Acquired resistance to cetuximab is mediated by increased PTEN instability and leads cross-resistance to gefitinib in HCC827 NSCLC cells

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EGFR inhibitors, including the small-molecule tyrosine kinase inhibitors such as gefitinib, and the monoclonal antibodies directed at the receptor such as cetuximab, have demonstrated promising effects in non-small cell lung cancer (NSCLC). In this study, we generated cetuximab-resistant cell lines (HCC827-CR) from HCC827 NSCLC cells to investigate acquired resistance mechanisms to cetuximab. In HCC827-CR cells, Akt was hyperactivated and its activity was persistent upon cetuximab treatment. Blockade of PI3K/Akt activity restored cetuximab sensitivity in HCC827-CR cells. Further investigation revealed that increased PTEN instability mediates constitutive Akt activation. By 1 \textmu M proteosomal inhibitor, MG-132, PTEN protein levels were restored and Akt activity was dramatically reduced. Overexpression of PTEN by transfection could not restore cetuximab sensitivity in HCC827-CR because overexpressed PTEN was degraded rapidly (\textless{}72 h). The increased PTEN instability was confirmed by the treatment of HCC827-CR with a protein synthesis inhibitor, cycloheximide. In the presence of cycloheximide, overexpressed PTEN was degraded more rapidly (\textless{}12 h) in HCC827-CR cells. Interestingly, HCC827-CR cells also revealed de novo resistance to gefitinib. Inhibition of PI3K/Akt signaling pathway restored sensitivity to gefitinib in HCC827-CR cells. Taken together, these data show that PTEN instability-mediated constitutive Akt activation is involved in acquired resistance mechanisms to cetuximab and also induces de novo resistance to gefitinib. Importantly, these findings suggest emergence of cross-resistance between two agents as a potential serious problem in the clinical setting.

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1. Introduction

The epidermal growth factor receptor (EGFR) is a member of the HER family of receptor tyrosine kinases (RTKs) and consists of four members: EGFR (HER1), HER2, HER3 and HER4. Binding of ligands to the extracellular domain of the EGFR induces receptor homo- or heterodimerization with other ErbB receptors, which results in intracellular autophosphorylation of specific tyrosine residues. EGFR autophosphorylation leads to a downstream signaling network, including MAPK and PI3K/Akt activation that influence cell proliferation, angiogenesis, invasion and metastasis [1,2]. EGFR overexpression, gene amplification,
mutation, or rearrangement are frequently observed in several human malignancies and correlated with poor prognosis and worse clinical outcomes. Hence, the EGFR is an attractive target for cancer therapy [1,3,4].

Among the several EGFR-targeting agents, monoclonal antibodies (MoAbs) and small-molecule tyrosine kinase inhibitors (TKIs) are the most promising and well studied. These agents target the same receptor, but they differ in action mechanisms. The anti-EGFR MoAbs prevent receptor dimerization/activation by blocking ligand binding to the extracellular domain of EGFR, whereas TKIs bind to the ATP-binding site in the tyrosine kinase domain (TKD) of the receptor, thereby inhibiting EGFR autophosphorylation and activation of the downstream signaling network [5,6].

Cetuximab is a recombinant anti-EGFR human/mouse chimeric MoAb and known to induce antibody-dependent cell cytotoxicity and sensitize tumor cells to chemotherapy and radiotherapy [7–10]. With these advantages, the clinical benefit of using cetuximab as a monotherapy or in combination with chemotherapy and/or radiation has been demonstrated in head and neck cancer and metastatic colorectal cancer [5].

Recently, adding cetuximab to chemotherapy has been shown survival benefit compared with chemotherapy alone in the first-line treatment of advanced non-small cell lung cancer (NSCLC) [11]. After failure of first-line therapy with cetuximab, gefitinib, a small molecule TKI, is a well-established option for the second-line treatment of advanced NSCLC [12]. In several studies, clinical responses to gefitinib are highly dependent on the presence of EGFR mutations in the ATP-binding cleft of EGFR TKD. These mutations lead to increased EGFR signaling and confer susceptibility to the TKI. Consequently, the mutant EGFR is inhibited 10- to 100-fold more effectively than wild-type EGFR by the TKI [13]. The two most common EGFR mutations include exon 19 deletions and L858R missense mutations, which are responsible for approximately 85% of all EGFR mutations identified in NSCLC [14]. However, these EGFR mutations in NSCLC cells are not associated with sensitivity to cetuximab [15]. Although cetuximab and gefitinib have different mechanisms of blockade of the EGFR and there is no evidence to suggest cross-resistance between the two agents, it is possible that resistance to cetuximab will subsequently affect the sensitivity to gefitinib.

Despite significant advances in anti-cancer drugs, long term control of cancer is difficult to achieve because many patients develop acquired resistance to the drugs. Therefore, it will be essential to identify mechanisms of resistance that develop to these agents as a means of rationally designing the subsequent treatment [16,17]. To date, two main mechanisms of acquired resistance to cetuximab have been identified. The EGFR ubiquitination/degradation and Src activation was identified as a resistance mechanism to cetuximab in DiFi colorectal cancer cells [18]. Recently, acquired resistance to cetuximab in H226 NSCLC cells was accompanied by EGFR upregulation and subsequent EGFR-dependent activation of HER3 [19].

In this study, we established cetuximab-resistant NSCLC (HCC827-CR) cell lines, which harbor a deletion mutation in exon 19 of the EGFR, to investigate additional mechanisms of acquired resistance to cetuximab. In contrast to the results from previous studies, we could not find the activation of Src or HER family members in HCC827-CR cells. Our results show that increased PI3K/Akt activation plays a key role in acquired resistance mechanisms to cetuximab in HCC827-CR cells. Importantly, cetuximab resistance results in de novo resistance to gefitinib in HCC827-CR cells with the gefitinib-sensitizing EGFR mutation (delE746_A750), suggesting the emergence of cross-resistance between these two agents as a potential serious problem in this clinical setting.

2. Materials and methods

2.1. Cell lines and establishment of acquired cetuximab-resistant cell lines

The HCC827 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% fetal bovine serum and cultured in a humidified atmosphere of 5% CO2 at 37 °C. To establish cetuximab-resistant cell lines, HCC827 cells were exposed to increasing concentrations (10–100 nM) of cetuximab for 6 months. To validate whether cells acquired resistance, cell viability was examined by MTT assay each time cetuximab concentrations were increased. Cell culture media and supplements were obtained from HyClone (Logan, UT).

2.2. Reagents and antibodies

Cetuximab (IMC-225, Erbitux®) and gefitinib were provided by ImClone System Inc. (New York, NY) and Astra-Zeneca (Macclesfield, UK), respectively. LY294002 and cycloheximide were purchased from Sigma (St. Louis, MO). MC-132 was purchased from Calbiochem (San Diego, CA). Antibodies against PTEN, β-actin and NEDD4-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were purchased from Cell Signaling (Danver, MA).

2.3. Cell viability assay

Cells were seeded at a density of 1 × 10^4 cells/well into a 96-well culture plate and incubated for 24 h. After cells were exposed to drugs for 72 h, 0.5 mg/ml of MTT was added to the medium in the well. After incubation for 4 h at 37 °C, formazan crystals in viable cells were solubilized with 100 μl DMSO. The optical density of the MTT formazan product was read at 565 nm on a microplate reader. All experiments were performed in triplicate. MTT was purchased from Amresco (Solon, OH).

2.4. Phospho-RTK array

Phospho-RTK arrays (R&D Systems, Minneapolis, MN) were used according to the manufacturer’s instructions. Briefly, following treatment, cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton
X-100, 0.5% deoxycholate, 0.1 mM EGTA, 1.0 mM EDTA, 0.1% SDS) in the presence of protease inhibitor, 1 mM NaF, 1 mM sodium orthovanadate, 0.5 mM DTT and 0.2 mM PMSF. A total of 150 μg fresh protein lysates were incubated overnight with phospho-RTK array membranes. Thereafter, bound phospho-RTKs were detected with a pan antiphosphotyrosine antibody conjugated to HRP using chemiluminescence.

2.5. Western blot analysis

After cells were lysed with RIPA buffer, protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were fractionated by SDS–PAGE and then transferred onto a nitrocellulose membrane (BioRAD, Richmond, CA). After blocking with 5% skim milk in PBS + 0.1% Tween 20, the membrane was incubated with the appropriate primary antibody at 4 °C overnight. Proteins were detected using HRP-conjugated secondary antibodies and ECL chemiluminescence detection system (Amersham-Pharma Biotech, Buckinghamshire, England).

2.6. Measurement of EGFR levels on the cell surface by flow cytometry

To measure levels of cell surface EGFR, cells were serum starved for 18 h at 0.5% serum and then treated with 50 ng/ml of EGF (R&D Systems, Minneapolis, MN). Thereafter, cells were washed with cold PBS and harvested by trypsinization. After washing with PBS containing 0.2% BSA, cells were incubated with PE-conjugated control IgG or PE-conjugated anti-EGFR antibodies in PBS containing 2% BSA for 1 h on ice. After incubation, cells were washed three times with PBS containing 0.2% BSA followed by flow cytometry. All antibodies were purchased from Abcam (Cambridge, MA). The data were analyzed with the WinMDI program (The Scripps Research Institute, La Jolla, CA).

2.7. Transient transfection

Wild type-PTEN (pcDNA3-PTEN) plasmid was obtained from Dr. Hong-Duck Um (Korea Institute of Radiological and Medical Sciences, Seoul, Republic of Korea). Transient transfections with pcDNA3 or pcDNA3-PTEN were performed with Lipoctamine™2000 reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, cells were seeded at a density of 2.5 × 10^5 cells/well in a 6 well plate. After 24 h, the cells were transfected with 2 μg/well of DNA for 6 h with Lipoctamine™2000 reagent and replaced with fresh growth medium. At 24 h after transfection, cells were treated with drugs as indicated in individual experiments and then harvested for analysis.

2.8. RT (reverse transcription)-PCR analysis of PTEN

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen, Dusseldorf, Germany). After RNA concentrations were determined with a Nanodrop Spectrophotometer ND-100, 1 μg of total RNA was converted to cDNA using SuperScript™III reverse transcriptase (Invitrogen, Carlsbad, CA). PTEN specific cDNA was amplified by PCR using PTEN specific primer pairs and were detected on 1% agarose gel running. GAPDH was used as an internal control of PTEN. Primer sequences were as follows: PTEN, sense 5′-ATGACAGCCATCATCAAAGAG-3′ and antisense 5′-GTGCC ACTGGCTCATAACTCCAG-3′; GAPDH, sense 5′-TGATGACAT CAAGAAGGTGAGAAG-3′ and antisense 5′-TCTTGGAGCGCATGTTGCCC-3′.

2.9. siRNA transfection

NEDD4-1 specific siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). siRNA transfection was performed with lipofectamine™RNAiMAX reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, cells were seeded at seeding density of 2.5 × 10^5 cells/well in a 6 well plate. After 24 h, the cells were transfected with 50 μM of siRNAs for 6 h with Lipofectamine™RNAiMAX reagent and replaced with fresh growth medium. At 48 h after transfection, cells were harvested.

![Fig. 1](image-url)  
**Fig. 1.** HCC827-CR cells have resistance to cetuximab compared with HCC827-P cells. A, Cells were treated with the indicated concentration of cetuximab (CTX) in 0.5% FBS-containing medium for 72 h. Cell viability was measured using the MTT assay and plotted as a percentage of cell viability relative to untreated control cells. B, After treatment of cetuximab (CTX) for 72 h, cells were harvested. Cell lysates were subjected to Western blot analysis for the indicated proteins. β-actin was used as a loading control.
3. Results

3.1. Establishment of acquired cetuximab-resistant HCC827 cell lines

We established cetuximab-resistant HCC827 NSCLC cell lines (HCC827-CR). The cell line was chosen because, in addition to its sensitivity to cetuximab, it is highly sensitive to gefitinib due to the presence of the sensitizing mutation in the TKD of EGFR. First, we examined resistance to cetuximab in HCC827-CR cells by MTT assay (Fig. 1A). HCC827 parental cells (HCC827-P) indicated approximately 80% inhibition of cell viability upon exposure to 1 nM cetuximab. In contrast, HCC827-CR cells displayed considerable resistance to cetuximab. Also, cetuximab induced the cleavage of caspase-3 and PARP, markers of apoptosis, in HCC827-P but not HCC827-CR cells (Fig. 1B).

3.2. Alteration of EGFR status is not involved in acquired resistance to cetuximab

To investigate mechanisms of acquired resistance to cetuximab, we first determined whether HCC827-CR cells have additional mutations in TKD of EGFR compared with HCC827-P cells. As a result of sequencing, no additional mutations were found in the TKD of EGFR in HCC827-CR cell lines (data not shown).

Cetuximab is known to block the EGFR signaling pathway through the induction of EGFR internalization/degradation [7]. Therefore, we hypothesized that impaired binding to cetuximab or impaired internalization of EGFR was involved in the acquired resistance mechanisms to cetuximab. To examine this hypothesis, we tested the effects of cetuximab on EGF-induced EGFR activation and analyzed patterns of EGF-induced EGFR activation through Western blotting (Fig. 2).

![Fig. 2](image2.png)

**Fig. 2.** In HCC827-CR cells, EGFR inhibition by cetuximab and EGF-induced internalization of cell surface EGFR were similar to those of HCC827-P cells. A, Cells were incubated with 10 nM cetuximab (CTX) for 24 h. After stimulation with 50 ng/ml EGF for 15 min, cells were harvested. EGFR phosphorylation and total EGFR protein levels were analyzed by Western blot. B, The levels of cell surface EGFR were monitored following 0–24 h of EGF (50 ng/ml) stimulation by flow cytometry. Outlined peaks show flow cytometry analysis of the number of EGFR receptors on the cell surface. Shaded peaks represent a negative control.

![Fig. 3](image3.png)

**Fig. 3.** Other RTKs including the HER family are not involved in acquired resistance to cetuximab in HCC827-CR cells. A, HCC827-P and HCC827-CR cells were treated with 100 nM cetuximab, and the cell lysates were hybridized to a phospho-RTK array. B, HCC827-P and HCC827-CR cells were seeded and incubated in the absence of cetuximab for 24 h. Cells were harvested and subjected to Western blot analysis for the indicated proteins.
internalization in HCC827-P and HCC827-CR cells. The basal EGFR activity of HCC827-CR cells was lower than that of HCC827-P cells. Upon EGF treatment, EGFR activation was induced in both HCC827-P and HCC827-CR cells. After cetuximab treatment, EGFR-induced EGFR activation was inhibited and EGFR protein levels were reduced in both cell lines (Fig. 2A). We then assessed EGF-induced EGFR internalization by flow cytometry. In HCC827-P cells, EGFR was rapidly internalized in response to EGF stimulation within 6 h. In HCC827-CR cells, EGF-induced EGFR internalization was similar to that of HCC827-P (Fig. 2B). These data suggest that HCC827-CR cells do not have impaired binding to cetuximab or impaired internalization of EGFR, and that other mechanisms are involved in cetuximab resistance.

3.3. The activation of other RTKs including the HER family is not involved in acquired resistance to cetuximab

Previous studies revealed that activation of alternative RTKs that bypass the EGFR pathway is involved in acquired resistance to anti-EGFR drugs [19–21]. To identify involvement of activation of other RTKs in acquired resistance to cetuximab, we performed phospho-RTK arrays in HCC827-P and HCC827-CR cells. In HCC827-CR cells, basal activities of most RTKs including the HER family were lower than those of the HCC827-P cells and were not changed upon treatment with 100 nM cetuximab (Fig. 3A). Recently, Wheeler et al. reported that activation of the HER family plays a key role in acquired resistance to cetuximab in NSCLC [19]. To confirm the reduced activity of the HER family in our cetuximab-resistant cells, we performed immunoblot analysis for the phospho-HER family. Consistent with the results of the phospho-RTK array, activities of EGFR, HER2 and HER3 in HCC827-CR cells were lower than those in HCC827-P cells (Fig. 3B). These data showed that activation of other RTKs do not play a role for acquired resistance to cetuximab in HCC827-CR cells.

3.4. Hyperactivation of the PI3K/Akt pathway plays a role in acquired cetuximab resistance and inhibition of PI3K/Akt pathway restores sensitivity to cetuximab

Another common molecular mechanism of resistance is the constitutive activation of downstream effectors of EGFR [22,23]. Therefore, we determined whether certain signaling molecules were activated in HCC827-CR cells. The activities of Akt and ERK, which are two main downstream effectors of EGFR, were examined by immunoblot analysis in HCC827-P and HCC827-CR cells. ERK activity in HCC827-CR cells was similar to that in HCC827-P cells. Because it was reported that Src activation mediates acquired resistance to cetuximab in colorectal cancer cells [18], we analyzed Src activity in HCC827-P and HCC827-CR cells. The Src

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**Fig. 4.** Constitutive Akt activation plays a role in acquired resistance to cetuximab. A, Cells were incubated with 100 nM cetuximab (CTX) in 0.5% FBS-containing RPMI1640 for 24 h. Activities of Akt, ERK and Src were determined by Western blot analysis. B, Cells were treated for 1 h with vehicle or 20 μM LY294002 and then incubated with vehicle or 100 nM cetuximab. After 72 h, cell viability was examined by the MTT assay. C, Cells were treated as described in Fig. 4B. After 24 h, cells were harvested and then subjected to Western blot analysis.
activity in HCC827-CR cells was similar to that in HCC827-P cells. In HCC827-P cells, phosphorylation of Akt was reduced in response to 100 nM cetuximab. In contrast to HCC827-P cells, Akt was strongly activated and sustained upon treatment of cetuximab in HCC827-CR cells (Fig. 4A). To examine that hyperactivation of Akt was involved in cetuximab resistance, we tested whether blockade of the PI3K/Akt pathway would restore cetuximab sensitivity in HCC827-CR cells. In the presence of 20 μM LY294002 (PI3K/Akt inhibitor), cetuximab reduced cell viability in HCC827-CR cells (Fig. 4B). In cetuximab-treated HCC827-CR cells, the cleavage of caspase-3 was induced only in the presence of LY294002 (Fig. 4C). Together, these findings suggest that activation of the PI3K/Akt pathway contributes to cetuximab resistance in HCC827-CR cells.

3.5. Increased PTEN instability mediates constitutive activation of Akt in HCC827-CR cells

The major underlying mechanism for an increased Akt activation is known to be an inactivation of the PTEN [24]. In order to investigate the mechanism of constitutive Akt activation in HCC827-CR cells, we first analyzed mRNA and protein levels of PTEN in HCC827-P and HCC827-CR cells. Although no remarkable difference was found in PTEN mRNA levels between HCC827-P and HCC827-CR cells, PTEN protein levels were obviously lower in HCC827-CR cells than those in HCC827-P cells (Fig. 5A). To clarify whether similar levels of PTEN mRNA from both cell lines was due to saturation of the amplification system used, PCR amplification was repeated with various cycle numbers. Similar levels of PTEN mRNA from both cell lines were repeatedly detected (data not shown). We therefore hypothesized that the low PTEN protein levels were due to increased ubiquitin-mediated proteosomal degradation in HCC827-CR cells. To examine this hypothesis, HCC827-CR cells were treated with a proteosomal inhibitor, MG-132, and PTEN protein levels were evaluated by immunoblot analysis. After treatment of 1 μM MG-132, PTEN protein levels increased and showed the characteristic ladder pattern of polyubiquitinated protein. At that time, Akt activity was reduced sharply (Fig. 5B). To confirm increased PTEN instability in HCC827-CR cells, wild-type PTEN was transiently overexpressed in HCC827-P and HCC827-CR cells and then analyzed by immunoblot analysis at 24 h intervals. In HCC827-P cells, overexpressed PTEN levels were sustained for 72 h. In contrast, overexpressed PTEN levels in HCC827-CR cells were sustained for 48 h and decreased dramatically at 72 h (Fig. 5C). To clarify that the reduction of overexpressed PTEN protein levels was not due to a reduction of protein synthesis but was a result of protein degradation in HCC827-CR cells, we induced PTEN overexpression and treated with an inhibitor of protein biosynthesis, cycloheximide (CHX) (Fig. 5D). In HCC827-P cells, overexpressed PTEN levels were reduced after 3 h and hardly detected at 12 h in the presence of CHX. In the absence of CHX, Akt activity was inhibited in PTEN overexpressed HCC827-CR cells. After treatment of CHX, according as overexpressed

Fig. 5. Increased PTEN instability mediates constitutive Akt activation in HCC827-CR cells. A, HCC827-P and HCC827-CR cells were seeded and incubated in the absence of cetuximab for 24 h. Cells were harvested for RT-PCR and Western blot analysis. For RT-PCR, total RNA was extracted from cells and converted to cDNA by reverse transcription. PTEN transcript levels were determined by PCR using PTEN specific primer pairs (upper). For Western blot analysis, cells were lysed and fractionated on SDS–PAGE for indicated proteins (lower). B, HCC827-CR cells were incubated with 1 μM MG-132 in 10% FBS-containing medium for 24 h. Cell lysates were subjected to Western blot analysis. C, Cells were transfected with control vector (MOCK) or plasmids expressing wild-type PTEN (WT-PTEN). At 24, 48 and 72 h post-transfection, cells were harvested and subjected to Western blot analysis for detection of PTEN protein expression levels. P, HCC827-P cells; CR, HCC827-CR cells. D, To confirm the increased PTEN instability in HCC827-CR cells, cells were transfected with indicated constructs. At 24 h post-transfection, cells were treated with 50 μg/ml cycloheximide (CHX). After CHX addition, cells were harvested at serial time points for Western blot analysis.
PTEN protein levels were reduced, Akt activity was recovered in PTEN overexpressed HCC827-CR cells. These findings were repeatedly detected in CHX-treated samples at 24 h and 48 h post-transfection with PTEN construct (Fig. S1). After treatment of CHX for 3 h, reduction of overexpressed PTEN protein levels was only detected in HCC827-CR cells but not in HCC827-P cells. In CHX-treated HCC827-CR cells, overexpressed PTEN at 48 h post-transfection was more rapidly degraded than that at 24 h post-transfection. In absence of CHX, Akt activity was inhibited in both HCC827-P and HCC827-CR cells. On the other hand, restoration of Akt activity was only detected in HCC827-CR cells after treatment of CHX. These data suggest that in HCC827-CR cells, PTEN instability is due to increased ubiquitin-mediated proteosomal degradation, thereby leading to the constitutive activation of Akt.

Although the mechanisms reducing PTEN protein levels remain poorly understood, the c-terminus of PTEN has been known to play a critical role in maintaining PTEN stability [25]. For that reason, we analyzed the full sequence of PTEN cDNA from HCC827-P and HCC827-CR cells. However, no mutations were detected in PTEN from either cell line (data not shown). In recent studies, it was reported that an E3 ubiquitin ligase, NEDD4-1, negatively regulates PTEN stability by ubiquitination [26,27]. To identify a role of NEDD4-1 in increased PTEN instability within HCC827-CR cells, we compared NEDD4-1 protein levels between HCC827-P and HCC827-CR cells. There was no remarkable difference in NEDD4-1 levels between HCC827-P and HCC827-CR cells (Fig. 6A). In addition, siRNA-mediated knockdown of NEDD4-1 did not affect PTEN protein levels in HCC827-CR cells (Fig. 6B). These data indicate that NEDD4-1 is dispensable for the increased PTEN instability in HCC827-CR cells.

To confirm involvement of PTEN loss in the acquired cetuximab resistance, we tested sensitivity to cetuximab after knockdown of PTEN using siRNA in HCC827-P cells (Fig. S2). As shown in Fig. S2A, knockdown of PTEN expression in HCC827-P cells resulted in Akt activation. Sensitivity to cetuximab was significantly decreased in siPTEN-transfected cells compared with siControl-transfected cells (Fig. S2B). Thus, these results strongly suggest that PTEN loss and subsequent Akt activation is involved in the acquired resistance to cetuximab.

3.6. HCC827-CR cells have de novo resistance to gefitinib and inhibition of the PI3K/Akt signaling pathway overcomes gefitinib resistance

In recent study, it was suggested that inhibition of HER family with HER family TKIs or blocking antibodies may be an effective therapeutic strategy to overcome cetuximab-acquired resistance because activation of the HER family is a main cetuximab-resistance mechanism [19]. Because PTEN instability-mediated Akt activation is an EGF-independent intracellular event, HCC827-CR cells may not respond to EGFR TKIs. To examine the sensitivity to EGFR TKIs, HCC827-CR cells were exposed to increasing concentrations of gefitinib and cell viability was examined by MTT assay. HCC827-CR cells revealed resistance to gefitinib even at high concentrations (10 μM) (Fig. 7A). We then tested whether a blockade of the PI3K/Akt signaling pathway also overcomes gefitinib resistance in HCC827-CR cells. In the presence of 20 μM LY294002, cell viability was significantly reduced by 10 nM gefitinib in HCC827-CR cells (Fig. 7B). Even though Akt activity was partially inhibited by 10 nM gefitinib alone in HCC827-CR cells, it was still higher than basal Akt activity in HCC827-P cells. Only the combination treatment of 20 μM LY294002 and 10 nM gefitinib induced complete inhibition of the Akt activity in HCC827-CR cells. Cleavage of caspase-3 was also detected in HCC827-CR cells by combination treatment with 20 μM LY294002 and 10 nM gefitinib, but not by gefitinib alone (Fig. 7C). These data suggest that HCC827-CR cells acquired de novo resistance to gefitinib as well as cetuximab and the resistance to gefitinib can be overcome by inhibition of the PI3K/Akt signaling pathway.

Although activities of HER family in HCC827-CR cells were lower than those in HCC827-P cells, we tested whether inhibition of HER family overcomes acquired resistance to cetuximab in HCC827-CR cells (Fig. S3). In contrast to HCC827-P cells, HCC827-CR cells showed resistance to BIBW2992, dual EGFR/HER2 inhibitor. When 10 nM BIBW2992 was co-treated with 100 nM cetuximab in HCC827-CR cells, resistance to cetuximab was not disappeared. Sensitivity to BIBW2992 or cetuximab in HCC827-CR cells was restored upon treatment with LY294002.

4. Discussion

In this study, our findings suggest that increased PTEN instability-mediated Akt activation plays a critical role in acquired cetuximab resistance. We further found that the acquired resistance to cetuximab could also induce de novo resistance to an EGFR TKI, gefitinib. PTEN instability in HCC827-CR cells resulted from increased ubiquitin-mediated proteosomal degradation which subsequently induced constitutive Akt activation. Inhibition of the PI3K/Akt pathway restored sensitivity to gefitinib as well as cetuximab in HCC827-CR cells. The findings of PTEN instability by increased proteosomal degradation and cross-resistance to EGFR TKIs in cetuximab-resistant cells are novel and have not been reported previously as acquired resistance mechanisms to cetuximab.

PTEN is a tumor suppressor and negatively regulates the PI3K/Akt pathway by conversion of phosphatidylinositol 3,4,5-triphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) [28]. The allelic loss or mutation of PTEN is common in many human malignancies and PTEN abnormality-associated Akt activation has been known to play an important role in chemotherapy resistance [29,30]. With regard to resistance to EGFR inhibitors, there have been several reports on the role of PTEN loss in de novo and/or acquired resistance to EGFR TKIs and cetuximab. In those reports, the mechanisms causing PTEN loss included...
homozygous deletion of PTEN, inactivating mutations, and epigenetic downmodulation of PTEN. These mechanisms causing PTEN loss induced reduction of mRNA levels as well as protein levels. Reintroduction of PTEN in all of the above mechanisms restored sensitivity to EGFR inhibitors in resistant cells [31–35]. However, unlike previous studies, in our study, PTEN loss in HCC827-CR cells was due to increased protein instability. Although we induced PTEN overexpression by transient transfection, we could not overcome the resistance to cetuximab in HCC827-CR cells (data not shown). This was probably because overexpressed PTEN levels were not sustained long enough to affect cell viability due to the shortened half-life of PTEN in HCC827-CR cells. Similar to our findings, PTEN loss at the protein level has been demonstrated in breast cancer with acquired resistance to HER2 MoAb, trastuzumab, which suggests that PTEN loss may be a common resistance mechanism to MoAbs targeting EGFR family members [36].

To overcome acquired resistance to cetuximab, it is important to identify acquired resistance mechanisms and determine a possible therapeutic target. Recently, Wheeler et al. reported that increased EGFR activity contributes to acquired resistance to cetuximab in the human NSCLC cell line NCI-H226 harboring no EGFR mutations [19]. Their study showed that cetuximab could not inhibit the activity of EGFR and inhibition of EGFR activity by treatment of EGFR TKIs can overcome cetuximab resistance. However, in our study, EGF-induced EGFR activation was inhibited and EGFR protein levels were reduced in both cell lines following cetuximab treatment. Furthermore, resistance to cetuximab allowed cross-resistance to gefitinib. The differences of acquired resistance mechanisms between the two cell lines might be associated with activation of different intracellular signaling pathways based on EGFR mutation status. Mutant EGFR-expressing cells, such as HCC827, selectively activate Akt and signal transducer and activator of transcription (STAT) pathway which promote cell survival, whereas wild-type EGFR-expressing cells, such as NCI-H226, activate ERK signaling which induces cell proliferation [37]. Because Wheeler’s NCI-H226 and our HCC827-CR cell lines are addicted to fundamentally different activated signaling pathways for survival, the acquired resistance mechanisms to cetuximab in both cells may be different. Our findings are extremely

![Fig. 7. HCC827-CR cells have de novo resistance to gefitinib. A, Cells were treated with the indicated concentration of gefitinib in 10% FBS-containing medium for 72 h. Cell viability was measured using the MTT assay and plotted as a percentage of cell viability relative to untreated control cells. B, Cells were treated for 1 h with vehicle or 20 μM LY294002 and then incubated with vehicle or 10 nM gefitinib. After 72 h, cell viability was examined by the MTT assay. C, Cells were treated as described in Fig. 6B. After 24 h, cells were harvested and then subjected to Western blot analysis for the indicated proteins.](image)
important regardless of the reasons because they suggest that gefitinib, which has been a standard therapeutic option for advanced NSCLC patients, particularly for those with sensitizing EGFR mutations, may not be effective in all patients after progression on cetuximab treatment.

In conclusion, our study demonstrates that increased PTEN instability-mediated constitutive Akt activation plays a critical role in acquired resistance to cetuximab in HCC827-CR cells. Importantly, constitutive Akt activation also induces de novo resistance to gefitinib in HCC827-CR cells with sensitizing EGFR mutations. These results imply that PI3K/Akt could be an attractive target if cetuximab or gefitinib is combined with an agent targeting the PI3K/Akt inhibitor. Further studies on a novel regulator of PTEN stability will help to identify a new therapeutic target for the effective therapy for NSCLC with acquired resistance to cetuximab.

Conflict of interest

None of the authors of this study has a conflict of interest.

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Appendix A. Supplementary material


References


