Inducible EGFR T790M-Mediated Gefitinib Resistance in Non-small Cell Lung Cancer Cells Does Not Modulate Sensitivity to PI103 Provoked Autophagy

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Introduction: Non-small cell lung cancer (NSCLC) with certain activating mutations in the epidermal growth factor receptor (EGFR) is sensitive to the small molecule EGFR tyrosine kinase inhibitors gefitinib and erlotinib, although acquired resistance eventually develops. Resistance is often mediated by acquisition of the T790M mutation in the activated *EGFR* allele. The aim of this study was to investigate in an EGFR tyrosine kinase inhibitor sensitive NSCLC cell line model, the impact of induced *EGFR* T790M expression on the cell biology and sensitivity to novel therapeutic strategies.

Methods: Doxycycline inducible EGFR T790M-mediated drug resistance was generated in the clinically relevant HCC827 NSCLC cell line. Cell fate, the activities of EGFR and downstream signaling molecules, and the sensitivity to downstream inhibition of EGFR signaling networks were examined in the presence or absence of induced EGFR T790M expression.

Results: Inducible EGFR T790M expression generated acquired resistance to EGFR inhibitors in HCC827 cells as expected. However, induced EGFR T790M expression did not affect activity of EGFR downstream signaling pathways or cell proliferation under the conditions tested. Moreover, sensitivity to inhibition of signaling molecules downstream of EGFR was unaffected by induced EGFR T790M. Importantly, HCC827 cells remained sensitive to class I phosphatidyl-inositol-3-kinase and mammalian target of rapamycin inhibition, which provoked pronounced autophagy, without significant apoptosis.

Conclusions: Phosphatidyl-inositol-3-kinase /mammalian target of rapamycin inhibition is a potentially effective therapeutic strategy against NSCLC with acquired resistance to EGFR inhibition. However, the implications of drug-induced autophagy in NSCLC need further exploration.

Key Words: NSCLC, EGFR T790M, PI-3K/mTOR inhibition, Autophagy

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Epidermal growth factor receptor (EGFR) activity is frequently deregulated in solid tumors affecting signaling networks important for tumor formation, maintenance, and development. This is mediated through activation of downstream signaling components including protein kinase B (PKB or Akt)/phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK)/ERK kinase (MEK).[1] Patients with non-small cell lung cancer (NSCLC) whose tumors harbor somatic and activation mutations in EGFR exons 18–21 show dramatic clinical responses to reversible EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib.[2–4] Most prevalent of these are a single amino acid substitution L858R and short in-frame deletions in exon 19.[5] These mutations are more common in females with adenocarcinoma classed as "never-smokers."[5]

Despite initial response, patients with NSCLC with activating EGFR mutations develop resistance to EGFR TKIs.[6] Approximately half of acquired resistance cases are accounted for by acquisition of an EGFR T790M mutation, which occurs in *cis* with the exons 18–21 activating mutation.[7,8] MET amplification is the second most prevalent event identified in this patient population,[9,10] and rare EGFR mutations L747S, D761Y, and T854A also decrease sensitivity to gefitinib and erlotinib.[11–13]

EGFR T790M mutation mediated resistance to gefitinib and erlotinib has been confirmed in vitro and in mouse models.[14–18] The target site for EGFR TKIs is the T790 residue in EGFR located at the back of the adenosine triphosphate (ATP) binding cleft. Mutation of this so-called "gatekeeper" residue to a bulkier methionine was believed to sterically hinder TKI binding.[7] However, in vitro, T790M mutation increases affinity of activated EGFR mutants to ATP, while having a modest negative effect on drug affinity for EGFR.[19] These results implicate

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competitive inhibition in favor of ATP (rather than steric hindrance) as the major contributor to T790M-mediated drug resistance.[19]

Rare familial cases of lung cancer are associated with germline T790M mutation in EGFR,[20] and introduction of T790M mutation into wild-type EGFR provided modest increase in catalytic activity[19] and enhanced its oncogenic potential.[18] In contrast, introduction of T790M mutation on activated (L858R) EGFR decreased catalytic activity.[19] Despite these results, when constitutively or transiently expressed in model cell lines, T790M-containing double mutants of EGFR display enhanced oncogenic properties compared with single mutants.[16,21,22] Although these effects should be mediated by enhanced activation of downstream signaling pathways, this was not observed in all cell lines.[16,23]

Although cells expressing double mutants with EGFR T790M are resistant to reversible EGFR TKIs, they retain sensitivity to high concentrations of irreversible EGFR inhibitors.[24] The latter demonstrated limited activity in mouse models of EGFR T790M-expressing lung cancer and against clinical NSCLC with acquired resistance to gefitinib or erlotinib.[17,25] Combination of the irreversible EGFR inhibitor HKI-272 with mammalian target of rapamycin (mTOR) inhibitor rapamycin or Hsp90 inhibition has shown promise in preclinical models against T790M-mediated drug resistance.[15,17,23]

Another probable route to overcoming EGFR T790M-mediated drug resistance would be direct pharmacological inhibition of PI3K or MEK, i.e., vertical attack on the EGFR pathway. MEK inhibition induces changes in the proapoptotic protein BIM_{EL} identical to those observed during gefitinib/erlotinib induced apoptosis in NSCLC cells sensitive to these agents.[12,26,27] However, NSCLC cells expressing activated EGFR mutants were insensitive to MEK inhibitor PD0325901.[26–28]

Class I PI3Ks are also activated downstream of ligand-bound EGFR, resulting in activation of PKB/Akt and mTOR.[29] In contrast to MEK inhibition, treatment with the pan-PI3K inhibitors LY294002 and wortmannin did not elicit apoptosis-associated changes in BIM_{EL} in NSCLC cells sensitive to EGFR TKIs. This was perhaps surprising, because inhibition of PI3K/Akt signaling has been correlated with decreased cell survival.[30] However, this concept relies mainly on experiments using PI3K inhibitors with relatively broad selectivity. Recently, inhibitors targeted more specifically to different classes of PI3K have been developed. One example, PI103 inhibits class I PI3K and mTOR and showed promising preclinical activity against malignant glioma.[31,32]

Treatment of different cancer cell lines with PI103 does not always result in apoptosis but often induces cell cycle arrest.[31,32] PI103 was also shown to induce autophagy in U87MG and PC3 cancer cell lines.[33] Autophagy, ("self-eating" stimulated by unfavorable microenvironments), is negatively regulated by Class I PI3Ks, through activation of Akt and mTOR.[34] Although a cell survival role for autophagy has been established, autophagy-dependent cell death has also been described as "type II programmed cell death."[35] Once autophagy is initiated, a subsequent block of late stages in the process might drive cells into apoptosis.[33]

To study EGFR T790M activity and drug sensitivity in a clinically relevant cell line, inducible expression of an activated EGFR mutant containing the T790M mutation was generated in EGFR TKI sensitive HCC827 NSCLC cells. This is, to our knowledge, the first report of inducible expression of EGFR T790M in a relevant cell line. Inducible systems have the advantage of being truly isogenic, allowing specific effects of the expressed gene to be dissected from underlying genetic differences between control and expressing cells. The system of inducible resistance to EGFR TKIs described here was used to study the impact of EGFR T790M expression on cell proliferation, the activation of signaling pathways downstream of EGFR, and the resultant sensitivity to inhibition of MEK, PI3K, and mTOR. The ability of PI3K and mTOR inhibition to induce cytostasis, apoptosis, or autophagy in this cellular context was evaluated, and the implications of autophagy induction in the clinic will be discussed.

MATERIALS AND METHODS

Drugs and Antibodies

Gefitinib (Iressa) was obtained from AstraZeneca (Alderley Park, UK). PI103, U0126, PD98059, and CL-387785 were from Calbiochem. All drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Doxycycline (Invitrogen, Paisly, UK) was dissolved in H_2O, stored at −20°C, and used at 100 ng/ml unless stated otherwise. Antibodies against the following proteins were from cell signaling: Akt, phospho-Ser-473-Akt, phospho-EGFR (all phosphorylated residues), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204); p70 S6-kinase and phospho-Thr389-p70 S6 kinase and poly ADP ribose polymerase (PARP). The following antibodies were sourced as follows; EGFR (Santa Cruz, Santa Cruz, CA), BIM (Calbiochem, Darmstadt, Germany), LC3 (Nanotools, Hamburg, Germany), actin and FLAG-tag (Sigma, St. Louis, MO), Src (Upstate, Waltham, MA), and Phospho-Tyr529-Src and phospho-tyr418-Src (Invitrogen).

DNA Constructs

The *EGFR* coding sequence was amplified from the pBSII-SK-EGFR plasmid kindly provided by Dr. William Pao (MSKCC, New York, NY). EGFR coding sequences were inserted into pTre2hyg (Clontech, Palo Alto, CA) using the *Nhe*I and *Not*I cloning sites to produce pTre2hyg-EGFR-Δ746-750-FLAG (control) or pTre2hyg-EGFR-Δ746-750-T790M-FLAG. To insert the *Nhe*I site at the 5′ of the *EGFR* coding sequence, the following forward primer was used: 5′-GCA ATG GCT AGC ACC ATG CGA CCC TCC GGG ACG-3′. A FLAG-tag (DYKDHD) followed by a *Not*I site were inserted at the 3′ end of the *EGFR* coding sequence using the following primer: 5′-GCA ATG GCG GCC GCC TAC TTG TCA TCG TCA TCC TTG TAA TCA CCT GCT CCA ATA AAT TC 3′.