The Effect of Acquired Cisplatin Resistance on Sensitivity to EGFR Tyrosine Kinase Inhibitors in EGFR Mutant Lung Cancer Cells

Jin Kyung Rho,* Yun Jung Choi,*† You Ri Choi,* Sun Ye Kim,* Su Jin Choi,‡ Chang-Min Choi,*§ Im Il Na,‡ and Jae Cheol Lee†§

*Department of Pulmonary and Critical Care Medicine, Asan Medical Center, University of Ulsan, Seoul, Korea
†University of Science & Technology, Daejeon, Korea
‡Department of Internal Medicine, Korea Cancer Center Hospital, Seoul, Korea
§Department of Oncology, Asan Medical Center, University of Ulsan, Seoul, Korea

Although epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) are used as first-line agents for treating nonsquamous cell lung cancer with EGFR mutation, there are many patients who have to receive these drugs following platinum-based chemotherapy. This study was designed to define whether exposure to cisplatin could affect the sensitivity to EGFR TKIs because conflicting results have been presented. We established sublines that are resistant to cisplatin from EGFR wild-type cells (A549 and H460) and EGFR mutant cells (PC-9 and HCC827). The EGFR-related signals were examined by Western blotting. MTT assay and the trypan blue exclusion method were used for the in vitro study, while tumor size and the SUV of the 18FDG-PET scans were measured in animal models. The IC50 value and apoptotic fractions after exposure to EGFR TKIs, such as gefitinib, erlotinib, and BIBW 2992, were almost the same in the cisplatin-resistant sublines compared to that of the parent cells. Although the baseline PTEN expression was reduced in the resistant cells, as was indicated in a previous study, the EGFR-related signals similarly responded to the EGFR TKIs. Furthermore, the reduced tumor size and SUV of the 18FDG-PET of the implanted tumor in nude mice according to erlotinib treatment were not different between the resistant sublines and the parent cells. In conclusion, the acquired resistance to cisplatin did not affect the sensitivity to EGFR TKIs in the EGFR mutant lung cancer cells, and this should abrogate any concerns about the use of EGFR TKIs following platinum-based chemotherapy.

Key words: Lung cancer; Epidermal growth factor receptor (EGFR) inhibitor; Chemoresistance; Cisplatin

INTRODUCTION

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) such as gefitinib and erlotinib in the treatment of non-small cell lung cancer (NSCLC) result in clinical benefits, especially in patient with EGFR TK mutations of G719A on exon 18, a deletion mutation on exon 19, and L858R on exon 21 (1,2). Clinical characteristics such as never-smoker, female, adenocarcinoma histology, and Asian heritage also could help to select patients responding better, although they are not completely matched with EGFR TK mutations (3). Currently EGFR TKIs are used as first-line agents for treating NSCLC with EGFR mutation after a phase III trial, open-label study (the IRESSA Pan-Asia Study) demonstrated significantly longer progression-free survival (PFS) and higher objective response rates (p < 0.0001) among EGFR mutation-positive patients who received gefitinib than among those who received carboplatin-paclitaxel (1). However, there are still many patients who have to receive EGFR TKIs following platinum-based chemotherapy because platinum-based cytotoxic chemotherapy is recommended in case of unknown EGFR mutation status or squamous cell lung cancer.

One of major concerns is whether the platinum-based first-line chemotherapy would influence the subsequent responsiveness of cancer cells to EGFR TKIs. To address this issue, Chin et al. performed a preclinical study using NSCLC cells with acquired resistance to cisplatin (4). They found that those cells exhibited reduced sensitivity to erlotinib through downregulation of PTEN, leading to persistent EGFR-independent PI3K/Akt signaling. From these results, they suggested that NSCLC patients with first-line EGFR TKIs might have more favorable clinical outcomes compared to patients treated with second-line or further EGFR TKIs in terms of progression-free interval. However, there has been no report demonstrating that longer progression-free or
overall survival could be achieved by first-line EGFR TKIs, although some recent studies showed the very impressive response rate in patients with EGFR TK mutations (5). Rather, most studies containing results of comparison between first-line and second-line EGFR TKIs showed no difference in clinical outcomes (6). In addition, there were contradictory preclinical experiments showing that chemotherapy-resistant tumor cell lines acquired enhanced sensitivity to EGFR TKIs (7,8).

Our study was performed to answer this controversial issue of whether the sensitivity to EGFR TKIs would be reduced by acquisition of resistance to cisplatin, which has important clinical implication.

MATERIALS AND METHODS

Cell Culture and Reagents

Four human NSCLC cell lines were used in this study. A549, H460, and HCC827 cells were purchased from the American Type Culture Collection. PC-9 cells were a gift from F. Koizumi and K. Nishio (National Cancer Center Hospital, Tokyo, Japan). Cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37°C in an atmosphere of 5% CO₂.

Apoptosis Assay

Apoptosis was quantified using the Annexin V-FITC apoptosis kit (BD Biosciences, San Diego, CA) in accordance with the manufacturer’s instructions. Briefly, cells were trypsinized (Invitrogen), pelleted by centrifugation, and resuspended in Annexin V binding buffer (150 mmol/L NaCl, 18 mmol/L CaCl₂, 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl₂). FITC-conjugated Annexin V (1 µg/ml) and propidium iodide (50 µg/ml) were added to cells and incubated for 30 min at room temperature in the dark. Analyses were done on a FACScan (Becton Dickinson, Mountain View, CA). The data were analyzed with CellQuest software (Becton Dickinson).

Xenograft In Vivo Study

Female severe combined immunodeficiency (SCID) mice (18–20 g, 6 weeks old) were purchased from the Animal Resources Centre. Tumors were grown by implanting cells (5 × 10⁶) in Matrigel (BD Biosciences) into the mouse shoulders. The mouse carried a PC-9 xenograft on the left shoulder and a PC-9/CR xenograft on the right shoulder. Treatment of the three mice per group was started when the tumors had reached a volume of 50–100 mm³ with vehicle control, erlotinib (100 mg/kg, 5 days a week). Erlotinib were orally administered. Treatment was stopped after 11 days and mice were followed up for tumor recurrence. For tumor size measurements, the length (L) and width (W) of the
tumor were measured with calipers, and tumor volume (TV) was calculated as $TV = (L \times W^2)/2$. Positron emission tomography (PET) scans were done before and after administration of drugs (0 and 7 days).

**PET Image Acquisition**

$^{18}$F-FDG PET imaging was performed with a dedicated small animal PET scanner (MicroPET® R4 scanner, Concorde Microsystems, Knoxville, TN) using LSO crystals, 350–750 keV energy window and timing windows of 6 ns. PET imaging was started 60 min after the administration of $7.4 \text{ MBq}/0.1 \text{ ml}$ of $^{18}$F-FDG via tail vein injection. Mice were anesthetized with 2% isoflurane in 100% oxygen (Forane solution; ChoongWae Pharma, Seoul, South Korea) for uptake and scanning. After position scanning, each mouse was scanned for 20 min to obtain a static image. The acquired three-dimensional emission list-mode data was reconstructed to temporally framed sinograms using Fourier rebinning (FORE) and ordered subsets expectation maximization (OSEM2D) reconstruction algorithm with four iterations. PET values were first converted to standardized uptake values (SUV) using the MicroPET® ASIPro software (Concorde Microsystems). Image visualization and analysis were performed using the nonproprietary Amide’s a Medical Image Data Examiner (AMIDE) software.

**RESULTS**

**Acquisition of Resistance to Cisplatin Did Not Affect the Sensitivity to EGFR TKIs**

To determine whether acquisition of resistance to cisplatin influences the sensitivity to EGFR TKIs, we established cell line models of acquired resistance to cisplatin. We selected four cell lines, including cells with wild-type EGFR (A549 and H460) and mutant EGFR (HCC827 and PC-9). Cisplatin-resistant cells were established, as described in Materials and Methods. The cisplatin-resistant cell lines are referred to as A549/CR, H460/CR, HCC827/CR, and PC-9/CR, respectively. In addition, two sublines were isolated from each resistant cell. All resistant cells showed above fourfold resistance to cisplatin (Table 1, Fig. 1a). Besides the sensitivity to paclitaxel in PC-9/CR cells, other resistant cells showed no cross-resistance against conventional anticancer drugs such as gemcitabine and paclitaxel (Table 1). In addition, all cisplatin-resistant cell lines showed no cross-resistance against reversible EGFR TKIs (gefitinib and erlotinib) and BIBW2992, an irreversible EGFR TKIs (Table 1, Fig. 1b). A longer term clonogenic survival assay also showed similar results (Fig. 1c).

**Reduced PTEN Expression Was Commonly Found in Cisplatin-Resistant Cells Without Affecting the Capability of EGFR TKIs to Modulate EGFR Akt Signaling and Induce Apoptosis**

One study showed that EGFR mutant NSCLC cells with acquired cisplatin resistance reduced erlotinib sensitivity (4). They demonstrated that these results were correlated with reduced PTEN function. Thus, we examined the basal level of PTEN as well as EGFR-related molecules. As shown in Figure 2a, the reduction of PTEN was observed in all cisplatin-resistant cells except for HCC827/CR cells. Nonetheless, all EGFR TKIs could modulate EGFR and downstream Akt activity.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cisplatin</th>
<th>Gemcitabine</th>
<th>Paclitaxel</th>
<th>Gefitinib</th>
<th>Erlotinib</th>
<th>BIBW 2992</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>9.6 (0.8)</td>
<td>4.0 (0.2)</td>
<td>&lt;0.01</td>
<td>4.2 (0.2)</td>
<td>4.8 (0.5)</td>
<td>1.2 (0.5)</td>
</tr>
<tr>
<td>A549/CR-1</td>
<td>53.9 (3.2)</td>
<td>3.8 (0.2)</td>
<td>&lt;0.01</td>
<td>6.7 (3.0)</td>
<td>6.5 (1.6)</td>
<td>3.0 (1.0)</td>
</tr>
<tr>
<td>A549/CR-2</td>
<td>61.2 (2.9)</td>
<td>3.6 (0.1)</td>
<td>&lt;0.01</td>
<td>5.2 (0.6)</td>
<td>5.4 (0.5)</td>
<td>2.6 (0.5)</td>
</tr>
<tr>
<td>H460</td>
<td>9.3 (0.6)</td>
<td>2.7 (0.2)</td>
<td>0.09 (0.01)</td>
<td>20.1 (2.2)</td>
<td>30.9 (2.7)</td>
<td>11.6 (2.0)</td>
</tr>
<tr>
<td>H460/CR-1</td>
<td>48.3 (2.0)</td>
<td>2.9 (0.1)</td>
<td>&lt;0.01</td>
<td>14.5 (2.2)</td>
<td>32.3 (3.6)</td>
<td>6.6 (1.1)</td>
</tr>
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<td>H460/CR-2</td>
<td>51.5 (3.6)</td>
<td>2.8 (0.4)</td>
<td>&lt;0.01</td>
<td>16.1 (3.8)</td>
<td>29.3 (4.3)</td>
<td>8.9 (1.0)</td>
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<td>PC-9</td>
<td>10.9 (1.8)</td>
<td>26.2 (4.0)</td>
<td>0.11 (0.03)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PC-9/CR-1</td>
<td>61.5 (4.8)</td>
<td>39.5 (0.9)</td>
<td>18.06 (1.74)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>PC-9/CR-2</td>
<td>55.4 (8.9)</td>
<td>26.3 (3.2)</td>
<td>21.33 (0.61)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>HCC827</td>
<td>13.7 (2.3)</td>
<td>34.9 (5.8)</td>
<td>0.14 (0.04)</td>
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<td>&lt;0.01</td>
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<tr>
<td>HCC827/CR-1</td>
<td>54.6 (6.3)</td>
<td>34.5 (4.4)</td>
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<td>&lt;0.01</td>
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<td>HCC827/CR-2</td>
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<td>32.8 (7.4)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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Values are mean IC$_{50}$ with SD in parentheses. The drug concentration (µM) is that responsible for 50% growth inhibition as determined in the MTT assay at 72 h. Data represent the mean from at least three independent experiments.
Figure 1. Cytotoxic effects of EGFR TKIs in cisplatin-resistant cells. (a, b) Cells were treated with the indicated concentrations of cisplatin or EGFR TKIs (gefitinib, erlotinib, and BIBW2992) for 72 h. The viability of cells was determined using the MTT assay. (c) Cells were incubated with indicated concentrations of EGFR-TKIs (gefitinib, erlotinib, BIBW2992) for 72 h and then the culture medium was changed every 3 days within 10 days. Cells were trypsinized and cell numbers were determined with ADAM Cell Counter. Bars represent the mean ± SD of three wells. G, gefitinib; E, erlotinib; B, BIBW2992.

dose and time dependently regardless of PTEN expression in EGFR mutant cells (Fig. 2b, c). Consistent with this, induction of p27, Bim, and subsequent apoptosis similarly occurred in cisplatin-resistant cells (Fig. 2d).

Antitumor Effects of Erlotinib Were Also Similar in Cisplatin-Sensitive and Cisplatin-Resistant Tumor Xenografts

Next, we established PC-9 and PC-9/CR tumor xenografts to confirm in vitro results. The sensitivity to erlotinib was evaluated by $^{18}$F-FDG uptake changes and tumor growth inhibition. In PC-9 and PC-9/CR tumor xenografts, erlotinib treatment led to the reduction of $^{18}$F-FDG uptake and tumor size reduction between them. In addition, the growth of cisplatin-resistant tumor resumed at a similar rate to that seen in parental tumor after administration of erlotinib was discontinued. These findings suggest that acquisition of resistance to cisplatin has no influence on the efficacy of EGFR TKIs.

DISCUSSION

We provided preclinical data suggesting that second-line EGFR TKIs would have comparable efficacy to first-line treatment. EGFR signaling is important for cancer cell proliferation and survival. Various kinds of cancer cells increased EGFR expression for survival as a response to cytotoxic chemotherapeutic agents, which made them more susceptible to the inhibition of EGFR...
Figure 3. The effect of erlotinib in a cisplatin-resistant cell xenograft model. SCID mice carried a PC-9 xenograft on the left shoulder (white arrows) and a PC-9/CR xenograft on the right shoulder (red arrows). Three mice used in each group were treated daily with 100 mg/kg erlotinib for 11 days. (a) PET images of tumors from PC-9 and PC-9/CR tumor-bearing mouse were obtained before (0 day) and after (7 day) erlotinib treatment. The graph shows $^{18}$FDG uptake change on day 7 after the first injection. (b) Length and width of the tumors were measured at days indicated, and tumor volumes were calculated. Bars present mean tumor volume $\pm$ SD.

Signals (8). It was also proved in xenograft animal models. In addition, Servidei et al. showed that altered signaling of receptor EGFR family, such as overexpression and constitutive phosphorylation of ErbB2 and ErbB3, altered HER ligand expression and enhanced activation of EGF-triggered EGFR pathway might contribute to increased sensitivity to gefitinib in chemoresistant tumor cell lines (7). However, because these earlier studies did not use EGFR mutant lung cancer cells, their result would not be clinically relevant. Therefore, the study conducted by Chin et al. with lung cancer cells harboring deletion mutation on exon 19 seems to have more clinical implication (4).

The sensitivity to EGFR TKIs is considered to be mostly dependent on Akt activation (11). Actually, resistant cells to EGFR TKIs commonly exhibit persistent Akt activation, although its activation could be decreased by treatment of drug, as so-called “Akt tail” (12). Chin et al. found that exposure to cisplatin in PC-9 cells resulted in reduction of PTEN contributing to persistent Akt phosphorylation. PTEN loss has been shown to be involved in EGFR TKIs resistance in some tumor cell lines (13–15). In addition, another study showed that PTEN loss contributes to erlotinib resistance in EGFR mutant lung cancer (16). However, they suggested that immediate and constitutive activation of Akt is more effective than PTEN loss to induce erlotinib resistance. Thus, the modulation of Akt activity plays a key role in determining sensitivity to EGFR TKIs. In our study, PTEN expression was also reduced in resistant
cells except HCC827 regardless of EGFR mutations whereas there was no significant induction of Akt activity. In addition, treatment of EGFR TKIs showed the complete inhibition of Akt activity in parental and cisplatin-resistant cells with EGFR mutation. The differences of these results may be due to degree of resistance to cisplatin and reduced PTEN expression. Previous study showed that cisplatin-resistant cells have high degree resistance to drug and a significant reduction of PTEN expression when compare to our resistant cells (4). Therefore, our findings indicate that although decreased PTEN expression is a common finding of cisplatin-resistant cells, it does not universally lead to Akt activation causing decreased sensitivity to EGFR TKIs.

In general, EGFR TKIs induce cell cycle arrest and/or apoptosis through the upregulation of its key target genes such as p27<sup>Kip1</sup> and Bim (17,18). These phenomena are associated with the efficacy of EGFR TKIs. We also observed the induction of p27<sup>Kip1</sup> and Bim in parental and cisplatin-resistant cells, and consequently, both cells showed a similar pattern of apoptosis after EGFR TKIs treatment. Consistent with these results, erlotinib treatment also shrunk cisplatin-resistant tumor implanted into SCID mice. Furthermore, once erlotinib treatment was halted, the growth of cisplatin-resistant tumor resumed at a rate similar to that seen in parental tumor.

In conclusion, the acquired resistance to cisplatin did not affect the sensitivity to EGFR TKIs in the EGFR mutant lung cancer cells, and this should abrogate any concerns about the use of EGFR TKIs following platinum-based chemotherapy.

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