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

Zofia Piotrowska<sup>1,4</sup>, Matthew J. Niederst<sup>1,4</sup>, Chris A. Karlovich<sup>2</sup>, Heather A. Wakelee<sup>1</sup>, Joel W. Neal<sup>1</sup>, Mari Mino-Kenudson<sup>1,4</sup>, Linnea Fulton<sup>1</sup>, Aaron N. Hata<sup>1,4</sup>, Elizabeth L. Lockerman<sup>1</sup>, Anuj Kalsy<sup>1</sup>, Subba Digumarthy<sup>1,4</sup>, Alona Muzikansky<sup>1,4</sup>, Mitch Raponi<sup>2</sup>, Angel R. Garcia<sup>1</sup>, Hillary E. Mulvey<sup>1</sup>, Melissa K. Parks<sup>1</sup>, Richard H. DiCecca<sup>1</sup>, Dora Dias-Santagata<sup>1,4</sup>, A. John Iafrate<sup>1,4</sup>, Alice T. Shaw<sup>1,4</sup>, Andrew R. Allen<sup>2</sup>, Jeffrey A. Engelman<sup>1,4</sup>, and Lecia V. Sequist<sup>1,4</sup>

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. The pretreatment fraction of T790M-positive cells affected response to rociletinib. Longitudinal circulating tumor DNA (ctDNA) analysis revealed an increase in plasma EGFR-activating mutation, and T790M heralded rociletinib resistance in some patients, whereas 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 used. To further improve outcomes, combination regimens that also target T790-wild-type clones are required.

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.

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See related commentary by Ichihara and Lovly, p. 694.
Historically, resistance mechanisms to targeted therapies have been conceptualized as binary variables; that is, 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 non–small cell lung cancer (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 third-generation EGFR inhibitors that block both activating EGFR mutations and T790M have shown promising results; rociletinib (CO-1686) and AZD9291 each elicit responses in approximately 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 with rociletinib at our institution 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, 12 T790M-positive patients ultimately had sufficient paired pretreatment and rociletinib-resistant biopsies (Supplementary Fig. S1 and Supplementary Table S1). One patient had two biopsies after developing progression on rociletinib (Table 1, 11a and 11b). Immediately after 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) whereas 9 had acquired resistance (defined as progression after initial tumor shrinkage: stable disease (n = 4) or partial response (n = 5) per RECIST criteria; Table 1; ref. 15). The time-to-progression on rociletinib ranged from 1 to 13 months. All biopsied lesions were radiographically enlarging (Fig. 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 (Supplementary Table S2).

Although all cases were T790M-positive prior to rociletinib, we were initially surprised to observe that six of the 13 resistant biopsies were T790–wild-type (Table 1 and Fig. 1B). Importantly, each resistant biopsy retained the original activating EGFR mutation. One of the six T790–wild-type cancers had intrinsic resistance, progressing after 1 month of rociletinib; the cancers from the remaining 5 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 tyrosine kinase inhibitors (TKI; Fig. 1C). Treatment with first-, second-, or third-generation TKIs all led to inhibition of EGFR phosphorylation, consistent with the absence of T790M, but all failed to suppress the AKT–mTOR pathway (Fig. 1D), suggesting a possible bypass mechanism of resistance (16). Because third-generation EGFR TKIs, such as 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 NGS (Supplementary Table S2). Two of the T790–wild-type cases underwent transformation to small cell lung cancer (SCLC; Fig. 1E and Supplementary Fig. 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 RB1 (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). NGS 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 (Supplementary Table S2).

Intratumoral T790M Heterogeneity
To understand why nearly half of the cases appeared to “lose” T790M, we examined radiographs from the time of each biopsy. Although it was 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 (Fig. 1A). This cancer’s “loss” of T790M suggests that the original lesion, although testing “positive” for T790M, may have contained both T790M-positive and T790–wild-type clones (Fig. 2A).
### Table 1. Results of pre- and post-rociletinib biopsies

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Time-to-progression on rociletinib, mos</th>
<th>Best RECIST response to rociletinib&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Site of biopsy</th>
<th>EGFR-activating mutation</th>
<th>T790M status</th>
<th>Site of biopsy</th>
<th>EGFR-activating mutation</th>
<th>T790M status (additional resistance mechanisms)</th>
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<td>Left pleura</td>
<td>+</td>
<td>WT (SCLC)</td>
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</table>

Abbreviations: amp, amplification of EGFR (number of copies); CNS, central nervous system; LLL, left lower lobe; LUL, left upper lobe; PD, progressive disease; PR, partial response; RLL, right lower lobe; RUL, right upper lobe; SD, stable disease; WT, T790–wild-type.

<sup>a</sup>Best RECIST response is the maximum percentage change in tumor burden compared with 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.

<sup>b</sup>Patient 4: progressed at 2.5 months in CNS, biopsy obtained at systemic progression (4 months).

<sup>c</sup>Patient 5: progressed at 4 months in CNS, biopsy obtained at systemic progression (8 months).

<sup>d</sup>Patient 8: a progressing lesion was biopsied at 3.5 months; however, time to systemic progression = 11 months.

<sup>e</sup>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 SCLC transformation and was T790-wild-type.

<sup>f</sup>Patient 2: EGFR copy number went from 1.4 pre-rociletinib to 6.4 post-rociletinib.

<sup>g</sup>Patient 6: EGFR copy number went from 10 pre-rociletinib biopsy to >25 post-rociletinib.

<sup>h</sup>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).
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...
T790 Heterogeneity in Rociletinib Resistance

Figure 2. Intratumoral T790M heterogeneity. 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), whereas in other cases the T790M-positive cells may make up the majority of the population (bottom). B, (I) two representative clones out of eight isolated from cell line MGH176, derived from an afatinib-resistant pleural effusion, demonstrate heterogeneity of T790M. Bright-field 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 (*), whereas the mutation is absent in clone 6. (II) Summary of the single-cell clone analyses showing that overall five of eight clones analyzed are T790M-positive and three of eight are T790-wild-type (see also Supplementary Fig. S3).

Baseline T790M Heterogeneity and Treatment Response

The observations above suggest that T790M-positive and T790-wild-type clones may coexist in some cancers with acquired resistance to initial EGFR TKIs (Fig. 2A and Supplementary Fig. 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 (Supplementary Fig. S3B). Thus, the “loss” of T790M at the time of acquired resistance to rociletinib is likely due to selection of T790M-positive cell lines harboring the original del19 mutation but T790M was present in only five of eight clones, whereas the remaining three were T790M-positive (Fig. 2B and Supplementary Fig. S3A). These results suggested that cancers with a higher baseline fraction of T790M-positive clones might have a better response to T790M-specific inhibitors such as rociletinib. We examined the genotyping results from pretreatment 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, because 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 those 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 pretreatment 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 with the maximum tumor shrinkage observed during rociletinib treatment (Fig. 3A and Supplementary Table S3). This analysis revealed a highly significant relationship showing cancers with a higher baseline fraction of T790M-positive cells had greater tumor shrinkage in response 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.
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 \( \text{EGFR} \) mutations and T790M within circulating tumor DNA (ctDNA) were quantified by BEAMing (Beads, Emulsions, Amplification, and Magnetics; ref. 20). Among the 6 patients for which complete plasma data for the duration of rociletinib was available, several mirrored the tissue biopsy results (Fig. 3B–D and Supplementary Fig. S4A–S4C). 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 \( \text{EGFR} \)-mutant cells. However, we observed two distinct longitudinal patterns of resistance.

The results from patient 7 (Fig. 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 (Fig. 3C and 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 T790M-wild-type, as predicted by plasma. Overall, among the 4 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 (Fig. 3C and D; Supplementary Fig. S4A and S4C). Thus, the plasma results

**Figure 3.** Plasma and tissue EGFR allele analyses. A, scatterplot showing the relationship between the allelic fraction of T790M (calculated as the percentage of T790M alleles/percentage 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 percentage change in the sum of the longest diameter (SLD) of RECIST target lesions]. Patients with the highest allelic fractions of T790M have the most significant reductions in tumor volume, whereas those with low (or absent) T790M were least likely to respond. The Spearman correlation coefficient (\( R \)) for this relationship is \(-0.596 (P = 0.0017)\). 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. Circulating tumor DNA analysis of patient 7 (B) shows an increase in both Del19 and T790M when radiographic progression develops. In contrast, patients 10 (C) and 11 (D) demonstrate increasing levels of the activating mutation at the time of radiographic progression while T790M remains suppressed. N/D, not detected.
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 whether rociletinib-resistant cancers maintain T790M.

**DISCUSSION**

The development of T790M-specific EGFR inhibitors, such as rociletinib, AZD9291, and others, has substantially benefitted patients with EGFR-mutant tumors with T790M-mediated acquired resistance to their initial EGFR treatments. Resistance mechanisms to third-generation EGFR inhibitors have not yet been described. Here, we report the results of 13 rociletinib-resistant biopsies and demonstrate that six 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 (Fig. 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 pretreatment biopsies had the most significant tumor shrinkage. This observation suggests that T790M-positive and T790–wild-type clones may be differentially sensitive to rociletinib, a hypothesis that is also supported by the differential response rates to rociletinib that 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 third-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 rociletinib, the growth advantage of the T790–wild-type SCLC population.

Note that many of the patients for whom baseline T790M allele frequency data were analyzed with respect to tumor shrinkage (Fig. 3A) still remain on treatment 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, whereas 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), whereas 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 makes 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 that 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, whereas a second biopsy obtained during more rapid clinical progression (11b) demonstrated T790–wild-type SCLC, indicating that 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 (Fig. 3D) suggest that T790M constituted a small fraction of the overall tumor burden at progression, further supporting our observation 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 (Fig. 2A). We observed evidence of increased EGFR amplification in three of the T790M-positive rociletinib-resistant biopsies, suggesting that 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 third-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 Massachusetts General Hospital (MGH) NGS assay that was used to genotype a majority of our resistant samples can detect somatic 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. Furthermore, the nine 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 toward 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. Furthermore, 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 pretreatment and posttreatment, nor had tissue analyzed with the same assay(s), due to tissue quantity and upgrading assays over time (e.g., 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 allele 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 oversimplification 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 (Supplementary Fig. S1). All patients included in the final cohort had pre- and post-rociletinib tissue available and were T790M-positive on their pretreatment biopsy, tested centrally by Clovis using an allele-specific PCR assay (Therascreen; Qiagen) and/or locally by one of the Clinical Laboratory Improvement Amendments (CLIA)–certified assays described for post-rociletinib biopsies below. All patients were treated with rociletinib at starting doses of 900 mg twice a day of the free-base formulation or 500 to 750 mg twice a day of the HBr formulation. Radiographic responses were assessed using RECIST version 1.1 (15). For each patient, the treating investigator determined clinically if a progression biopsy was indicated. In 11 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 Institutional Review Board–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 (Supplementary 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; ref. 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 insertions/deletions within 39 cancer-related genes (24). The Foundation One platform (Foundation Medicine) also uses 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 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 4 mL Vacuette), processed into plasma within 30 minutes (1,800 × g for 10 minutes at 18°C–23°C), and stored at −70°C or below until ready for processing. For each timepoint, ctDNA was extracted from 2 mL of plasma using the QIAamp DNA Circulating Nucleic Acid Kit. Plasma ctDNA was interrogated for EGFR mutations using BEAMing (20). Briefly, a preamplification step is conducted with a high-fidelity DNA polymerase, and an aliquot of the preamplified 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 this 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_S752delV, L747_A750delP, L747_T751del, and L747_P753Sdel], 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 ...
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ROCK inhibitor (27, 28). The MGH176 cells were derived from an afatinib-resistant pleural effusion obtained in July 2013, the 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-passage (passage 1–3) cells were plated at a density of 5,000 to 10,000 cells/10 cm 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 was 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 (1:1,000; Cell Signaling Technology), pERK (1:1,000; Cell Signaling Technology), pEGFR (1:1,000; y1068) was from Abcam. Antibodies for pT308, pT202/Y204, and pS6 were purchased from Cell Signaling Technology. pAKT (1:1,000; y308), and pERK (1:1,000; y204), pS6 (1:1,000; $240/244), and actin (1:1,000) were from Cell Signaling Technology. pEGFR (1:1,000; y1068) was from Abcam.

Cell Viability Assays

MGH700 cells were plated in RPMI supplemented with 10% FBS at a density of 3,000 cells per 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.

Disclosure of Potential Conflicts of Interest

Zofia Piotrowska has received speakers bureau honoraria from Clovis Oncology. C.A. Karlovich has ownership interest (including patents) in Clovis Oncology. H.A. Wakelee reports receiving commercial research grants from Clovis Oncology, AstraZeneca, and Genentech/Roche. J.W. Neal reports receiving commercial research grants from Genentech/Roche, Mek, ArQuie, Novartis, Exelixis, Boehringer-Ingelheim, and Nektar and is a consultant/advisory board member for Amgen. T. Shaw is a consultant/advisory board member Pfizer, Novartis, Genentech, Roche, Ignyta, Ariad, Chugai, and Blueprint. A.R. Allen has ownership interest (including patents) in Gatekeeper, and is a consultant/advisory board member for Novartis, Genentech, AstraZeneca, and Clovis Oncology. L.V. Sequist is a consultant/advisory board member for Boehringer-Ingelheim, AstraZeneca, Novartis, Genentech, Merrimack, Taiho, and Clovis Oncology. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Piotrowska, A. Kalsy, M. Raponi, D. Dias-Santagata

Study supervision: Z. Piotrowska, A.R. Allen, J.A. Engelman, L.V. Sequist

Other (cell line generation): E.L. Lockerman

Other (provided cells for experiments): M.K. Parks, R.H. DeCecca

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REFERENCES


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