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Heterogeneity Underlies the Emergence of EGFR T790 Wild-Type Clones Following Treatment of T790M-Positive Cancers with a Third Generation EGFR Inhibitor

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Abstract

Rociletinib is a third-generation EGFR inhibitor active in lung cancers with T790M, the gatekeeper mutation underlying most first-generation EGFR drug resistance. We biopsied patients at rociletinib progression to explore resistance mechanisms. Among 12 patients with T790M-positive cancers at rociletinib initiation, six had T790 wild-type rociletinib-resistant biopsies. Two T790 wild-type cancers underwent small cell lung cancer transformation; three T790M-positive cancers acquired *EGFR* amplification. We documented T790 wild-type and T790M-positive clones coexisting within a single pre-rociletinib biopsy. In fact, the pre-treatment fraction of T790M-positive cells impacted response to rociletinib. Longitudinal ctDNA analysis revealed an increase in plasma *EGFR* activating mutation and T790M heralded rociletinib resistance in some patients, while in others the activating mutation increased but T790M remained suppressed. Together, these findings demonstrate the role of tumor heterogeneity when therapies targeting a singular resistance mechanism are employed. To further improve outcomes, combination regimens that also target T790 wild-type clones are required.

Introduction

Epidermal growth factor receptor (EGFR) inhibitors have revolutionized the treatment of *EGFR*-mutant lung cancers; patients now achieve median survivals of 3–5 years compared to 1 year in those lacking a targetable mutation (1, 2). Nevertheless, initial therapy with currently-approved EGFR inhibitors (gefitinib, erlotinib, afatinib) eventually leads to drug resistance, most commonly via the T790M resistance mutation, observed in 50–65% of resistant biopsies (3–5).

Historically, resistance mechanisms to targeted therapies have been conceptualized as binary variables; i.e. a resistance biopsy is either positive or negative for a given mechanism (6). However, this simple approach may not accurately reflect the complexities of tumor heterogeneity, which has been well described among NSCLC and other malignancies (7–9). Indeed; among *EGFR*-mutant lung cancers with acquired resistance to their initial EGFR inhibitor, there is growing evidence that all resistant cells may not share the same resistance mechanism. We and others have previously reported that *EGFR*-mutant patients undergoing more than one post-resistance biopsy and/or an autopsy can exhibit different resistance mechanisms, likely reflecting both intratumoral and intertumoral heterogeneity, as well as dynamic changes in the relative populations of resistant clones over time (3, 4, 10–12).

Novel 3rd-generation EGFR inhibitors that block both activating *EGFR* mutations and T790M have shown promising results; rociletinib (CO-1686) and AZD9291 each elicit responses in ~60% of *EGFR*-mutant patients with T790M-mediated resistance to their initial EGFR inhibitor (13, 14). Thus, despite potential heterogeneity, if a single biopsy demonstrates T790M, most patients derive clinical benefit from T790M inhibition, suggesting that T790M clones may dominate the resistant cells in a majority of cases. However, heterogeneity may prove relevant when these and other therapies targeting specific resistance mechanisms enter the clinic, as subclones that are not targeted by next-generation drugs may ultimately promote resistance. In this study, we biopsied T790M-positive patients treated with rociletinib upon progression to explore rociletinib resistance.

Results

Post-rociletinib biopsies

To examine mechanisms of rociletinib resistance in *EGFR*-mutant, T790M-positive lung cancers, we prospectively biopsied progressing lesions in all patients treated at our institution with rociletinib on a phase I/II clinical trial (NCT01526928) who were willing and able to undergo repeat biopsies. We treated 64 patients with rociletinib between August 2012 and February 2015. Of these, twelve T790M-positive patients ultimately had sufficient paired pre-treatment and rociletinib-resistant biopsies (Figure S1, Table S1). One patient had two biopsies (11a, 11b) after developing progression on rociletinib (Table 1). Immediately before initiating rociletinib all patients were on regimens including an EGFR inhibitor and had a T790M-positive biopsy. Three had intrinsic resistance to rociletinib (defined as progression on the first restaging scan) while nine had acquired resistance (defined as progression after initial tumor shrinkage: stable disease (n=4) or partial response (n=5) per RECIST criteria) (Table 1) (15). The time-to-progression on rociletinib ranged from 1–13

months. All biopsied lesions were radiographically enlarging (Figure 1A). Eleven rociletinib-resistant biopsies were analyzed by next-generation sequencing (NGS) of a panel of cancer-related genes. One biopsy (#1) was obtained before NGS was adopted at our institution and was analyzed by allele-specific PCR targeting common mutations in 23 cancer-related genes. In a second case (#3), there was insufficient tissue for NGS so allele-specific PCR targeting T790M and activating EGFR mutations was performed instead (Table S2).

While all cases were T790M-positive prior to rociletinib, we were initially surprised to observe that six of the thirteen resistant biopsies were T790 wild-type (Table 1, Figure 1B). Importantly, each resistant biopsy retained the original activating *EGFR* mutation. One of the six T790 wild-type cancers had intrinsic resistance, progressing after one month of rociletinib; the cancers from the remaining five patients had acquired rociletinib resistance. We developed a cell line (MGH700) from the biopsy of one such patient (#10) with T790 wild-type acquired rociletinib resistance. As expected, these cells maintained resistance to all tested EGFR TKIs (Figure 1C). Treatment with 1st, 2nd or 3rd generation TKIs all led to inhibition of EGFR phosphorylation, consistent with absence of T790M, but all failed to suppress the AKT/mTOR pathway (Figure 1D), suggesting a possible bypass mechanism of resistance (16). Since 3rd-generation EGFR TKIs like rociletinib effectively inhibit both activating *EGFR* mutations and T790M, it is not surprising that the emerging T790 wild-type clones were cross-resistant to other EGFR inhibitors.

In our efforts to identify additional resistance mechanisms, the biopsied cancers were subjected to a broad set of analyses including next-generation sequencing (NGS) (Table S2). Two of the T790 wild-type cases underwent transformation to small cell lung cancer (SCLC) (Figure 1E, S2). This phenomenon has previously been observed in gefitinib and erlotinib-resistant patients who did not harbor T790M (3, 4). We have previously shown that these EGFR mutant SCLCs lose EGFR expression and dependence on EGFR activity and are resistant to EGFR inhibitors (17). Consistent with previous reports, the transformed SCLCs continued to harbor their original *EGFR* activating mutations and lost RB (17). Indeed, one SCLC (#12) developed a mutation in *RB1* (E587*) and the other (#11b) lost expression of RB1 by IHC. In addition to SCLC transformations in a subset of the T790 wild-type patients, we also observed increased *EGFR* amplification in three of the seven biopsies that retained T790M at resistance (Table 1). Amplification of *EGFR* was previously observed in T790M-positive cell lines with *in vitro* resistance to the EGFR inhibitor dacomitinib (18). Next-generation sequencing revealed no additional mutations in *EGFR* among our cohort of resistant samples, and no new putative resistance mutations have been identified in other genes through these analyses to date. *MET* amplification was not observed among 9 patients tested (Table S2.)

Intratumoral T790M heterogeneity

To understand why nearly half of cases appeared to “lose” T790M, we examined radiographs from the time of each biopsy. While not always feasible to serially sample the same lesion before and after rociletinib, patient 12 illustrates a case where the same lesion was biopsied at baseline, responded to rociletinib, subsequently progressed, and was then re-

biopsied (Figure 1A). This cancer's "loss" of T790M suggests that the original lesion, while testing "positive" for T790M, may have contained both T790M-positive and T790 wild-type clones (Figure 2A).

To examine whether such intratumoral heterogeneity with respect to T790M exists, we studied a patient with an *EGFR* del19 mutation who was treated with first-line afatinib (note: he was not part of our rociletinib-resistant cohort.) After 16 months, he developed afatinib resistance including a malignant pleural effusion. Clinical testing of the thoracentesis cell-block revealed del19 and T790M. We established a cell line from this thoracentesis sample (MGH176) and isolated eight single-cell clones early in its development. Direct sequencing demonstrated that all eight single-cell clones harbored the original del19 mutation but T790M was only present in five of eight clones, while the remaining three were T790 wild-type (Figures 2B, S3A). These results were verified by an independent genotyping assay and serve as an example of both T790M-positive and T790 wild-type cells residing within a single T790M-"positive" biopsy. Two additional cell lines (MGH706 and MGH748) were assessed using the same approach and in both cases all clones were T790M-positive (Figure S3B). Thus, the "loss" of T790M at the time of acquired resistance to rociletinib is likely due to selection of T790 wild-type clones present at treatment initiation and resistant via an independent mechanism rather than rociletinib-resistant cells mutating or deleting their T790M alleles.

Baseline T790M heterogeneity and treatment response

The observations above suggest T790M-positive and T790 wild-type clones may coexist in some cancers with acquired resistance to initial *EGFR* TKIs (Figure 2A). We speculated that the fraction of cells harboring T790M might impact treatment response to T790M-specific inhibitors like rociletinib. We examined the genotyping results from pre-treatment biopsies of all patients at our institution treated with rociletinib to determine the relative frequency of the T790M and activating mutation alleles. We hypothesized that this T790M/activating mutation ratio could serve as a surrogate for the proportion of cells within the biopsy that were T790M-positive since it is well-understood that every cancer cell harbors the *EGFR* activating mutation (19). In order for this estimate to be accurate, we needed to exclude cases where extra *EGFR* copies could skew the relative allele frequency calculation. Hence, we excluded patients with *EGFR* amplification (*EGFR*:*CEP7* ratio ≥ 1.5) by FISH, those who did not have *EGFR* FISH testing performed on the pre-rociletinib biopsy, and those with an *EGFR* activating mutation fraction of $>60\%$, which similarly suggests >2 copies of *EGFR*. This yielded 25 patients with pre-treatment T790M/activating mutation allele frequencies that could be used to estimate the fraction of T790M-positive cells within the biopsy. For each patient, we compared this ratio to the maximum tumor shrinkage observed during rociletinib treatment (Figure 3A; Table S3). This analysis revealed a highly significant relationship showing cancers with a higher baseline fraction of T790M-positive cells had greater tumor shrinkage to rociletinib ($p=0.0017$). Importantly, this result suggests that a more quantitative assessment of T790M burden (as opposed to a binary positive/negative test) may provide additional information regarding the degree to which T790M-positive patients are likely to respond.

Longitudinal Plasma analyses

To assess the evolution of these cancers through treatment and compare plasma and tissue results, we analyzed the plasma prospectively collected as part of the phase I/II trial for the 12 patients with paired biopsies in our cohort. The allelic fractions of the activating *EGFR* mutations and T790M within circulating tumor DNA (ctDNA) were quantified by BEAMing (20). Among the six patients for which complete plasma data for the duration of rociletinib was available, several mirrored the tissue biopsy results (Figures 3B–D, S4A–C). In all patients with radiographic tumor shrinkage, we observed a dramatic decline in both activating mutation and T790M within days of initiating rociletinib, suggesting initial effective suppression of *EGFR*-mutant cells. However, we observed two distinct longitudinal patterns of resistance.

The results from patient 7 (Figure 3B) demonstrate that both T790M and del19 remained suppressed in plasma during a sustained radiographic response but, eventually, both del19 and T790M began to rise as radiographic progression developed. As predicted by plasma, the resistance tumor biopsy was T790M-positive. This pattern is distinct from that observed in patients 10 and 11 (Figures 3C–D) in which there were initial declines in both plasma T790M and activating mutations but at radiographic progression the prevalence of activating mutations rose steadily while T790M remained low. In both of these cases, the resistance biopsies were T790 wild-type, as predicted by plasma. Overall, among the four patients who “lost” T790M on their resistance biopsies and were included in the plasma cohort, all four had low plasma T790M concentrations (<10 copies/mL) at the time of biopsy (Figures 3C–D, S4A and S4C). Thus, the plasma results support the biopsy results and demonstrate that rociletinib resistance can emerge from a population of T790M-positive or T790 wild-type cells. Moreover, the concordance between plasma and biopsy T790M status suggests that genetic analyses from plasma could be sufficient to determine if rociletinib-resistant cancers maintain T790M.

Discussion

The development of T790M-specific *EGFR* inhibitors like rociletinib, AZD9291, and others has substantially benefitted *EGFR*-mutant patients with T790M-mediated acquired resistance to their initial *EGFR* treatments. Resistance mechanisms to 3rd-generation *EGFR* inhibitors have not yet been described. Here, we report the results of thirteen rociletinib-resistant biopsies and demonstrate that 6 of 13 cases were T790 wild-type at the time of resistance, suggesting that heterogeneity with respect to T790M may be critically important. In fact, it may be outgrowth of T790 wild-type subclones that were present prior to rociletinib that leads to resistance in nearly half of cases. In other words, rociletinib may effectively suppress the growth of T790M-positive cells, but a population of T790 wild-type cells within the baseline T790M-“positive” tumor may have a proliferative advantage under rociletinib therapy and could mediate the development of resistance (Figure 2A).

Moreover, we observed that the degree of heterogeneity within a T790M-“positive” tumor may be an important predictor of rociletinib response. Patients with the highest fraction of T790M-positive cells on their pre-treatment biopsies had the most significant tumor shrinkage. This observation suggests that T790M-positive and T790 wild-type clones may be

be differentially sensitive to rociletinib, a hypothesis which is also supported by the differential response rates to rociletinib which have been reported among T790M-positive and T790-wild type patients (13). More than a simple “positive” or “negative” T790M result, quantification of the allele frequencies within a tumor may serve as a predictive biomarker capable of identifying those most likely to have a dramatic tumor shrinkage on rociletinib and likely other 3rd-generation EGFR inhibitors. In other words, if only a small minority of the resistant cancer cells harbor T790M, even if these cells are exquisitely sensitive to a 3rd generation inhibitor, the response may be minimal. Thus, thinking of T790M solely as a binary variable may not be the most inclusive model when making treatment decisions in the clinic. Moreover, in a preliminary analysis of ten non-*EGFR* amplified patients who had both tumor and plasma T790M fractions available at the start of rociletinib, we observed a significant relationship between the two modalities (Figure S5), suggesting that plasma may be a useful surrogate of tumor T790M burden in the future.

Note that many of the patients for whom baseline T790M allele frequency data was analyzed with respect to tumor shrinkage (Figure 3A) remain on treatment still and hence are not included in our rociletinib-resistant biopsy cohort. However, two patients (#2 and #3) are included in both cohorts and illustrate that the degree of baseline T790M heterogeneity may predict the type of resistance that develops to rociletinib. Patient #2 had a high T790M fraction prior to rociletinib while patient #3 had a low T790M fraction prior to starting on treatment. Both had intrinsic resistance to rociletinib and progressed within < 2 months. However, the resistance biopsy of patient #2 remained T790M-positive and developed *EGFR* amplification (not present at baseline), while patient #3 was T790-wild type at resistance. This suggests that perhaps selective outgrowth of T790-wild type clones may be more likely to occur in tumors where T790M made up only a small fraction of the population at the start of treatment. However, this potential relationship needs to be further assessed in a larger cohort.

We also found that longitudinal analyses of the relative allelic burden of activating *EGFR* mutations and T790M within patient plasma provide further evidence of two distinct patterns of emerging rociletinib resistance. In some cases, resistance corresponded to an increase in both T790M and the activating mutation, suggesting the emergence of a T790M-positive clone which may have developed a novel mechanism of rociletinib resistance. In other cases, only the activating mutation was detected as clinical resistance developed, suggesting that a T790 wild-type population within a heterogeneous tumor may have been the driver of rociletinib resistance. Importantly, in both scenarios the emergence of resistance was detectable on plasma ctDNA analysis prior to the appreciation of radiographic progression, as has previously been observed in other malignancies (21). Moreover, the dominant driver of resistance (T790M-positive or T790 wild-type) identified within plasma was confirmed on the corresponding tumor biopsy. In addition to substantiating the role of tumor heterogeneity as an important factor in rociletinib resistance, these results highlight the clinical utility of plasma ctDNA, which could play a key role in evaluating response and indicating emerging resistance in the clinic.

We also describe two cases of SCLC transformation at acquired resistance to rociletinib. Although this phenomenon has been well-described upon resistance to erlotinib and

gefitinib, to our knowledge this is the first report of this histologic transformation occurring on a T790M-targeted EGFR inhibitor (3, 4, 17). In patient 11, the first post-rociletinib biopsy (11a) showed T790M-positive adenocarcinoma, while a second biopsy obtained during more rapid clinical progression (11b) demonstrated T790 wild-type SCLC, indicating the clinical deterioration may have been due to the selective growth advantage of the T790 wild-type SCLC population. In this case, the plasma results (Figure 3D) suggest that T790M constituted a small fraction of the overall tumor burden at progression, further supporting that the T790 wild-type population was likely driving resistance.

The cases that remained T790M-positive post-rociletinib serve as evidence that T790M-positive cells may survive EGFR inhibition and promote acquired resistance in some patients (Figure 2A). We observed evidence of increased *EGFR* amplification in three of the T790M-positive rociletinib-resistant biopsies, suggesting additional expression of EGFR may be able to overcome rociletinib inhibition. Amplification of gene targets is a common mechanism of resistance to targeted therapies, especially when the concentration of the drug in the patient is not in great excess of the concentrations needed to adequately suppress the target. Thus, we speculate that higher concentrations of rociletinib or more potent 3rd-generation EGFR TKIs may not be as susceptible to this resistance mechanism.

Previous studies have proposed a point mutation to the cysteine residue (C797) that covalently interacts with irreversible EGFR inhibitors as a putative resistance mechanism to this class of drugs (22). Although the MGH NGS assay that was used to genotype a majority of our resistant samples can detect mutations at residue 797, we did not observe this or any other novel *EGFR* mutation in our cohort. However, it would not be surprising if this mutation emerges as more patients are analyzed. Further, the 9 samples that were tested for *MET* amplification were all negative and no variants detected on NGS were felt to be strong putative candidates for underlying acquired resistance to rociletinib. Identification of the resistance mechanisms in these cases is the subject of ongoing evaluation.

Our study is limited to a relatively small cohort of patients including both intrinsic and acquired resistance to rociletinib. Because many patients remain on rociletinib on the phase I/II study at our center, and in fact those with the most durable responses are still on treatment, this cohort may be biased towards sampling patients who did relatively poorly on therapy. As we expand our cohort of repeat biopsies among patients with acquired resistance to rociletinib and include some of the best responders, the pattern of the findings may shift. For example, in the cases in which T790 wild-type cells drove the resistance, it is likely that these cells were present prior to rociletinib treatment and were therefore preferentially selected upon initiation of rociletinib. If this holds true, then T790 wild-type driven resistance could be less commonly observed among patients with longer durations of response. Further, as with any cohort of patients undergoing repeat biopsies, our study is also limited in that not all patients had biopsies at the same site pre- and post-treatment, nor had tissue analyzed with the same assay (s), due to tissue quantity and upgrading assays over time (for example, MGH shifted from the allele-specific SNaPshot assay to an NGS platform in 2014). Larger, prospective cohorts will be needed to validate our observations about T790M allelic frequency predicting response to treatment and longitudinal plasma patterns of T790M and activating mutations.

Our findings demonstrate that the current paradigm of considering acquired resistance mechanisms in a binary positive/negative fashion may be an over-simplification of the true biology. Rociletinib and other T790M-targeted drugs appear to be most effective in T790M-“positive” patients by initial reports, but there is significant molecular heterogeneity among these patients. Although selectively targeting a T790M-positive population can lead to dramatic responses, some T790M-“positive” cancers have distinct clones that survive the initial EGFR TKIs due to mechanisms other than T790M. Our findings support the growing lines of evidence that treatment strategies that combine T790M-targeted agents such as rociletinib with other drugs to target T790 wild-type cells will ultimately be required to promote even more durable remissions.

Methods

Patients and treatment

Any *EGFR*-mutant lung cancer patient treated with rociletinib on the phase I/II trial (NCT01526928) at MGH and Stanford who underwent a biopsy while on rociletinib was eligible (Figure S1). All patients included in the final cohort had pre- and post-rociletinib tissue available and were T790M-positive on their pre-treatment biopsy, tested centrally by Clovis using an allele-specific PCR assay (Therascreen®, Qiagen) and/or locally by one of the CLIA-certified assays described for post-rociletinib biopsies below. All patients were treated with rociletinib at starting doses of 900mg BID of the free-base formulation or 500–750 mg BID of the HBr formulation. Radiographic responses were assessed using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (15). For each patient, the treating investigator determined clinically if a progression biopsy was indicated. In eleven patients, the rociletinib-resistant biopsy was obtained at the time of systemic disease progression. Patient 8 developed a pleural effusion after 3.5 months, while her systemic response remained favorable. The characteristics of her rociletinib-resistant pleural effusion are reported here. All patients provided signed informed consent under an IRB-approved protocol allowing NGS and exploratory research on the biopsies. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Genotype assessments

All post-rociletinib biopsies were analyzed with CLIA-certified genotyping assays (Table S2). Testing methodologies included the MGH NGS platform, the FoundationOne NGS platform and the SNaPshot allele-specific assay (23–25). The SNaPshot allele-specific assay detects somatic mutations in 23 cancer-related genes via multiplex PCR followed by single base extension sequencing (SNaPshot, Applied Biosystems) (23). A separate PCR reaction using primers flanking *EGFR* exons 19 and 20 detects in-frame insertions or deletions in *EGFR*. The newer MGH NGS platform uses anchored multiplex PCR to detect single nucleotide variants and insertion/deletions within 39 cancer-related genes (24). The Foundation One platform (Foundation Medicine) also utilizes NGS and includes the entire coding sequence of 315 cancer-related genes and select introns of 28 additional genes (25).

Samples with sufficient material were tested for *EGFR* and *MET* amplification via fluorescent *in situ* hybridization (FISH) using standard protocols as described previously (3, 26)

Plasma BEAMing Methods

Blood for plasma *EGFR* analysis was collected at baseline, treatment day 15, and at the start of each 21-day cycle on the rociletinib phase I/II trial. Blood was collected in K2 EDTA tubes (up to four, 6mL Vacuettes), processed into plasma within 30 minutes (1800 xg for 10 min at 18–23 °C) and stored at –70 °C or below until ready for processing. For each timepoint, ctDNA was extracted from 2mL of plasma using the QIAamp DNA Circulating Nucleic Acid Kit (Manchester, U.K.).

Plasma ctDNA was interrogated for *EGFR* mutations using BEAMing (Beads, Emulsions, Amplification and Magnetics) (20). Briefly, a pre-amplification step is conducted with a high-fidelity DNA polymerase, and an aliquot of the pre-amplified product is used to perform single molecule PCR on magnetic beads in water-in-oil emulsions. Beads with mutant products are distinguished from wild-type using allele-specific, fluorescently-labeled probes and the bead populations are counted by flow cytometry. For the present study, primers and probes were designed to interrogate the *EGFR* T790M and L858R mutations and several of the most common deletions in exon 19 (E746_A750del (13), E746_S752>V, L747_A750>P, L747_T751del, and L747_P753>S) which account for approximately 90% of all exon 19 deletions. The assays have been validated to detect allelic variants at a level of 0.02%.

Cell Line Development, Single cell cloning and sequencing

The MGH176, MGH700, MGH706 and MGH748 cell lines were initially established on an irradiated fibroblast feeder layer isolated from normal human tissue and cultured in tumor cell media plus rock inhibitor (27, 28). MGH176 cells were derived from an afatinib-resistant pleural effusion obtained in July 2013, MGH706 cells from an erlotinib-resistant adrenal metastasis (February 2014), the MGH700 cells from a rociletinib-resistant liver biopsy (July 2014) and the MGH748 cells from an afatinib/cetuximab-resistant lymph node (July 2014.) Cell lines were sequenced to confirm the presence of the *EGFR* activating mutation that was identified by clinical testing of the corresponding biopsy sample. For single cell clone isolation, early in development (passage 1 – 3) cells were plated at a density of 5–10,000 cells/10cm on a feeder free culture plate. After several weeks of growth, colonies that emerged were imaged and isolated independently by trypsinization using cloning rings. DNA was extracted from each clone using the DNeasy kit (Qiagen) and Sanger sequencing performed on PCR products of *EGFR* exon 19, exon 20 and exon 21 at the Sequencing group of the CCIB DNA Core Facility at MGH. PCR products were also ligated into the Topo TA for sequencing vector (Invitrogen) and transformed into competent bacteria. Plasmid DNA from individual colonies which each represent a single PCR product were individually sequenced to confirm the T790M status of each clone.

Reagents

Antibodies for pAkt (T308), pERK (T202/Y204), pS6 (S240/244) and actin were from Cell Signaling. pEGFR (y1068) was from Abcam.

Cell Viability Assays

MGH700 cells were plated in RPMI supplemented with 10% FBS at a density of 3000 cells/well in a 96-well plate. The following day, the cells were treated with gefitinib, afatinib, rociletinib or AZD9291 (all purchased from Selleck) for 72 hours and then cell viability was assessed by CellTiter-Glo (Promega) using standard protocols.

Calculation of T790M/Activating Mutation Allele Frequency Ratio

We obtained the allelic fraction of T790M and *EGFR* activating mutations from clinical testing of all rociletinib-treated patients with pretreatment biopsies submitted for allele-specific PCR or NGS at MGH. Patients who did not have concurrent *EGFR* FISH testing, those with *EGFR* amplification (EGFR:CEP7 ratio of ≥ 1.5 by FISH), and those with an activating mutation allelic fraction of $> 60\%$ were excluded. The ratio of T790M:activating mutation was calculated for each patient and plotted against the maximum reduction in tumor volume by RECIST. The significance of the relationship was tested with the Spearman correlation coefficient. For plasma testing, patients with <10 copies of the activating mutation were excluded from the analysis because of high intrinsic variability in quantitation at very low copy numbers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

This report documents that half of T790M-positive *EGFR*-mutant lung cancers treated with rociletinib are T790 wild-type upon progression, suggesting that T790 wild-type clones can emerge as the dominant source of resistance. We show that tumor heterogeneity has important clinical implications and that plasma ctDNA analyses can sometimes predict emerging resistance mechanisms.

Figure 1. Results of rociletinib-resistant biopsies

Panel A.

Representative images from a CT of the chest showing (I) thickening of the left pleura prior to the start of rociletinib, (II) resolution of the left pleural thickening due to disease response on day 44 of rociletinib and (III) progressive thickening of the left pleural at rociletinib progression (day 372.) Arrows depict the site of biopsies obtained at baseline and at progression.

Panel B.

Pie-chart showing distribution of findings on rociletinib-resistant biopsies. 6 of 13 biopsies had “lost” T790M and 2 of these underwent transformation to small cell lung cancer. 7 remained T790M-positive, and 3 of these developed *EGFR* amplification.

Panel C.

Rociletinib-resistant cell line MGH700 (derived from the rociletinib-resistant biopsy of patient 10) demonstrates *in vitro* cross-resistance to all tested EGFR inhibitors. MGH700 cells were treated with the indicated EGFR TKIs over a range of doses for 72 hours. CellTiter Glo was used to measure cell viability. Experiments were carried out in quadruplicate and the error bars depict standard error of the mean.

Panel D.

Western blot showing that treatment of the MGH700 cell line with 1st, 2nd or 3rd generation EGFR TKIs leads to inhibition of EGFR phosphorylation, but fails to suppress the AKT/mTOR pathway. Lysates from MGH700 cells treated with DMSO or 1 μ M of Gefitinib, Afatinib, or Rociletinib for 6 hours were probed with the indicated antibodies.

Panel E.

Pre and post-rosciletinib biopsies of patient #11. Histologic analyses illustrate that the pre-rosciletinib biopsy shows moderately differentiated adenocarcinoma with an acinar pattern. The post-rosciletinib biopsy (11b) exhibits nests of tumor cells with a high nuclear to cytoplasmic ratio and no gland formation. Immunohistochemical studies reveal the tumor cells to be negative for chromogranin and synaptophysin pre-rosciletinib and positive for both neuroendocrine markers in the post-rosciletinib liver biopsy. The results, in conjunction with the morphology, are consistent with the diagnosis of small cell carcinoma in the rosciletinib-resistant specimen.

Figure 2. Intratumoral T790M heterogeneity

Panel A.

A conceptual model showing that tumors with T790M “positive” resistance to erlotinib, gefitinib or afatinib may be heterogeneous, consisting of both T790M-positive (blue) and T790 wild-type (white) populations. In some cases of rociletinib resistance, T790 wild-type cells may be the dominant driver of tumor growth and may lead to a predominantly T790 wild-type tumor (top), while in other cases the T790M-positive cells may make up the majority of the population (bottom.)

Panel B.

(I) Two representative clones out of eight isolated from cell line MGH176, derived from an afatinib-resistant pleural effusion, demonstrate heterogeneity of T790M. Brightfield images of the clones are shown on the left and nucleotide sequences on the right. Both clones are positive for the *EGFR* exon 19 deletion mutation. The nucleotide sequence of clone 2 reveals the presence of T790M (*), while the mutation is absent in clone 6.

(II) Summary of the single-cell clone analyses showing that overall 5 of 8 clones analyzed are T790M positive and 3 of 8 are T790 wild-type (see also supplemental figure S3.)

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Figure 3. Plasma and Tissue EGFR allele analyses
Panel A.

Scatterplot showing the relationship between the allelic fraction of T790M (calculated as the percent of T790M alleles/percent of *EGFR* activating mutation alleles as quantified by allele-specific PCR or NGS) in the pre-rociletinib biopsy and the maximum reduction in tumor volume (maximal percent change in the sum of the longest diameter of RECIST target lesions.) Patients with highest allelic fractions of T790M have the most significant reductions in tumor volume, while those with low (or absent) T790M were least likely to respond. The Spearman correlation coefficient (R) for this relationship is -0.596 (p=0.0017.)

Panels B–D.

Longitudinal quantitative analyses of *EGFR* mutations are shown with the relative copies of T790M and activating mutation per milliliter of plasma depicted by solid and dashed red lines, respectively. The corresponding radiographic response is depicted with a solid blue line, measured as the sum of the longest diameters of the target lesions as per the RECIST method. Arrows indicate the timing of rociletinib-resistant biopsies. ctDNA analysis of

patient 7 (panel B) shows an increase in both Del19 and T790M when radiographic progression develops. In contrast, patients 10 (panel C) and 11 (panel D) demonstrate increasing levels of the activating mutation at the time of radiographic progression while T790M remains suppressed.

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Table 1

Results of pre and post-rosciletinib biopsies

Rociletinib Response			Pre-Rociletinib Biopsy			Post-Rociletinib Biopsy		
Patient ID	Time to Progression on Rociletinib (Months)	Best RECIST response to Rociletinib §	Site of Biopsy	EGFR Activating Mutation	T790M status	T790M status (Additional resistance Mechanisms)	EGFR Activating Mutation	Site of Biopsy
INTRINSIC RESISTANCE								
1	1	+41% (PD)	Lung (RUL)	+	+	+	+	Lung (RUL)
2	1.5	+11% (PD)	Lung (RLL)	+	+	+ amp (6.4) ¶	+	Lung (RUL)
3	1	-6% (PD)	Mediastinal Lymph Node	+	+	WT	+	Lung (RUL)
ACQUIRED RESISTANCE								
4	2.5*	-11% (SD)	Left Pleura	+	+	+	+	Left Pleura
5	4 [†]	-21% (SD)	Right Mainstem Bronchus Tumor	+	+	WT	+	Lung (RUL)
6	5	-25% (SD)	Lung (LUL)	+	+	+ amp (>25) **	+	Lung (LUL)
7	13	-29% (SD)	Pleural Fluid (Right)	+	+	+	+	Pleural Fluid (Right)
8	3.5°	-30% (PR)	Lung (LLL)	+	+	WT	+	Pleural Fluid (Left)
9	4	-31% (PR)	Lung (LUL)	+	+	+	+	Lung (RLL)
10	4	-32% (PR)	Liver (Right Lobe)	+	+	WT	+	Liver (Left Lobe)
11a [‡]	7	-35% (PR)	Left Adrenal Mass	+	+	+ amp (>25) ††	+	Lung (RLL)
11b [‡]						WT (SCLC)	+	Liver (Right Lobe)
12	12	-71% (PR)	Left Pleura	+	+	WT (SCLC)	+	Left Pleura

EGFR – Epidermal Growth Factor Receptor; RECIST – Response Evaluation Criteria in Solid Tumors; WT – T790 wild-type; SD- Stable Disease; PR- Partial Response; PD- Progressive Disease; RUL- Right Upper Lobe, RLL – Right Lower Lobe, LUL – Left Upper Lobe, LLL- Left Lower Lobe, SCLC – Small Cell Lung Cancer; amp – amplification of EGFR (number of copies)

§ Best RECIST response is the maximum percent change in tumor burden compared to baseline as measured by the sum of the longest diameters of the target lesions, according to the RECIST method. Tumors must shrink by at least 30% to be classified as partial response and tumors may shrink and still be classified as progression if new lesions arise.

* Patient 4: Progressed at 2.5 months in CNS, biopsy obtained at systemic progression (4 months)

† Patient 5: Progressed at 4 months in CNS, biopsy obtained at systemic progression (8 months)

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°Patient 8: A progressing lesion was biopsied at 3.5 months, however, time to systemic progression = 11 months

[‡]Patient 11: Two rociletinib-resistant biopsies were obtained; the first (11a) showed adenocarcinoma that was T790M-positive and EGFR-amplified. One month later, a second biopsy (11b) showed small cell lung cancer transformation and was T790 wild-type.

[¶]Patient 2: *EGFR* copy number went from 1.4 pre-rociletinib to 6.4 post-rociletinib

** Patient 6: *EGFR* copy number went from 10 pre-rociletinib biopsy to > 25 post-rociletinib

[‡] Patient 11: *EGFR* copy number went from 2.6 pre-rociletinib to > 25 on her first post-rociletinib biopsy (11a). The second post-rociletinib biopsy (11b, SCLC transformation) was not *EGFR* amplified (1.1 copies).