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Nrf2 but not Autophagy Inhibition is Associated with the Survival of Wild-type Epidermal Growth Factor Receptor Non-small Cell Lung Cancer Cells

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Abstract

Non-small cell lung cancer (NSCLC) is one of the most common malignancies in the world. Icotinib and Gefitinib are two epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) that have been used to treat NSCLC. While it is well known that mutations of EGFR can affect the sensitivity of NSCLC to the EGFR-TKI, other mechanisms may also be adopted by lung cancer cells to develop resistance to EGFR-TKI treatment. Cancer cells can use multiple adaptive mechanisms such as activation of autophagy and Nrf2 to protect against various stresses and chemotherapeutic drugs. Whether autophagy or Nrf2 activation contributes to the resistance of NSCLC to EGFR-TKI treatment in wild-type EGFR NSCLC cells remains elusive. In the present study, we confirmed that Icotinib and Gefitinib induced apoptosis in EGFR mutant HCC827 but not in EGFR wild-type A549 NSCLC cells. Icotinib and Gefitinib did not induce autophagic flux or inhibit mTOR in A549 cells. Moreover, suppression of autophagy by chloroquine, a lysosomal inhibitor, did not affect Icotinib- or Gefitinib-induced cell death in A549 cells. In contrast, Brusatol, an Nrf2 inhibitor, significantly suppressed the cell survival of A549 cells. However, Brusatol did not further sensitize A549 cells to EGFR TKI-induced cell death. Results from this study suggest that inhibition of Nrf2 can decrease cell vitality of EGFR wild-type A549 cells independent of autophagy.

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Conflict of Interest Statement

All of the authors have no conflict of interest to claim for the manuscript entitled “Nrf2 But Not Autophagy Activation Is Associated with Resistance to EGFR Inhibitor-Induced Lung Tumor Cell Apoptosis”.

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Keywords

autophagy; EGFR; Nrf2; Lung cancer; apoptosis

1. Introduction

Lung cancer is the most diagnosed cancer with the highest mortality in the world (Stewart and Wild 2014). In lung cancer cells, the tyrosine kinase activity of epidermal growth factor receptor (EGFR) is commonly dysregulated by EGFR gene mutation, increased EGFR gene copy number, or EGFR protein overexpression, resulting in aberrant EGFR signaling that is associated with increased tumor cell survival, proliferation, invasion, and metastasis (Ciardiello and Tortora 2008). As a result, EGFR has become an important target for developing targeted therapy for non-small cell lung cancer (NSCLC). Icotinib (Ico, formerly BPI-2009H), a potent small-molecule inhibitor of EGFR tyrosine kinase (Zhejiang Bata Pharma Ltd, Hangzhou, Zhejiang, China, Patent No. WO2003082830), has been approved by the State Food and Drug Administration (SFDA) of China to treat NSCLC. Compared with the Gefitinib (Gef), a widely used EGFR tyrosine kinase inhibitor (TKI), Ico shows similar positive clinical antitumor activities and better safety in advanced NSCLC patients with EGFR mutations (Liu, Jiang et al. 2011; Ren, Zhao et al. 2011; Shi, Zhang et al. 2013). However, most NSCLC patients develop intrinsic and acquired resistance to EGFR-TKI. In addition, how to target to the TKI insensitive EGFR wild-type NSCLC is largely undetermined. Hence, there is an urgent need to develop new combination of treatments to simultaneously target multiple pathways that enhance the efficacy of EGFR-TKI for treating NSCLC, in particular, the EGFR wild-type NSCLC.

Autophagy is a lysosomal degradation pathway that regulates cellular homeostasis by removing damaged organelles and misfolded proteins to protect cells including cancer cells against various adverse stresses (Galluzzi, Pietrocola et al. 2015). Accumulating evidence indicates that EGFR signaling regulates autophagy (Levine and Kroemer 2008; Dragowska, Weppler et al. 2013; Wei, Zou et al. 2013; Tan, Thapa et al. 2015). EGFR-TKI induces autophagy in multiple cancer cell lines by inhibiting the downstream PI3K/AKT/mTOR pathway and/or inhibiting EGFR-mediated Beclin 1 phosphorylation (Dragowska, Weppler et al. 2013; Wei, Zou et al. 2013). Despite the evidence that TKI induces autophagy in lung cancer cells is compelling, conflicting results regarding the role of autophagy in the response to the treatment of EGFR-TKI have been reported. Some studies showed that pharmacological inhibition of autophagy enhanced EGFR-TKI-mediated cell death in lung cancer cells (Han, Pan et al. 2011; Tang, Wu et al. 2015), while Wei Y et al. reported that inhibition of autophagy by expressing a tyrosine phosphomimetic Beclin 1 mutant in NSCLC cells resulted in resistance of EGFR-TKI treatment (Wei, Zou et al. 2013). The underlying mechanisms for these different observations are not clear. Moreover, it is largely elusive how modulating autophagy would affect wild-type EGFR NSCLC cells in response to other stress or drug treatment.

Nuclear factor, NF-E2-related factor 2 (Nrf2), is an important transcription factor. Many of the Nrf2 target genes are cellular important antioxidant genes, drug metabolism enzymes

and transporters, which can protect against the detrimental effects induced by the insult of oxidative and electrophilic stresses (Lau, Villeneuve et al. 2008; Taguchi, Motohashi et al. 2011). In keeping with its ability to protect against chemical and oxidative stress, constitutive activation of Nrf2 has also been shown to contribute to the development of drug resistance in cancer cell lines and tissues (Slocum and Kensler 2011; Taguchi, Motohashi et al. 2011; Son, Pratheeshkumar et al. 2015). Moreover, persistent activation of Nrf2 also contributes to tumorigenesis in mouse livers with impaired autophagy (Ni, Woolbright et al. 2014). These results indicate that persistent activation of Nrf2 may promote tumorigenesis and cancer cell survival (Lau, Villeneuve et al. 2008; Taguchi, Motohashi et al. 2011). In addition to Keap1-mediated regulation of Nrf2 activation, Nrf2 can also be activated by the non-canonical pathway through SQSTM1/p62 (hereafter referred to as p62), a protein which generally accumulates in cells with impaired autophagy (Bjorkoy, Lamark et al. 2005; Ni, Boggess et al. 2012; Ni, Woolbright et al. 2014). p62 directly interacts with Keap1 resulting in Nrf2 accumulation and activation (Lau, Wang et al. 2010; Okatsu, Saisho et al. 2010). Importantly, both p62 and Nrf2 are commonly accumulated in various human cancers including lung cancer (Inami, Waguri et al. 2011; Inoue, Suzuki et al. 2012). Therefore it is possible that inhibiting p62-mediated persistent activation of Nrf2 may help to sensitize cancer cells to other chemotherapy such as EGFR-TKI.

As discussed above, both autophagy and Nrf2 are important cellular protective mechanisms in cancer cells, but how these two cellular adaptive mechanisms are affected by EGFR-TKI in lung cancer cells and whether they contribute to the resistance to EGFR-TKI in wild-type EGFR NSCLC are elusive. In the present study, using A549 (which is naturally resistant to EGFR-TKI due largely to the wild-type EGFR nature), we investigated the effects of EGFR-TKI on autophagy and Nrf2 activation in A549 cells. We demonstrated that EGFR-TKI did not affect mTOR and autophagic flux in A549 cells. Moreover, pharmacological inhibition of autophagy by chloroquine did not affect EGFR-TKI-induced cell death in A549 cells. In contrast, pharmacological inhibition of Nrf2 decreased the cell viability of A549 cells although simultaneously inhibition of Nrf2 and autophagy together with EGFR-TKI did not show synergistic effects on cell viability in A549 cells.

2. Materials and Methods

2.1 Reagents and antibodies

Icotinib (Betta Pharmaceuticals Co., Ltd, Zhejiang, China), Gefitinib (LC laboratories, G-4408), and chloroquine (CQ) were from Sigma. Brusatol was purchased from Tauto Biotech (#14907-98-3, Shanghai, China). The Primary antibodies used in this study were: p62 (Abnova, Cat. # H00008878-M01), phospho-S6 (Cell Signaling, #4858S), total-S6 (Cell Signaling, #2217S), phospho-AKT(S473) (Cell Signaling, #4058), total AKT (Cell Signaling, #9272), Phospho-4E-BP1 (Cell Signaling, #9451), total-4E-BP1 (Cell Signaling, #9452), NQO1 (Santa Cruz), caspase-3 (Cell Signaling, #9661L), and β -actin (Sigma, #a5541). The microtubule-associated protein 1 light chain 3 (LC3) antibody was developed as described previously (Ding, Ni et al. 2009). The rabbit anti-GCLC and GCLM antibodies were kindly provided by Dr. Terrance Kavanagh from The University of Washington. The secondary antibodies were HRP conjugated goat-anti-rabbit (Jackson ImmunoResearch, Cat.

#111-035-045) and goat-anti-mouse IgG antibodies (Jackson ImmunoResearch, Cat. #115-035-062).

2.2 Cell cultures

The human lung cancer cell lines (A549 and HCC827) were maintained in RPMI 1640 supplemented with 10% (v/v) FBS and antibiotics. Stock solution of Icotinib or Gefitinib was prepared in dimethyl-sulphoxide (DMSO) (Sigma) and diluted with medium before use. Final concentration of DMSO was 0.1%.

2.3 Cytotoxicity assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to determine cell viability/growth as we described previously (Yang, Peng et al. 2015). Briefly, cells were seeded into 24-well plates (cultured overnight) and treated with different concentrations of EGFR-TKIs for different time points. MTT (1.25 mg/mL) was added into each well 2 hours prior to the end of the experiments. After that, the supernatant was removed and 500 μ L DMSO was added into each well to solubilize the blue-purple crystals of formazan. The absorbance was then measured using a plate reader (Tecan GENios) at 550 nm. All the experiments were repeated at least 3 times. In addition, after the designated treatments with TKIs or autophagy inhibitor CQ (20 μ M) or Brusatol (40 nm), cells were stained with Hoechst 33342 (1 μ g/mL) for apoptotic nuclei or propidium iodide (PI, 1 μ g/mL) for secondary necrosis or necrosis followed by fluorescence microscopy as previously described (Ding, Ni et al. 2009).

2.4 Analysis of Caspase-3 Activation and Activities

Caspase-3 activation was determined via immunoblotting with an antibody against cleaved caspase 3 (Cell Signaling, #9661L). Analysis of the caspase-3 activity was performed using 20 μ g of protein and 20 μ M of a specific caspase-3 fluorescent substrate (Ac-DEVD-AFC), and the fluorescence signals were detected using a fluorescence plate reader (Tecan GENios) at 400/510 nm (excitation/emission) as we described previously (Williams, Hou et al. 2013).

2.5 RNA Isolation and Real-Time qPCR

RNA was isolated from cultured A549 and HCC827 cells using Qiagen RNeasy kit (Qiagen, Valencia, CA) after designated treatments and reverse transcribed into cDNA by RevertAid reverse transcriptase (Fermentas). Real-time PCR was performed on an Applied Biosystems Prism 7900HT real-time PCR instrument (ABI, Foster City, CA) using Maxima SYBR green/rox qPCR reagents (Fermentas). Primer sequences were as follows: *β -actin* forward: 5'-AGAGCTACGAGCTGCCTGAC-3'; *β -actin* reverse: 5'-AGCACTGTGTTGGCGTACAG-3'; *Atg8/Map1lc3b* forward: 5'-AGCAGCATCCAACCAAATC-3'; *Atg8/Map1lc3b* reverse: 5'-CTGTGTCCGTTACCAACAG-3'; *Sqstm1/p62* forward: 5'-TGCCCAGACTACGACTTGTG-3'; *Sqstm1/p62* reverse: 5'-CTCTCCCCAACGTTCTTCAG-3'; *Nqo1* forward: 5'-CAAATCCTGGAA GGATGGAA-3'; and *Nqo1* reverse: 5'-GGTTGTCAGTTGGGATGGAC-3'; *Keap1* forward: 5'-

CTCATCCAGCCCTGTCTTCA-3'; *Keap1* reverse: 5'-GGTACATGACAGCACCGTTC-3'.

2.6 Electron Microscopy

For electron microscopy (EM) studies, A549 and HCC827 cells were seeded on plastic coverslips in petri-dishes and treated with Ico (1 μ M) and Gef (1 μ M) for 6 hours. The cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), followed by 1% OsO₄. The cells were further dehydrated followed by cutting of thin sections and staining with uranyl acetate and lead citrate. All images were obtained using a JEM 1016CX electron microscope with a digital camera.

2.7 Western blot analysis

Cells were washed in PBS and lysed in RIPA buffer. Protein (20 μ g) from the total cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blotted with the indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce). Images were obtained and analyzed using Image J software (National Institute of Health, USA). Each experiment was performed in triplicate.

2.8 Statistical Analysis

Experimental data were subjected to One-way analysis of variance analysis (ANOVA) or student t test where appropriate. $p < 0.05$ or $p < 0.01$ was considered significant.

3 Results

3.1 EGFR-TKI decreases cell viability in HCC827 but not in A549 cells

Two human lung cancer cell lines A549 (wild-type EGFR) and HCC827 (mutant EGFR) were treated with EGFR-TKI Ico and Gef with different time points and concentrations. As revealed by the MTT assay, both Ico and Gef treatments decreased cell viability in HCC827 but not in A549 cells in a time- and dose-dependent manner (Fig. 1A & B). In HCC827 cells, treatment with both inhibitors reduced cell viability to 60% at 24 hours, which further decreased to 40% and 20% at 48 and 72 hours, respectively. However, neither inhibitor had a significant effect on the viability of A549 cells even after 72 hours treatment. To determine whether decreased viability in HCC827 cells after EGFR-TKI treatment could be due to apoptosis, we next determined the activation of caspase-3, which plays an important role in apoptosis. Consistent with the decreased cell viability, EGFR-TKI treatment increased cleaved caspase-3 levels and caspase-3 activity in HCC827 but not A549 cells in a time- and dose-dependent manner (Fig. 1C-E). These results confirm that EGFR-TKI induces caspase-mediated apoptosis in EGFR mutant HCC827 cells but not in EGFR wild type A549 cells (Paez, Janne et al. 2004).

3.2 Autophagy may not be essential for the resistance of A549 cells to EGFR-TKI

In addition to the expression of wild-type EGFR in A549 cells, whether other additional mechanisms could also contribute to the resistance of EGFR-TKI is not clear. Emerging

evidence suggests that many cancer cells can utilize autophagy as a cell survival mechanism and EGFR-TKI has been shown to induce autophagy in some lung cancer cells (Han, Pan et al. 2011; Wei, Zou et al. 2013). The levels of LC3-II increased in both A549 and HCC827 cells after treatment with various concentrations of Gef and Ico (Fig. 2A). p62, an autophagy substrate protein, was decreased after Ico or Gef treatment more evidently in HCC827 cells than in A549 cells (Fig. 2A). Moreover, the levels of LC3-II were much higher in HCC827 cells than in A549 cells in the absence of TKI. When cells were treated with CQ alone, a lysosomal inhibitor, the levels of LC3-II increased in both A549 cells and HCC827 cells. Moreover, the levels of LC3-II further increased in Gef plus CQ- and Ico plus CQ-treated HCC827 cells compared to treatment with CQ, Gef, or Ico alone-treated HCC827 cells. In contrast, the levels of LC3-II were almost identical in Gef plus CQ- or Ico plus CQ-treated A549 cells compared with CQ alone-treated A549 cells (Fig. 2B). These results suggest that Gef and Ico increase autophagic flux in HCC827 cells but not in A549 cells. EM studies showed that there were increased numbers of autophagic vacuoles (AV) in control, Gef and Ico-treated HCC827 cells compared to A549 cells, although only the Ico treatment group reached statistical significance (Fig. 3A & B). Taken together, these results suggest that EGFR-TKI-induced autophagic flux in HCC827 cells but not in A549 cells.

We next determined whether a combination of EGFR-TKI with an autophagy inhibitor may affect cell viability in A549 cells. We found that inhibition of autophagy by CQ alone had no significant effects on cell viability in A549 cells. Moreover, the combination of CQ with Ico or Gef treatment had no effects on cell viability or caspase-3 cleavage compared with Ico or Gef treatment alone (Fig. 4A–D). Despite EGFR-TKI induced autophagic flux in EGFR mutant HCC827 cells, we found that the combination of CQ with Ico or Gef treatment also had no effects on cell viability compared with Ico or Gef alone treatment (data not shown). These results indicate that pharmacological inhibition of autophagy does not sensitize either EGFR wild type A549 or mutant HCC827 cells to EGFR-TKI. These data suggest that autophagy may not be essential for the resistance of A549 cells to EGFR-TKI, and inhibition of autophagy by CQ has no additional effects on cell viability after EGFR-TKI treatment regardless of EGFR status.

3.4 EGFR-TKI does not affect Akt-mTOR pathways in A549 cells

Akt-mTOR pathway plays a critical role in regulating tumor cell growth, and activation of Akt-mTOR is seen in many NSCLC patients resistant to EGFR-TKI treatment (Fumarola, Bonelli et al. 2014). We next determined the role of EGFR-TKI on Akt-mTOR changes in A549 cells. The levels of phosphorylated Akt only slightly decreased in A549 cells after either Gef or Ico treatment for the various time points that we assessed (Fig. 5A). After either Ico or Gef treatment, the levels of phosphorylated S6 and 4EBP1, two downstream substrates of mTOR, did not change cells except that the phosphorylated levels of S6 decreased at 24 hours (Fig. 5A). We previously showed that mTOR was inhibited in prolonged cultured hepatoma cells likely due to the depletion of nutrients (Yang, Peng et al. 2015). We thus determined the changes of mTOR in A549 cells during their culture for various time points up to 24 hours without any treatments. The levels of phosphorylated Akt and phosphorylated S6 decreased at 24 hours in A549 cells (Fig. 5B). Moreover, the total protein levels of S6 and 4EBP1 also decreased after Gef treatment (Fig. 5B). These results

are in general agreement with our previous findings in cultured hepatoma cells (Yang, Peng et al. 2015). Collectively, these results indicate that Ico or Gef does not affect Akt-mTOR in A549 cells.

3.5 Persistent activation of Nrf2 in A549 cells

Increasing evidence indicates that many tumor cells including lung cancer cells have somatic mutations of Nrf2 and Keap1 resulting in persistent activation of Nrf2 (Hayes and McMahon 2009). The basal mRNA level of *Nqo1*, one of the bona fide target genes of Nrf2, was almost 60-fold higher in A549 than that of HCC827 cells (Fig. 6A). These data indicate that A549 cells have much higher Nrf2 activation than HCC827 cells. Ico or Gef treatment alone increased the mRNA levels of *Nqo1* 4- to 6-fold in HCC827 cells, which was still remarkably lower compared with A549 cells. There were no significant changes in *Keap1* mRNA levels after either Ico or Gef treatment except that Gef significantly increased the expression of *Keap1* in HCC827 cells. The basal mRNA levels of *Atg8*, one of the key autophagy-related genes, were comparable between A549 and HCC827 cells and remained unchanged after Ico or Gef treatment in A549 cells. However, the mRNA levels of *Atg8* increased 3-fold in Ico or Gef-treated HCC827 cells. The mRNA level of *p62* in A549 cells was 2-fold higher than in HCC827 cells, which did not change by either Ico or Gef treatment (Fig. 6A). The protein levels of Keap1 were comparable between A549 and HCC827 cells based on immunoblot analysis. Keap1 levels did not change after treatment with Ico or Gef in neither A549 nor HCC827 cells except that Keap1 levels decreased in HCC827 cells that were treated with Gef (1 μ M). It is possible that decreased Keap1 in HCC827 cells is through autophagic degradation since Gef increased autophagic flux in HCC827 cells (Fig. 2) and Keap1 has been shown to be degraded via autophagy (Taguchi, Fujikawa et al. 2012). A549 cells had higher basal protein levels of NQO1, GCLC, and GCLM, which are all authentic Nrf2 target genes, than HCC827 cells regardless of Ico or Gef treatment (Fig. 6B). These data indicate that A549 cells have higher Nrf2 activity than HCC827 cells, although Nrf2 activity is not affected by Ico or Gef treatment in A549 cells.

3.6 Inhibition of Nrf2 activity by Brusatol decreases cell viability in A549 cells

To investigate whether persistent activation of Nrf2 in A549 cells was one of the underlying mechanisms for its resistance to EGFR-TKI, we next determined the effects of Brusatol, a pharmacological inhibitor of Nrf2 (Ren, Villeneuve et al. 2011), on Nrf2 inhibition and cell viability in A549 cells. Brusatol decreased the protein levels of Nrf2 in a dose-dependent manner at 4 hours in A549 cells (Fig. 7A). Brusatol decreased the protein levels of Nrf2 and GCLC but had less effect on NQO1 and GCLM when A549 cells were treated for 24 hours (Fig. 7B). Brusatol reduced 20% and 45% of cell viability at 20 nM and 40 nM in A549 cells (Fig. 7C). Brusatol did not increase cleavage of caspase-3 and caspase-3 activity in A549 cells (Fig. 7D & E). These data suggest that inhibition of Nrf2 by Brusatol may induce caspase-3 independent cell death and/or cell growth inhibition in A549 cells.

3.7 Pharmacological inhibition of Nrf2 or simultaneous inhibition of Nrf2 and autophagy does not improve the sensitivity of A549 to EGFR-TKI treatment

To examine whether inhibition of Nrf2 activation would sensitize A549 cells to Ico or Gef treatment, we determined cell morphology and nuclear changes as well as cell viability after

the cells were treated with Ico or Gef with or without Brusatol. Apoptotic cells normally display fragmented or condensed nuclei by Hoechst 33342 staining whereas necrotic or secondary necrotic cells (late apoptosis) have PI positive stained nuclei. As a positive control, HCC827 cells were treated with Ico for 24 hours. We found that there were a number of HCC827 cells detached and round up, which accompanied with fragmented nuclei Hoechst 33342 staining. In contrast, Ico, Brusatol or Ico with Brusatol did not cause any significant morphological changes in A549 cells (Fig. 8). Ico or Gef treatment alone caused only around 2~4% apoptosis in A549 cells (Fig. 9A). Brusatol alone treatment induced around 7% apoptosis in A549 cells but it did reach statistical significance. The combination of Ico or Gef with Brusatol did not further increase the number of apoptotic cells compared with Ico or Gef alone treatment in A549 cells. The percentages of PI positive cells were all below 5% in Ico-, Gef- or Brusatol-treated A549 cells (Fig. 9B). These data suggest that apoptosis as well as secondary necrosis or necrosis may not be critical for cell death induced by these treatments in A549 cells. Ico or Gef treatment did not increase the cleavage of caspase-3 and caspase-3 activity in A549 cells, and the combination of Ico or Gef with Brusatol did not further increase caspase-3 activation in A549 cells (Fig. 9C & D). These results suggest that the combination of EGFR-TKI and Brusatol does not have further beneficial effects in enhancing apoptosis in A549 cells compared to the use of either of these agents alone. Therefore, we next determined the effects of the combination of EGFR-TKI and Brusatol (Nrf2 inhibitor) and CQ (autophagy inhibitor) on cell viability/growth in A549 cells. While Ico alone treatment had no effect on cell viability/growth in A549 cells, Brusatol alone decreased the cell viability/growth in A549 cells for about 30% compared with control cells. The combination of Ico with Brusatol or Ico with Brusatol and CQ together did not further change the cell viability/growth in A549 cells (Fig. 9E). These results indicate that inhibition of Nrf2 alone by Brusatol reduces the cell viability/growth independent of EGFR-TKI sensitivity. However, the combination of Brusatol with EGFR-TKI and an autophagy inhibitor (CQ) does not have additional beneficial effects on the reduction of cell viability/growth compared to the use of Brusatol alone in A549 cells.

4 Discussion

EGFR is a receptor tyrosine kinase belonging to the ErbB family, which is abnormally activated in many human malignancies including NSCLC. Increasing evidence shows that EGFR-TKI (such as erlotinib and gefitinib) are effective in NSCLC tumors that have activating mutations in EGFR but are not effective in tumors with wild type EGFR (Paez, Janne et al. 2004; Mendelsohn and Baselga 2006). However, it is not clear whether other mechanism would also contribute to the resistance of wild-type EGFR NSCLC tumors in addition to the status of EGFR.

Increasing evidence suggests that a variety of mechanisms may be responsible for the development of drug resistance in NSCLC in addition to EGFR. One of the mechanisms is autophagy, which has been shown to be a cell protective mechanism for both cancer and normal cells against various stress conditions and chemotherapeutic drugs. Autophagy acts as an adaptive mechanism to remove damaged mitochondria via mitophagy, relieve ER (endoplasmic reticulum) stress by removing misfolded proteins and provide nutrients for cancer cells against chemotherapeutic drug-induced cell death (Ding, Ni et al. 2007;

Amaravadi, Lippincott-Schwartz et al. 2011). Indeed, it has been reported that EGFR-TKI induces autophagy in various cancer cells *in vitro* including NSCLC (Fung, Chen et al. 2012; Wei, Zou et al. 2013; Tang, Wu et al. 2015). EGFR may regulate autophagy in cancer cells through EGFR kinase-dependent and independent mechanisms. Wei *et al* (Wei, Zou et al. 2013) reported that kinase-active EGFR inhibits autophagy in NSCLC. Kinase-active EGFR can directly interact with Beclin1 resulting in increased phosphorylation of multiple tyrosine sites on Beclin 1, which leads to decreased Beclin-1 associated VPS34 kinase activity. EGFR-TKI treatment inhibits the tyrosine phosphorylation of Beclin 1 and in turn induces autophagy in NSCLC (Wei, Zou et al. 2013). In contrast, kinase-inactive EGFR has been reported to activate autophagy by physically interacting with the oncoprotein LAPT4B, which stabilizes both kinase-inactive EGFR and LAPT4B at endosomes to further recruit Sec5, an exocyst subunit. The EGFR-LAPT4B-Sec5 complex disassociates Beclin 1 from its negative regulator Rubicon to initiate autophagy (Tan, Thapa et al. 2015). Therefore, it is possible that EGFR-TKI block EGFR kinase signaling, but they may still activate autophagy through an inactive EGFR pathway, which could potentially provide a survival advantage and TKI resistance in wild type EGFR NSCLC. In addition to the Beclin 1-VPS34 complex, mTOR is another well-known player in regulating autophagy. In the presence of nutrients and growth factors, mTOR is activated and negatively regulates autophagy by directly phosphorylating serine757 on the Unc-51 like kinase 1 (ULK1, the yeast Atg1 homolog), the core protein of the upstream autophagy regulating complex that is composed of ULK1, FIP200, Atg13 and Atg101 (Mizushima 2010). Increased serine757 phosphorylation decreases ULK1 kinase activity and in turn inhibits autophagy (Kim, Kim et al. 2013). In addition to EGFR, the PI3K/Akt pathway is also often aberrantly activated in various cancers including lung cancer (Brognard, Clark et al. 2001; Osaki, Oshimura et al. 2004). Interestingly, Akt also negatively regulates autophagy by either directly activating mTOR or inhibiting the Beclin1-VPS34 complex through AKT-mediated phosphorylation of TSC2 or Beclin 1, respectively (Inoki, Li et al. 2002; Wang, Wei et al. 2012). We found that EGFR-TKI (Ico and Gef) differentially regulate autophagy in EGFR wild-type A549 cells and EGFR mutant HCC827 cells. Specifically, EGFR-TKI increased autophagic flux in HCC827 but not in A549 cells. To our surprise, inhibition of autophagy by CQ failed to sensitize either HCC827 (data not shown) or A549 cells to EGFR-TKI-induced cell death or cell growth inhibition. This is in contrast to the general notion that autophagy is a cell survival mechanism in cancer cells including lung cancer cells. Our results are also not in agreement with a recent report that CQ enhanced Gef-induced cell growth in Gef-resistant PC9 NSCLC cells that also have EGFR mutations (Tang, Wu et al. 2015). The difference between our findings and Tang *et al* (Tang, Wu et al. 2015) could be due to the different NSCLC cells we used. However, it should be noted that inhibition of autophagy by CQ only reduced 20% cell survival/viability in the Gef-resistant PC9 cells in the report by Tang *et al* (Tang, Wu et al. 2015), indicating that the improvement of CQ on the resistance of NSCLCs is limited. Moreover, NSCLC tumors that carry a tyrosine phosphomimetic Beclin 1 mutation leading to reduced autophagy are resistant to EGFR-TKI treatment (Wei, Zou et al. 2013). These observations suggest that reduction of autophagy may not necessarily always sensitize NSCLC cells to EGFR-TKI, which is in agreement with our findings in the present study. Therefore, the role of autophagy in NSCLC seems complex, and further studies are required in order to modulate autophagy for treating drug-resistant NSCLC.

In addition to autophagy, increasing evidence supports that Nrf2 activation also contributes to tumorigenesis and cancer drug resistance including in NSCLC (Hayes and McMahon 2009; Jaramillo and Zhang 2013; Ni, Woolbright et al. 2014). Nrf2 is a key transcription factor that regulates the gene expression for critical antioxidants and drug metabolism enzymes. Emerging evidence suggests that Nrf2 may have a dual role in cancer (Jaramillo and Zhang 2013). On one hand, Nrf2 functions as a tumor suppressor since Nrf2 knockout mice are more susceptible to chemical carcinogen-induced tumor formation (Ramos-Gomez, Kwak et al. 2001). This anti-tumor activity of Nrf2 (“the good side”) is likely due to its antioxidant function, which protects cells from oxidative stress-mediated DNA damage and cell transformation. On the other hand, persistent activation of Nrf2 has been shown to promote liver tumorigenesis (Ni, Woolbright et al. 2014). Moreover, somatic mutations of Nrf2 and Keap1 have been identified in many human cancers including NSCLC resulting in persistent activation of Nrf2 in these cancer cells (Hayes and McMahon 2009; Jaramillo and Zhang 2013). The pro-tumor activity of Nrf2 (“the dark side”) is likely due to the ability of transformed tumor cells to utilize the Nrf2 antioxidant function to protect themselves against oxidative stress-induced damage. Therefore, it is likely that at the early stage of tumorigenesis, activation of Nrf2 may be beneficial for preventing tumorigenesis. However, once the tumor is formed, inhibition of Nrf2 may be beneficial to make these cells more susceptible to chemotherapeutic drugs. Loss-of-function mutations in Keap1 have been identified in A549 cells (Hayes and McMahon 2009), and in the present study we confirmed that A549 cells indeed have persistent high Nrf2 activation compared with HCC827 cells. This is likely one of the mechanisms contributing to EGFR-TKI resistance in A549 cells in addition to their wild-type EGFR status. Indeed, pharmacological inhibition of Nrf2 by Brusatol inhibited up to 40% of cell growth in A549 cells, which further supports the notion that NSCLC cells can use Nrf2 as a cell survival mechanism. The cell survival pathway of Nrf2 seems to overlap with the EGFR as well as the autophagy pathway because simultaneously blocking these three pathways did not show additional beneficial effects compared to the inhibition of EGFR or Nrf2 alone. However, it has been reported that the Nrf2 inhibition effects of Brusatol may be transient and reversible after prolonged treatment (Ren, Villeneuve et al. 2011; Olayanju, Copple et al. 2015). Therefore, while our studies to modulate Nrf2 and autophagy using Brusatol and CQ did not overcome the resistance of A549 cells to EGFR-TKI, future studies to identify more potent Nrf2 and autophagy inhibitors may be helpful to improve drug resistance of A549 cells. In conclusion, our studies indicate that the drug resistance of NSCLC is very complex and involves multiple signaling pathways that include EGFR, Nrf2 and autophagy. To improve EGFR-TKI resistance in NSCLC patients, future research may be focused on the identification of new drugs to target Nrf2 (more potent than Brusatol) and autophagy (more potent and specific than CQ), or new strategies for the combination of Nrf2 inhibition with other chemotherapeutic drugs.

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Abbreviations

CQ	Chloroquine
EGFR	Epidermal growth factor receptor
EM	electron microscopy
Gef	Gefitinib
Ico	Icotinib
Keap1	Kelch-Like ECH-Associated Protein 1
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NSCLC	Non-small cell lung cancer
Nrf2	NF-E2-related factor 2
TKI	tyrosine kinase inhibitors
ULK1	Unc-51 like kinase 1

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Highlights

- Cancer cells use adaptive mechanisms against chemotherapy.
- Autophagy is not essential for the drug resistance of lung cancer A549 cells.
- Inhibition of Nrf2 decreases cell survival of lung cancer A549 cells.

Figure 1. A549 cells are resistant to EGFR-TKI-induced apoptosis

A549 or HCC827 cells were incubated with Gef (1 μM) or Ico (1 μM) for various time points (A) or different concentrations of Gef or Ico for 24 hours (B) followed by MTT assay. Data were expressed as mean ± SEM from at least three independent experiments. ** p<0.01, Ico or Gef group vs control groups (One way anova analysis). Total cell lysates were subjected to western blot analysis for cleaved caspase-3 (C & D) and (E) caspase-3 activity. Data were expressed as mean ± SEM. The results were from at least three independent experiments. * p<0.05, **p<0.01 Ico or Gef group vs control groups; # p<0.05, ## <0.01, HCC827 cells vs A549 cells (One way anova analysis).

Figure 2. EGFR-TKI induces autophagic flux in HCC827 but not in A549 cells

A549 and HCC827 cells were treated with Ico or Gef at the indicated doses for 6 hours. (A) Total cell lysates were subjected to western blot analysis with the indicated antibodies. (B) A549 and HCC827 cells were treated with Ico (1 μM) and Gef (1 μM) with or without CQ (20 μM) for 6 hrs. Total cell lysates were subjected to immunoblot with the indicated antibodies. Densitometry analysis was conducted and data are presented underneath the blots. Data were expressed as fold of control (mean ± SEM) from at least three independent experiments.

Figure 3. EM study of the formation of autophagic vacuoles in A549 and HCC827 with or without EGFR-TKI treatment

A549 and HCC827 cells were treated with vehicle control DMSO (control) or Gef (1 μM) or Ico (1 μM) for 6 hours. Then cells were fixed and further processed for EM analysis. **(A)** Representative EM photographs are shown. Arrows denote autophagic vacuoles (AV, autophagosomes and autolysosomes). N: Nucleus. Bar: 500 nm. **(B)** The number of autophagic vacuoles (AV, including both autophagosomes and autolysosomes) per 100 μm^2 cytosol was quantified. Data are presented as means $\pm$ SE (more than 15 cell sections). # $p < 0.05$, HCC827 vs A549 cells (Student t test).

Figure 4. Pharmacological inhibition of autophagy does not affect Ico or Gef-induced cell death in A549 cells

A549 cells were incubated with Ico (1 μ M, **A**) or Gef (1 μ M, **B**) with or without CQ 20 μ M for 24 hours. Cell viability was determined by MTT assay. Data were expressed as mean $\pm$ SEM from at least three independent experiments. Total cell lysates were subjected to western blot analysis for cleaved caspase-3 (**C & D**). Lysates from Gef (1 μ M)-treated HCC827 cells were used as a positive control.

Figure 5. Gef or Ico does not inhibit mTOR in A549 cells

(A) A549 cells were treated with Gef (1 μ M) or Ico (1 μ M) for the indicated time. Total cell lysates were subjected to western blot analysis with the indicated antibodies. Representative blots from three independent experiments are shown. (B) A549 cells were split and cultured overtime for attachment in full culture medium. Cell lysates were prepared from the indicated culture time points and subjected to western blot analysis with the indicated antibodies. Representative blots from three independent experiments are shown.

Figure 6. Persistent Nrf2 activation in A549 cells

(A) A549 and HCC827 cells were treated with Gef (1 μ M) or Ico (1 μ M) for 6 hours. Real-time quantitative PCR was conducted and data were expressed as fold change to HCC827 control group. Data were presented as mean $\pm$ SEM from at least three independent experiments. * p <0.05. A549 vs HCC827. ** p <0.01 Gef vs control (HCC827, One way anova analysis). (B) A549 and HCC827 cells were treated with various concentrations of Ico (1 μ M) or Gef for 6 hours and total cell lysates were subjected to western blot analysis. Representative blots from 3 independent experiments are shown.

Figure 7. Brusatol inhibits Nrf2 and reduces cell growth/viability in A549 cells

A549 cells were treated with different concentrations of Brusatol for 4 hours. (A) Total cell lysates were subjected to western blot analysis. Representative blots from 3 independent experiments are shown. (B) A549 cells were treated with different concentrations of Brusatol for 24 hours. Total cell lysates were subjected to western blot analysis. Representative blots from 3 independent experiments are shown. (C) Cell viability was determined by MTT assay. Data were expressed as mean $\pm$ SEM from at least three independent experiments. # $p < 0.05$; ## $p < 0.01$ A549 Brusatol vs control (One way anova analysis). (D). Total cell lysates were subjected to western blot analysis for cleaved caspase-3. PC (positive control): lysates from Gef (1 μ M)-treated HCC827 cells were used as a positive control. (E) Caspase-3 activity was measured using a specific z-DEVD-AMC substrate. Data were expressed as mean $\pm$ SEM from at least three independent experiments.

Figure 8. Cellular morphological changes of A549 cells treated with Ico and Brusatol

A549 cells were pre-treated with 40 nM Brusatol for 3 hours. Then the cells were treated with Ico (1 μ M) for another 24 hours. The cells were stained with Hoechst 33342 (1 μ g/mL) and PI (1 μ g/mL) followed by fluorescence microscopy. Representative phase-contrast and Hoechst 33342 and PI staining images are shown from three independent experiments. HCC827 cells (treated with Ico (1 μ M) for 24 hours) were used as positive controls for cellular morphological changes. The boxed areas are enlarged photographs denoting apoptotic nuclei.

Figure 9. Effects of Brusatol, CQ and EGFR-TKI on cell viability in A549 cells

A549 cells were treated as in Figure 8. Apoptotic (A) and PI positive cells (B) were counted. At least four different fields were counted in each experiment from a total of three independent experiments. Data were expressed as mean $\pm$ SEM. Total cell lysates were subjected to western blot analysis (C) and caspase-3 activity assay (D). PC (positive control): lysates from Gef (1 μ M)-treated HCC827 cells were used as a positive control. Data were expressed as mean $\pm$ SEM from at least three independent experiments. (E) A549 cells were treated as in (A) with or without CQ (20 μ M) for 24 hours followed by MTT assay. Data were expressed as mean $\pm$ SEM from at least three independent experiments. # $p < 0.05$; ## $p < 0.01$ A549 treatment groups vs control (One way anova analysis).