFR*, *FGF2*, *p21* and *GAPDH* in A549 and A549/ER cells was measured using RT-PCR. *GAPDH* expression was detected as a control.

EGFR mutation in A549/ER cells. EGFR mutations, including the threonine-to-methionine substitution at position 790 (T790M, in exon 20) and the leucine-to-glutamine (L861Q, in exon 21) have been reported to be resistance mutations to erlotinib (12). To examine T790M and L861Q mutations in A549/ER cells, pyrosequencing was used. The *EGFR* T790M and L861Q mutations were not present in the A549/ER cells (data not shown).

Cross resistance to erlotinib. The drug sensitivity of each cell line was tested using a MTT assay. Fig. 2 shows the sensitivity of the parental and resistant cell lines to various anti-cancer agents. Notably, A549/ER cells were resistant to paclitaxel and gemcitabine as compared to A549 cells (Fig. 2A and B).

PCR array analysis and RT-PCR analysis of A549 and A549/ER cells. The temporal pattern of gene expression in A549 and A549/ER cells was compared by a PCR array that covered 84 genes. A number of genes including *EGFR* and *MET*

were down-regulated in A549/ER compared to A549 cells. A total of five genes were up-regulated more than 1.4-fold in A549/ER cells. The genes, including the ones for *FGF2* and *p21/CDKN1A*, were increased in A549/ER compared to A549 cells (Fig. 3A). The expression of *insulin-like growth factor-1 receptor (IGF1R)* and *IGF2R* was not altered between A549 and A549/ER cells. To validate the PCR array using independent methods, RT-PCR analysis was performed using specific primers for each gene. Consistent with the array data, *FGF2* and *p21* expression was increased in A549 cells, while *EGFR* expression was decreased (Fig. 3B).

Discussion

Lung cancer is the most common cause of cancer-related mortality in developed nations. NSCLC normally presents as incurable locally advanced or metastatic disease. Despite significant research efforts, survival prospects remain poor and 14% of all patients with lung cancer are expected to live 5 years after diagnosis (1). EGFR-specific TKIs, including gefitinib and erlotinib, have been developed as therapeutic agents for NSCLC treatment (6,7). Although the benefits of erlotinib were statistically significant, resistance to erlotinib reduces its efficacy. To investigate the basis of resistance to erlotinib, we isolated erlotinib-resistant human NSCLC A549 cells, termed A549/ER cells.

One mechanism of acquired resistance occurs when EGFR is mutated. Mutations at T790M and L861Q are normally associated with erlotinib resistance (12). Therefore, we performed a sequence of genome in A549 cells and A549/ER cells. Results showed that neither A549 nor A549/ER cells carried a mutation of T790M and L861Q; thus, the acquired resistance identified in this study was not caused by EGFR mutation.

To investigate the basis of resistance to erlotinib, we performed a PCR array in A549 and A549/ER cells. The expression of *EGFR* and *MET* in A549/ER cells was decreased when compared to the sensitive A549 cells. Additionally, similar levels of *IGF1R* and *IGF2R* were expressed in the two cell lines. EGFR signaling is linked to multiple intracellular pathways that inhibit apoptosis and promote survival and proliferation. Upon ligand-induced activation, EGFR and MET generate phosphotyrosine sites for the recruitment of Ras and phosphatidylinositol-3 kinase (PI3K), resulting in the classic mitogen-activated protein kinase and Akt pathway (13). These results suggest that A549/ER cells are independent of EGFR signaling for their growth and survival. Notably, the expression of *FGF2* in A549/ER cells was increased compared to that in A549 cells. Through the activation of FGFR1 β signaling, FGF2 promotes the survival and proliferation of tumors. Stimulation of FGFR1 β results in PI3K/Akt activation and causes resistance to anti-cancer agents (14,15). Neutralizing FGFR1-specific antibody abrogates the physiologic and chemoprotective effects of FGF2/FGFR1 β signaling (15). The A549/ER cells may have alternative means of maintaining PI3K/Akt signaling, including FGF2/FGFR overexpression, that activates PI3K/Akt signaling in an EGFR- or MET-independent manner. Strategies, including the neutralizing FGFR1 or FGF2-specific antibody, or inhibitors of FGFR1, may be effective in restoring sensitivity to erlotinib-resistant cells.

On the other hand, the overexpression of p21 inhibits proliferation in mammalian cells and has been found to inhibit all cyclin-CDK complexes, suggesting that it is a cyclin-CDK inhibitor (16). p21 has demonstrated that the inhibitory control on cyclin-CDK complexes is mediated through its N-terminal domain and is distinct from its ability to bind PCNA (16). p21 plays an essential role in growth arrest after DNA damage, while its overexpression leads to cell cycle arrest, which prevents DNA damage (17). Erlotinib-mediated signaling is involved in the up-regulation of p21 (18). We demonstrated the overexpression of p21 in erlotinib-resistant cells (A549/ER cells) when compared to A549 cells (Fig. 3). Additionally, Ferrandiz *et al* have reported that p21-deficient colon cancer cells were more sensitive towards imatinib and gefitinib than parental cells (19). These results suggest that the overexpression of p21 may prevent erlotinib signaling in A549/ER cells.

In conclusion, a better understanding of the characterization and mechanism of resistance to erlotinib in A549/ER cells may be useful in the identification of agents that reverse clinical erlotinib resistance in NSCLC.

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