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Inhibition of IGF1R Signaling Abrogates Resistance to Afatinib (BIBW2992) in EGFR T790M Mutant Lung Cancer Cells

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Abstract

Non-small cell lung cancer (NSCLC) patients with an epidermal growth factor receptor (EGFR) mutation have benefited from treatment of reversible EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib. Acquisition of a secondary mutation in EGFR T790M is the most common mechanism of resistance to first generation EGFR TKIs, resulting in therapeutic failure. Afatinib is a second generation of EGFR TKI that showed great efficacy against tumors bearing the EGFR T790M mutation, but it failed to show the improvement on overall survival of lung cancer patients with EGFR mutations possibly because of novel acquired resistance mechanisms. Currently, there are no therapeutic options available for lung cancer patients who develop acquired resistance to afatinib. To identify novel resistance mechanism(s) to afatinib, we developed afatinib resistant cell lines from a parental human-derived NSCLC cell line, H1975, harboring both EGFR L858R and T790M mutations. We found that activation of the insulin-like growth factor 1 receptor (IGF1R) signaling pathway contributes to afatinib resistance in NSCLC cells harboring the T790M mutation. IGF1R knockdown not only significantly sensitizes resistant cells to afatinib, but also induces apoptosis in afatinib resistance cells. In addition, combination treatment with afatinib and linsitinib shows more than additive effects on tumor growth in *in vivo* H1975 xenograft. Therefore, these findings suggest that IGF1R inhibition or combination of EGFR-IGF1R inhibition strategies would be potential ways to prevent or potentiate the effects of current therapeutic options to lung cancer patients demonstrating resistance to either first or second generation EGFR TKIs.

Keywords

Afatinib; EGFR; IGF1R; Lung cancer; Cellular response to anticancer drugs; Reversal of drug resistance

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Introduction

Non-small lung cancer (NSCLC) is the most common type of lung cancer in the United States [1]. Epidermal growth factor receptor (EGFR) mutation, one of the most frequent events associated with NSCLC, occurs in about 20% of all NSCLC cases in the United States [2,3]. EGFR is a 170 kDa transmembrane receptor tyrosine kinases (RTKs) [4]. Activating mutations in EGFR can lead to abnormal metabolism, increased cell proliferation and cell survival through activation of the downstream mitogen-activated protein kinase (MAPK) and v-akt murine thymoma viral oncogene homologue 1 (AKT) signaling pathways [5–9]. The most common EGFR mutations among NSCLC patients are EGFR exon 19 deletion (48%) and EGFR L858R mutation (43%) [10]. NSCLC patients with EGFR mutations have benefited significantly from first generation of EGFR TKIs such as gefitinib and erlotinib, but the vast majority of patients treated with these drugs eventually develop resistant to EGFR TKIs [8,11–13]. EGFR-T790M mutation is the most common acquired resistance mechanism to first generation of EGFR TKIs, and has been detected in about 50% of NSCLC patients with reported first generation EGFR TKIs resistance [8,12]. Recently, a second generation irreversible EGFR TKI, afatinib has been approved by the U.S. Food and Drug Administration (FDA) as a first-line therapy for NSCLC patients with EGFR exon 19 deletion or L858R mutations [14,15]. Afatinib is an irreversible EGFR TKI with strong affinity to the EGFR T790M mutation. However, clinical trials have revealed little difference in overall survival between patients treated with afatinib versus other first generation EGFR TKIs [14,16,17]. Given the nearly universal acquisition of resistance to EGFR TKIs, a broader approach is necessary to manage the resistance phenomenon despite enhancement in binding affinity and inhibitor activity over successive TKI generations [18,19].

Clinical data indicates that expression of EGFR and IGF1R are significantly correlated in a subset of lung cancers, and that co-expression of high levels of both EGFR and IGF1R is associated with significantly poor prognosis [20]. Both EGFR and IGF1R can both mediate down-stream MAPK and AKT signaling independently of one another or in concert, because of the existence of EGFR and IGF1R cross talk [21]. IGF1R has been demonstrated to have a role in gefitinib resistance in cancers including lung cancers in the absence of the EGFR T790M mutation [22,23]. However, the role of IGF1R signaling in the acquisition of resistance to irreversible EGFR TKIs, which have activity against the EGFR T790M mutation, has not been explored to date. Given the existence of EGFR and IGF1R compensatory signaling [24,25], we hypothesized that IGF1R signaling may contribute to acquired resistance to afatinib in lung cancers harboring the EGFR T790M mutation.

Here, we report that IGF1R signaling contributes to the acquired resistance to afatinib in lung cancers with pre-existing EGFR T790M mutation. IGF1R inhibition via either a small molecule inhibitor or shRNA mediated knockdown alone was sufficient to sensitize afatinib resistance. Also, concurrent inhibition of both IGF1R and EGFR overcome the resistance to afatinib in EGFR mutant lung cancers both *in vitro* and *in vivo*. Therefore, targeting multiple receptors signaling pathways represent a potential strategy to circumvent the development of resistance, and could result in significant synergies that could circumvent or prevent resistance to afatinib beyond EGFR T790M-driven resistance.

Materials and Methods

Cell culture and reagents

H1975 cells harboring EGFR L858R and T790M mutation were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). PC9 cells (EGFR exon 19del) were obtained from Dr. Joan Massague (Memorial Sloan Kettering Cancer Center, New York, NY) and H3255 cells (EGFR L858R) were obtained from NCI (National Cancer Institute, Bethesda, MD). PC9, H3255, H1975, and AR (afatinib resistant) cells were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) in a 37°C humidified 5% CO₂ incubator. Afatinib and linsitinib was purchased from LC laboratories (Massachusetts, USA).

Generation of afatinib resistance (AR) H1975 cells

Generation of resistance cells were developed as previously described [26,27]. H1975 cells were initially cultured with low concentration of afatinib (5 nM) and continuously cultured in increasing concentration of afatinib up to 1 μ M over three months. H1975 cells were considered as afatinib resistant when cells were normally grown in the presence of afatinib (1 μ M).

IGF1R Knockdown

The empty vector plasmid with and shRNA targeting endogenous *IGF1R* transcript were packaged in 293T cells (Orbigen, San Diego, CA) by transfection with helper plasmids using Lipofectamine LTX (Life Technologies, Carlsbad, CA). H1975 and H1975 afatinib resistance cells were transduced with lentiviral particles with 8 μ g/mL Polybrene (Sigma, St. Louis, MO). Media was replaced 24 hours after transduction, and cells were selected with 2 μ g/mL puromycin (Life Technologies, Carlsbad, CA) for 3 days.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cell lines using Tri-zol reagent and protocols (Life Technologies, Carlsbad, CA). Quantitative real-time PCR (qPCR) was conducted using the method as described previously [28]. Briefly, one microgram of total RNA per sample was converted to cDNA using the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). qPCR assay was done using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). 1 μ L of cDNA was added to a 25 μ L reaction mixture containing PCR master Mix. Primers used: human EGFR (Forward, 5'-TCG CAC TTC TTA CAC TTC CGG-3' and Reverse, 5'-CCC ACT CAR GCT CTA CAA CCC-3'), human IGF1R (Forward, 5'-TGA AAG TGA CGT CCT GCA ATC-3' and Reverse, 5'-GGT ACC GGT GCC AGG TTA C-3'), (Actin Forward, 5'-GGA CGA CAT GGA GAA AAT CT-3' and Reverse, 5'-GGA TAG CAC AGC CTG GAT AG-3'). The fold change of gene expression in tumor tissues as compared to the paired normal tissues was calculated as 2^d , where $d = C_{T \text{ normal}} - C_{T \text{ tumor}}$. P-values were determined using a two-tailed Student's T-Test.

Western Blotting

Cells were lysed with 200 μ L of 1X NP40 lysis buffer containing proteinase inhibitor cocktails (Fisher Scientific, Pittsburg, PA), sheared 10 times with a 28 gauge needle, spun at $16,000 \times g$ for 30 minutes, normalized by protein concentration as determined by the Bradford method, and boiled for 5 min. Normalized lysate was resolved by 4-12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, CA) and immunoblotting with indicated antibodies. The following antibodies were used: rabbit phospho-EGFR(#3777s), EGFR(#4267s), phospho-IGF1R(#3021s), IGF1R (#9750s), phospho-AKT (#4060s), AKT (#9272s), phospho-ERK1/2 (#4370s), ERK1/2 (#9012s) (Cell Signaling Technology, Danvers, MA), rabbit phospho-IGF1R(#bs-54471R) (Bioss, Woburn, MA) and mouse anti-Actin (sc-81178) (Santa Cruz Biotechnology, Santa Cruz, CA).

Kinetic growth and Caspase3/7 apoptosis assays

Cells were harvested by trypsinization, counted on a Countess automated cell counter (Life Technologies, Carlsbad, CA) and plated at 2,000 cells per well on 96 tissue culture plates in 3 replicates. Cells were treated with the indicated reagents after cells were attached to the plate at 24 hours post initial incubation. Photomicrographs were taken every two hour using an Incucyte live cell imager (Essen Biosciences, Ann Arbor, MI). Confluence and caspase3/7 apoptosis activity were measured using Incucyte software (Essen Biosciences, Ann Arbor, MI) over 3 days in culture. Apoptotic index was measured by dividing the number of caspase 3/7 positive cells as measured by fluorescence of caspase 3/7 substrate by total number of cells as measured using Vybrant Green DyeCyle (Life Technologies, Carlsbad, CA) at 3 days post indicated treatment. Data were analyzed using Incucyte software (Essen Biosciences, Ann Arbor, MI).

Immunohistochemical (IHC) analysis

The protein expression levels of Ki-67, p-IGF1R and cleaved caspase-3 were measured by IHC analysis, as described previously [29] and the index was assessed by counting positively stained cells in each 40 $\times$ field in the densest area of each tumor section. The number of positively-stained cells per total stained area represented an index of Ki67, p-IGF1R and cleaved caspase-3 index.

H1975 *In vivo* Xenograft

H1975 *in vivo* xenograft study was performed as described previously [30]. Five million cells suspended in 200 μ L PBS (phosphate-buffered saline) with 50% Matrigel (BD Biosciences) was subcutaneously injected into 8 week old nu/nu athymic mice (Charles River). Once tumor volumes reached 150 mm³, mice were randomly placed into 4 treatment groups: vehicle control (0.5% (w/v) methylcellulose containing 0.4% Tween 80) [31], afatinib (5 mg/kg), linsitinib (20 mg/kg) and a combination of afatinib (5 mg/kg), linsitinib (20 mg/kg). Each reagent was given daily by oral gavage for 15 days. Mice were weighed and tumor volumes were measured with calipers in every 2 days. The average tumor volume in each group was calculated as previously described [27]. At the end point, mice were euthanized by CO₂ asphyxiation and tumor tissues from xenograft mice were fixed in zinc formalin solution overnight and stored in 70% ethanol for histopathology evaluation.

Statistical analysis

A two-tailed Student's T-test was used to evaluate the differences between the control samples and the treated samples. * $P < 0.05$ and ** $P < 0.005$ was considered statistically significant.

Results

Establishment afatinib-resistant lung cancer cells harboring EGFR T790M mutation

In vitro tissue culture systems have been used extensively to study the mechanisms underlying EGFR TKI resistance against first generation inhibitors [32,33]. In order to identify the putative resistance mechanisms that may drive the clinical resistance phenotype observed with second generation EGFR TKI inhibitor, such as afatinib, we used the H1975 cell line harboring both an EGFR L858R activation mutation and a T790M gatekeeper mutation. H1975 cells were initially grown in the presence of a low concentration of afatinib (5nM), with an escalating dose of afatinib overtime, culminating in a final dose of 1 μ M (Fig 1A). The pooled population of H1975 cells were selected in the presence of afatinib over three months and considered resistant when they were grow similar to the control H1975 cells in the presence of 1 μ M of afatinib as previously demonstrated [32–34]. Independent pooled populations of afatinib resistant cells, AR1, AR2, and AR3 were passaged over two months under drug free conditions. The growth rate of both H1975 parental cells AR1, AR2, and AR3 were measured as described in the material and methods. The growth rates were not significantly different between H1975 and AR1, AR2 and AR3 (Fig 1B), which indicates that resistance mechanism to afatinib may affect on other cellular phenotype such as cell survival, migration, invasion or metastasis. Both H1975 parental and AR cells then were subjected to the growth assay in the presence of various doses of afatinib. H1975 parental cells were sensitive to afatinib whereas the growth rate of AR1, AR2 and AR3 cells were not sensitive to afatinib (Fig 1C). These data suggest that AR cells may depend on alternative signaling pathways rather than EGFR driven signaling pathways which make them resistant to afatinib.

Characterization of afatinib resistance mechanism in EGFR T790M mutant lung cancer cells

IGF1R signaling has been demonstrated to have a role in the resistance not only to the first generation of EGFR TKIs, and MEK (mitogen-activated protein kinase kinase, also known as MAP2K, MAPKK) inhibitors in tumors driven by mutant K-ras [35]. However, the role of IGF1R signaling pathways in resistance to second generation EGFR TKIs, such as afatinib has not yet been demonstrated in lung cancers harboring EGFR T790M mutation. Significant work has already shown that EGFR inhibitors (first and second generation) can result in a secondary EGFR T790M mutation which confers resistance [33,36]. These tumors are then sensitive to afatinib. However, other resistance mechanisms eventually develop which have not been characterized and seriously limit the duration of efficacious afatinib therapy. Given the existence of EGFR-IGF1R cross talk, we examined both EGFR and IGF1R signaling pathways in H1975 parental cells in the presence of various doses of afatinib for 24 hours (Fig 2A). Afatinib treatment increased IGF1R phosphorylation in dose dependent manner. We then, examined EGFR and IGF1R signaling pathways in H1975

parental and AR cell lines with and without afatinib treatment via western blot (Fig 2B). H1975 parental cells maintained EGFR signaling pathways, which was significantly attenuated upon treatment with afatinib. Conversely, AR cells exhibited increased basal levels of IGF1R phosphorylation while EGFR phosphorylation was significantly decreased compared to that in the H1975 parental cells (Fig 2B). We also observed the EGFR-IGF1R interaction via Immunoprecipitation in both H1975 parental and AR cells (Supplemental Fig S4). EGFR and its downstream MAPK and AKT phosphorylation was diminished upon afatinib treatment in H1975 parental cells while ARs cells showed increased IGF1R, MAPK and AKT phosphorylation levels even with afatinib treatment compared to that in H1975 parental cells (Fig 2B). Total endogenous mRNA and protein expression levels of EGFR and IGF1R were not affected in H1975 parental and ARs cells (Fig 2B and Supplemental Fig S1).

Concurrent Inhibition of IGF1R and EGFR can overcome afatinib resistance

Given the significant increase IGF1R phosphorylation in AR cell lines, we examined whether IGF1R inhibition with the small molecule inhibitor, linsitinib, would be sufficient to overcome afatinib resistance in these cell lines. H1975 parental and AR cells were subjected to the growth assay in the presence of various doses of linsitinib for 72 hours as demonstrated in the material and method. H1975 parental cells were not sensitive to linsitinib even with high dosage of linsitinib (20 μ M) whereas AR cells were relatively sensitive to linsitinib in dosage and time dependent manner compared to H1975 parental cells (Fig 2C). Although ARs cells were more dependent on IGF1R signaling pathways, IGF1R inhibition alone was not sufficient to overcome resistance. Effects of linsitinib on IGF1R, EGFR and downstream effectors were examined by western blot (Fig 2D). Linsitinib effectively inhibited IGF1R phosphorylation in AR cells as well as downstream IRS-1 and AKT phosphorylation in a dosage dependent manner, whereas linsitinib up-regulated p-IRS and p-AKT levels in H1975 parental cells (Fig 2D). These may explain potential mechanism on why AR cells were more sensitive to linsitinib treatment (Fig 2D). Interestingly, linsitinib up-regulated p-ERK levels both H1975 parental cells and AR cells, which may explain linsitinib alone was not sufficient to overcome afatinib resistance (Fig 2D). These data suggest that EGFR-IGF1R cross talk may compensate for the effects of IGF1R inhibition in afatinib resistance cells.

To verify our observation that IGF1R inhibition could partially re-sensitize afatinib resistance cells, we used shRNA approach to knockdown IGF1R expression. These experiments lend further credence to the argument that IGF1R signaling plays an important role in afatinib resistance, and represents a necessary level of evidence due to the off target effects that can occur with small molecule inhibitors and TKIs in particular. In order to examine the effects of IGF1R knockdown in H1975 and ARs cells, we performed the growth assay and apoptosis assay as outlined in the materials and method section. IGF1R knockdown did not significantly affect on the cell proliferation in either H1975 or AR cells (Fig 3A). However, afatinib treatment in IGF1R deficient ARs was sufficient to sensitize the resistance to afatinib (Fig 3A). Interestingly, IGF1R knockdown increased the apoptosis in both H1975 parental and AR cells (Fig 3B). Afatinib treatment with IGF1R knockdown in AR cells significantly inhibited the growth of ARs cells (Fig 3A) and induced apoptosis (Fig

3B). We also examined if concurrent inhibition of IGF1R and EGFR can abrogate afatinib resistance in AR cells. AR cells were then treated with either afatinib, linsitinib, and a combination of afatinib and linsitinib for 72 hours as indicated in the material and method. Single treatment was not effective on both cell growth and apoptosis in AR cells whereas combination of EGFR and IGF1R inhibition with afatinib and linsitinib significantly inhibit the growth and induced apoptosis in AR cells (Fig 3D-E). Effects of IGF1R knockdown on EGFR and IGF1R signaling pathways were examined via Western blot analysis with or without afatinib treatment (Fig 3C). IGF1R knockdown alone was partially down-regulated phospho-ERK and phospho-AKT levels in AR cells while phospho-ERK and phospho-AKT levels were up-regulated in H1975 parental cells that may be the existence of EGFR-IGF1R cross talk (Fig 3C). IGF1R signaling cascade via its downstream effector, AKT, is known to regulate cell proliferation and cell survival [37–39]. IGF1R knockdown with afatinib treatment together abolish both phospho-ERK and phospho-AKT levels in both H1975 and AR cells. Thus, Western blot analysis indicates that AR cells maintaining IGF1R-AKT signaling pathway that leads resistance to afatinib which diminished by IGF1R knockdown.

Synergistic Effects of EGFR and IGF1R inhibition in EGFR Mutant Lung Cancers

Given our data demonstrating that concurrent IGF1R and EGFR inhibition is capable of re-sensitizing tumor cells that are resistant to afatinib, we sought to evaluate whether this combination treatment could increase the efficacy of afatinib or prevent the development of afatinib resistance. To this end, we examined the effects of combined afatinib and linsitinib treatment in three EGFR mutant NSCLC cell lines: PC9 (EGFR exon 19del), H3255 (EGFR L858R mutation), and H1975 (EGFR L858R and T790M mutation). As shown in Supplementary Fig S2 A-C, linsitinib treatment alone had no significant effect on proliferation of any of these three EGFR mutant NSCLC cell lines while afatinib shows superior effects on all three EGFR mutant NSCLC cell lines in a dose and time dependent manner (Supplementary Fig S2 A-C) compared to linsitinib treatment. Although EGFR mutant human NSCLC cell lines were not sensitive to linsitinib alone, linsitinib sensitized these cell lines to the anti-proliferative effects of afatinib, which was reflected in synergistic decrease in proliferation seen across all three EGFR mutants human NSCLC cell lines tested (Fig 4A). Synergy between afatinib and linsitinib may also indicate compensatory cross talk between EGFR and IGF1R signaling pathways in EGFR mutant lung cancers. The combination of afatinib and linsitinib treatment also showed synergistic apoptotic effects in all three EGFR mutant NSCLC cell lines (Fig 4B). Apoptotic induction was significantly increased in all tested human EGFR mutant NSCLC cell lines upon inhibition of EGFR and IGF1R with afatinib (0.5 μ M), and linsitinib (2 μ M) respectively. The combination treatment resulted in a 2 fold, 1.3 folds, and 2.6 fold increased in apoptosis in PC9, H3255, and H1975 compared to that with afatinib alone. As expected, concurrent inhibition of IGF1R and EGFR with the first generation EGFR TKI, erlotinib, did not show any synergistic effect on cell growth in H1975 harboring T790M mutant NSCLC cell line because erlotinib has no effects due to the T790M mutation (Supplementary Fig S3). We also examined the effects of EGFR, IGF1R, and combined inhibition on the activity of downstream effectors MAPK and AKT. Interestingly, afatinib inhibited ERK phosphorylation robustly and AKT phosphorylation partially, while linsitinib inhibited AKT signaling partially (Fig 4C). Treatment with the combination of afatinib and linsitinib abolished both ERK and AKT

phosphorylation (Fig 4C). In addition, proliferation results were validated by similar results upon IGF1R knock-down and afatinib treatment in PC9 and H3255 EGFR mutant NSCLC cell lines (Supplementary Fig S5). IGF1R knockdown alone showed no effects on cell growth on the three EGFR mutant NSCLC cell lines while IGF1R knockdown with afatinib treatment abolished both ERK and AKT phosphorylation levels (Fig 4D) as we observed with concurrent inhibition of EGFR and IGF1R with afatinib and linsitinib in Fig 4C. As a result, concurrent inhibition of IGF1R and EGFR showed synergistic effects on cell growth and apoptosis via inhibition MAPK and AKT signaling pathways in EGFR mutant NSCLC cell lines.

Combination Treatment with Afatinib and Linsitinib Inhibits EGFR mutant NSCLC Progression *in vivo* in H1975 Xenograft Model

We further evaluated the combined effects of afatinib and linsitinib on EGFR mutant NSCLC in a H1975 xenograft an *in vivo* model. As predicted by *in vitro* studies, the effects of linsitinib alone on EGFR mutant NSCLC tumor progression *in vivo* was not significant ($p=0.06$) (Fig 5A-B). Afatinib significantly inhibited tumor growth by 27% compared with vehicle treated control group ($**p<0.005$) (Fig 5A-B). Combination treatment with afatinib and linsitinib significantly inhibited tumor growth by 56% *in vivo* compared to that in the group treated with vehicle control ($*p<0.05$) (Fig 5A-B). Anti-proliferative and pro-apoptotic effects of the combination of afatinib and linsitinib were examined by Ki67 and cleaved caspase-3 IHC staining which showed enhanced effects of the afatinib and linsitinib combination on EGFR mutant NSCLC xenografts, reflected by decreased proliferation and increased apoptosis (Fig 5C-E). We also confirmed elevation of p-IGF1R levels by afatinib treatment *in vivo* (Fig 5D, 5F). The increase in IGF1R phosphorylation in xenografted mice receiving afatinib closely mirrors the *in vitro* increase in p-IGF1R (Fig 2A) when H1975 cells were treated with afatinib. Specifically, afatinib treatment led to a 1.7-fold increase in p-IGF1R levels *in vivo*. ($**p<0.005$) (Fig 5F). Therefore, both our *in vitro* and *in vivo* data suggest that concurrent inhibition of EGFR and IGF1R may overcome or prevent resistance to afatinib in EGFR mutant NSCLCs.

Discussion

Patients with EGFR mutant lung tumors are routinely treated with first generation EGFR TKIs, such as gefitinib or erlotinib but development of resistance to the TKI is inevitable [40]. EGFR T790M mutation is detected in more than 50% of lung cancer patients within 12 months post TKI treatment [8,12]. The irreversible EGFR TKI, afatinib, has been approved by the U. S. FDA, and can abrogate EGFR T790M driven resistance. However, results from recent clinical trials indicate that afatinib is initially effective in patients with the EGFR T790M mutation, but tumor continues to evolve and eventually develops resistance rendering afatinib treatment ineffective [14,16,17]. Identifying new resistance mechanisms may enable the efficacy of afatinib to be extended, and could ultimately result in better outcomes for patients. In order to identify novel resistance mechanisms to afatinib in the setting of cancer that is resistant to first generation EGFR TKIs (EGFR T790M mutation), we developed afatinib resistant lung cancer cell lines from parental H1975 cells harboring the EGFR T790M mutation. Development of an *in vitro* resistance model to first generation

EGFR TKIs have been well-established [32,33] and these system have helped to elucidate the role of the EGFR T790M mutation in resistance to EGFR TKIs.

It has been reported that IGF1R signaling plays a role in the acquisition of resistance to first generation of EGFR TKIs [21,24,36,41]. However, the role of IGF1R signaling in acquired resistance to afatinib in patients who are already resistant to first generation EGFR TKIs due to the EGFR T790M mutation has not been addressed. Our efforts represent the first work to demonstrate that IGF1R signaling pathway plays a critical role in resistance to afatinib in lung cancer cells harboring EGFR T790M mutation. Using an escalating dose approach, we developed afatinib resistant cell lines from parental sensitive H1975 cells harboring the EGFR L858R activation mutation and the EGFR T790M mutation which is associated with resistance to first generation EGFR TKIs. These resistant cells exhibited high expression levels of p-IGF1R and p-IRS while p-EGFR expression levels were diminished compared to the H1975 parental cells. Cortot *et al* previously reported that the IGF1R signaling pathway may be involved in resistance to other irreversible EGFR TKIs such as PF299804 or WZ4002 in lung cancer cells in the absence of the EGFR T790M mutation [23]. We demonstrated that IGF1R signaling pathway is involved in the resistance to the irreversible EGFR TKI, afatinib, in EGFR mutant lung cancer cells harboring EGFR T790M mutation (Figs 2, 3). It is possible that IGF1R signaling activation may contribute to the acquired resistance to first generation TKIs in some tumors, subsequently affecting resistance to afatinib. However, the majority of resistance to first generation TKIs is mediated by EGFR T790M mutation [8,12]. In addition, our data indicate that IGF1R signaling activation was only observed in the afatinib resistant cell lines (AR1, AR2 and AR3), but not in parental H1975 cell lines, demonstrating that IGF1R signaling was a novel acquired afatinib resistance mechanism in lung cancer cells harboring the EGFR T790M mutation. IGF1R inhibition alone was not sufficient to overcome the resistance to afatinib (Figs 2–5); a phenomenon that may due to the existence of IGF1R-EGFR cross talk [21,24] or nature of cross talk between EGFR and other alternative receptor tyrosine kinases such as fibroblast growth factor receptors (FGFRs) [26,34]. Thus, it would be important to determine the detail mechanisms involved in EGFR and other receptor tyrosine kinases cross-talk which may play an important role in mediating drug resistance.

IGF1R inhibition alone via small molecule inhibitors or IGF1R monoclonal antibody has been tested in the clinic, but results were disappointing [42,43]. In addition, clinical trials with combination treatment of first generation of EGFR TKIs plus anti-IGF1R monoclonal antibody therapy did not show any increased benefit to lung cancer patients compared to first generation of EGFR TKI monotherapy [44]. These results may largely be a consequence of tumor heterogeneity or the lack of sufficient biomarkers to select patients that are likely to benefit from the therapy [42,43]. Our results suggest that concurrent inhibition of IGF1R and EGFR may be an effective strategy for the treatment of patients with resistance to afatinib to increase efficacy and minimize recurrence [19,45–47]. Concurrent inhibition of IGF1R and EGFR was capable of abrogate resistance to afatinib in H1975 derived resistant cells (Fig 3). It has been reported that concurrent inhibition of EGFR and IGF1R also increased the efficacy of radiation therapy on prostate cancer cells [48]. Both *in vitro* and *in vivo* data demonstrate that combined inhibition of EGFR and IGF1R showed significant effects on cell growth and induced apoptosis in lung cancer cell lines harboring the most

common EGFR mutations via inhibition of both MAPK and AKT signals pathways (Figs 4, 5). In the nature of tumor heterogeneity and ability of tumors to evolve, another possible mechanism(s) of resistance to irreversible EGFR TKIs will need to be determined. Study of the correlation between EGFR T790M mutation status and levels in the tumors of lung cancer patients would be useful in determining if IGF1R phosphorylation can be used as a biomarker for efficacy of concurrent inhibition of EGFR and IGF1R. Based on our data, we believe that combination of afatinib and IGF1R inhibition would be a better therapeutic option to the patients who developed the acquired resistance to afatinib. Whether resistance will develop upon combination treatment remains to be seen in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characteristics of Afatinib resistance H1975 lung cancer cell lines harboring EGFR T790M mutation

(A) Schematic representing the development of afatinib resistant H1975 lung cancer cell lines as indicated in Materials and Methods. H1975 cells were exposed to escalating dose of afatinib from 5nm to 1μM over three months. Cells were considered afatinib resistant when they could be normally cultured in the presence of 1μM of Afatinib.

(B) Cell confluence of H1975 and three H197t afatinib resistant cell lines, AR1, AR2, and AR3 were monitored over 48 hours. Growth rate of between H1975 and H1975 afatinib resistance cells were not statistically significant (ns, not significant; $p > 0.05$).

(C) The effects of afatinib on H1975, AR1, AR2, and AR3 cell growth was measured by live-cell imaging. H1975 cells were sensitive to afatinib in a dose and time dependent manner while AR1, AR2, and AR3 cells were not sensitive to afatinib up to 1 μM. (** $p < 0.005$)

Figure 2. IGF1R activation leads to resistance

(A) Effects of afatinib on EGFR and IGF1R Signaling Pathways. H1975 cells were treated with afatinib with various doses and cell lysates were subjected to Western blot analysis. Afatinib effectively inhibits p-EGFR and p-ERK while p-AKT was partially inhibited. IGF1R phosphorylation was increased upon afatinib treatment in H1975 cells.

(B) H1975 and AR1, AR2, and AR3 cells were treated with control (DMSO) or Afatinib (1μM) for 24 hours. Cell lysates were subjected to Western blot analysis with antibodies as indicated. Afatinib resistance cells exhibit activated IGF1R signaling whereas H1975 cells depend on EGFR signaling.

(C) H1975, AR1, AR2, and AR3 cell growth was measured in the presence of the IGF1R inhibitor, linsitinib, over 72 hours as indicated in material and method. H1975 cells were not sensitive to IGF1R inhibition via linsitinib up to 20 μM on cell confluence. Afatinib resistance cell lines, AR1, AR2, and AR3 cells were relatively sensitive to the IGF1R inhibition via linsitinib compared to that in the parental cell line. (* $p < 0.05$, ** $p < 0.005$)

(D) Effects of linsitinib on EGFR and IGF1R Signaling Pathways. H1975, AR1, AR2 and AR3 cells were treated with control DMSO or linsitinib (2 and 5 μM) for 24 hours. Cell lysates were subjected to Western blot analysis. IGF1R-IRS-1-AKT phosphorylation was

effectively inhibited by linsitinib in afatinib resistance cells whereas IRS-1-AKT phosphorylation was up-regulated by linsitinib in H1975 parental cells.

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Figure 3. Effects of IGF1R Knockdown and IGF1R Inhibition in H1975 and afatinib resistance (AR) cells

(A) Effects of IGF1R knockdown on the growth of H1975 parental and AR cells were measured as described in the materials and methods. IGF1R knockdown alone had no significant effects on the cell growth on both H1975 parental and AR cells. However, IGF1R knockdown significantly sensitizes the effects of afatinib on the growth of AR cells.

(B) Effects of IGF1R knockdown on the apoptosis of H1975 parental and AR cells were determined via measuring the caspase 3/7 activity by live-cell imaging. IGF1R knockdown significantly induced apoptosis in both H1975 parental and AR cells.

(C) Effects of IGF1R knockdown in the absence and presence of afatinib (1 μ M) in H1975 and AR cells on the EGFR and IGF1R signaling pathways were examined via Western blot analysis. H1975 and AR cells transfected with scramble vector control and shIGF1R were treated with control DMSO and afatinib for 24 hours and cell lysate was subjected to the Western blot as indicated in the Materials and Methods. IGF1R knockdown alone down-regulates p-ERK and p-AKT levels in AR cells compared to the H1975 parental cells. AR

cells maintained the p-ERK and p-AKT levels in the presence of afatinib, but IGF1R knockdown sensitized the resistance to afatinib via down-regulating p-ERK and p-AKT levels.

(D) Afatinib resistance cells were exposed to afatinib, linsitinib and combination over 48 hours. Effects of EGFR, IGF1R and combined EGFR and IGF1R inhibition on cell confluence were measured as indicated in the materials and methods. Combined EGFR and IGF1R showed significant effects on cell confluence afatinib resistance cells.

(E) Afatinib resistance cells were exposed to afatinib, linsitinib and combination over 48 hours. Effects of EGFR, IGF1R and combined EGFR and IGF1R inhibition on cell apoptosis were measured by caspase3/7 activity as indicated in the materials and methods. Combined EGFR and IGF1R showed significantly induced apoptosis via caspase3/7 in afatinib resistance cells. (ns, no significance; * $p < 0.05$; ** $p < 0.005$).

Figure 4. Synergistic effects of EGFR and IGF1R inhibition with afatinib and linsitinib in EGFR mutant NSCLC

(A)-(B) Combined EGFR and IGF1R inhibition in the NSCLC cell lines with the most common EGFR mutation, PC9 (EGFR exon19del), H3255 (EGFR L858R) and H1975 (EGFR L858R,T790M). The effects of combined EGFR and IGF1R inhibition on cell confluence and apoptosis were examined via live-cell imaging. Concurrent inhibition of EGFR and IGF1R with afatinib and linsitinib showed synergistic effects on EGFR mutant cell proliferation and apoptosis.

(C) PC9, H3255 and H1975 cells were treated with control (DMSO), afatinib (0.5μM), linsitinib (2μM) and combination for 24 hours. Cell lysates were analyzed by Western blot analysis. EGFR inhibition with afatinib induces IGF1R activation by up-regulating IGF1R phosphorylation levels. Combination of EGFR and IGF1R inhibition was effectively inhibited MAPK and AKT signaling pathway by down-regulating both ERK1/2 and AKT phosphorylation levels.

(D) Effects of IGF1R knockdown in the absence and presence of afatinib (0.5 μM) in PC9, H3255 and H1975 cell lines on the EGFR and IGF1R signaling pathways were examined via Western blot analysis. PC9, H3255 and h1975 cells transfected with scramble vector control and shIGF1R were treated with control DMSO and afatinib for 24 hours and cell lysate was examined by Western blot analysis. IGF1R knockdown in the presence of afatinib down-regulates both p-ERK and p-AKT levels in all three EGFR mutant PC9, H3255 and H1975 cell lines. (* p<0.05).

Figure 5. Combined EGFR and IGF1R inhibition in in vivo H1975 xenograft model

(A)-(B). H1975 Xenograft model were established as described in the materials and methods. The effects of vehicle control, afatinib, linsitinib and combination of afatinib and linsitinib on the tumor growth were monitored over 15 days. Afatinib alone treatment reduced the tumor volume by 27% and linsitinib treatment reduced the tumor volume by 18%. Concurrent inhibition of EGFR and IGF1R via afatinib and linsitinib combination reduced tumor volume by 56% which shows more than additive effects on tumor volume in in vivo H1975 xenograft model (* p<0.05; **p<0.005).

(C) Relative expression levels of Ki-67 from each group of mice after 15 days of treatment. Ki-67 positive cells were down-regulated by 36% in afatinib treatment group and down-regulated by 20% in linsitinib treatment group (* p<0.05; **p<0.005).

(D) Representative images of IHC staining of Ki-67, p-IGF1R and cleaved caspase3 of xenograft tumors from each group after 15 days of treatment.

(E) Relative expression levels of cleaved caspase 3 from each group of mice after 15 days of treatment. Relative expression levels of cleaved caspase 3 were increased by 4.7 fold in the afatinib treatment group, 1.2 fold in the linsitinib treatment group and 9.7 fold in the combination of afatinib and linsitinib treatment group (* p<0.05, **p<0.005).

(F) Relative expression levels of p-IGF1R from each group of mice after 15 days of treatment. The p-IGF1R levels were increased by 1.7 fold in afatinib treatment group compared to the vehicle control treatment group. (* $p<0.05$, ** $p<0.005$).

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