Tumor Analyses Reveal Squamous Transformation and Off-Target Alterations As Early Resistance Mechanisms to First-line Osimertinib in *EGFR*-Mutant Lung Cancer

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ABSTRACT

Purpose: Patterns of resistance to first-line osimertinib are not well-established and have primarily been evaluated using plasma assays, which cannot detect histologic transformation and have differential sensitivity for copy number changes and chromosomal rearrangements.

Experimental Design: To characterize mechanisms of resistance to osimertinib, patients with metastatic *EGFR*-mutant lung cancers who received osimertinib at Memorial Sloan Kettering Cancer Center and had next-generation sequencing performed on tumor tissue before osimertinib initiation and after progression were identified.

Results: Among 62 patients who met eligibility criteria, histologic transformation, primarily squamous transformation, was identified in 15% of first-line osimertinib cases and 14% of later-line cases. Nineteen percent (5/27) of patients treated with first-line osimertinib had off-target genetic resistance (2 *MET* amplification, 1 *KRAS* mutation, 1 *RET* fusion, and 1 *BRAF* fusion) whereas 4% (1/27) had an acquired *EGFR* mutation (*EGFR* G724S). Patients with squamous transformation exhibited considerable genomic complexity; acquired *PIK3CA* mutation, chromosome 3q amplification, and *FGF* amplification were all seen. Patients with transformation had shorter time on osimertinib and shorter survival compared with patients with on-target resistance. Initial *EGFR* sensitizing mutation, time on osimertinib treatment, and line of therapy also influenced resistance mechanism that emerged. The compound mutation *EGFR* S768 + V769L and the mutation *MET* H1094Y were identified and validated as resistance mechanisms with potential treatment options.

Conclusions: Histologic transformation and other off-target molecular alterations are frequent early emerging resistance mechanisms to osimertinib and are associated with poor clinical outcomes.

See related commentary by Piotrowska and Hata, p. 2441

Introduction

The identification of *EGFR* T790M as the dominant mechanism of resistance to first- and second-generation EGFR-tyrosine kinase inhibitors (TKI) resulted in the development of osimertinib, a third-generation EGFR-TKI (1, 2). Osimertinib's initial approval was in the setting of progression on initial EGFR-TKI in patients with tumors harboring *EGFR* T790M (3). More recently, osimertinib exhibited superior progression-free survival (PFS) compared with erlotinib or gefitinib as initial treatment in patients with *EGFR*-mutant non–small cell lung cancer, positioning osimertinib as the preferred first-line treatment where available (4). Nevertheless, acquired resistance to osimertinib invariably develops with a median PFS of 19 months (4).

Similar to earlier EGFR inhibitors, characterization of the landscape of resistance enables the development of subsequent therapies. Our knowledge about mechanisms of resistance to osimertinib is primarily derived from patients who received osimertinib after other EGFR-TKIs; these patients have preexisting *EGFR* T790M which may induce fundamentally distinct resistance mechanisms compared with patients receiving first-line osimertinib (5–9). In the later-line osimertinib setting, the most commonly reported acquired on-target *EGFR* mutation is EGFR *C797S*, with other *EGFR* mutations such as G724, L792, and L718/G719 also reported (5, 6, 8, 10–12). *MET* amplification is the most frequently identified off-target resistance mechanism, although alterations in *RET, ALK, BRAF*, and *FGFR* also occur (5–8, 10–15). Small cell and squamous cell histologic transformation have been reported as infrequent mechanisms of resistance (5, 8, 16–20).

Analysis of circulating tumor DNA (ctDNA) has been the predominant method for investigating resistance, but cannot detect histologic transformation and has differential sensitivity for copy number changes and chromosomal rearrangements compared with tissue analysis (5, 6, 11, 12, 21–25). Furthermore, published studies have lacked paired tumor samples pre- and post-osimertinib, which makes determination of acquired alterations and putative resistance

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Translational Relevance

Prior reports of mechanisms of resistance to osimertinib primarily focus on patients who received osimertinib after other EGFR-tyrosine kinase inhibitors and rely heavily upon circulating tumor DNA. We utilized paired tumor tissue with next-generation sequencing performed before osimertinib and after progression to analyze resistance mechanisms in a cohort of 62 patients with *EGFR*-mutant lung cancer treated with osimertinib, either as first-line or later-line treatment. We identified lineage plasticity and in particular, squamous histologic transformation, as unexpectedly frequent with first-line osimertinib and associated with considerable genomic complexity, highlighting the importance of tissue-based analyses to evaluated acquired resistance. We also detected a diverse array of off-target genomic resistance mechanisms that may be amenable to targeted therapy. Sensitizing *EGFR* mutation, time on osimertinib therapy, and line of therapy all may influence the resistance spectra identified. Finally, we validate two novel resistance alterations, *EGFR* S768 + V769L and *MET* H1094Y and explore relevant potential treatment options.

mechanisms challenging. Therefore, we sought to use paired tumor tissue to detect molecular and histologic mechanisms of resistance to osimertinib and identify potential associations with clinical outcomes.

Materials and Methods

In accordance with the Belmont report and following the Institutional Review Board (IRB)/Privacy Board at Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY) for retrospective review of records and waiver of consent, we retrospectively identified all patients with *EGFR*-mutant metastatic lung cancers who received osimertinib and had pretreatment and postprogression tumor samples (acquired resistance defined by Jackman criteria; ref. 26), where targeted hybrid capture, next-generation sequencing (NGS) of tumor DNA had been performed. The primary NGS platform was MSK-IMPACT (27), but other NGS platforms, such as MSK-Ampliseq (Supplementary Materials and Methods) and Foundation Medicine NGS (28), were occasionally utilized. For MSK-IMPACT, patients were consented to MSKCC IRB protocol 12-245. Patients were divided into two cohorts: (i) patients who received osimertinib without prior EGFR-TKI exposure ("first-line" osimertinib), and (ii) patients who received osimertinib after prior EGFR-TKIs ("later-line" osimertinib).

Patient records were reviewed to extract demographic information, clinical outcomes, and molecular and histologic data. Time-to-treatment discontinuation (TTD) was defined as time from start of EGFR-TKI to last administered dose prior to a treatment change (29). Overall survival (OS) was defined as date of osimertinib initiation to date of death or last follow-up as of May 1, 2019. Fisher exact and log-rank tests were used to identify associations between clinical, molecular, and histologic features, and Kaplan–Meier methodology was used for TTD and OS. In all cases of transformation, the original pathology samples were re-reviewed to confirm the absence of preexisting neuroendocrine, squamous/adenosquamous, or small cell histology using IHC performed for p40, TTF1, RB1, and p53.

MSK-Fusion Solid, a custom RNAseq panel, was used to detect fusions in cases where no resistance mechanism was identified by NGS and sufficient tissue was available (30). Single nucleotide variants and copy number variants identified by MSK IMPACT were analyzed on the cBioPortal (31). Specific mutations were assessed for enrichment with the McNemar test using paired samples and Fisher exact test in unpaired analyses. Additional methods are provided in the Supplementary Materials and Methods.

Results

Clinical characteristics

Sixty-two patients were identified with acquired resistance to osimertinib and paired tumor tissue available for analyses.

Table 1. Clinical characteristics of patients with acquired resistance to osimertinib by line of therapy.

Clinical characteristics	First-line osimertinib (%)		Later-line osimertinib (%)		Total *N* (%)	
Total	**27**		**35**		**62**	
Age						
Median (range)	58 (44–75)		59 (40–75)		58 (40–75)	
Sex						
Male	12 (44)		11 (31)		23 (37)	
Female	15 (56)		24 (69)		39 (63)	
Smoking						
Never smoker	17 (63)		22 (63)		39 (63)	
Former smoker	10 (37)		13 (37)		23 (37)	
Histology	Before Osi	After Osi	Before Osi	After Osi	Before Osi	After Osi
Adenocarcinoma	24 (89)	20 (74)	35 (100)	28 (80)	59 (95)	49 (79)
Squamous	2 (7)	4 (15)	0 (0)	3 (9)	2 (3)	6 (10)
Neuroendocrine	0 (0)	1 (4)	0 (0)	2 (6)	0 (0)	3 (5)
Other	1 (4)	2 (7)	0 (0)	2 (6)	1 (2)	4 (7)
EGFR mutation						
Exon 19 deletion	14 (52)		28 (80)		42 (68)	
L858R	11 (41)		6 (17)		17 (27)	
T790M	1 (4)		32 (91)		33 (53)	
Other	2 (7)		1 (3)		3 (5)	

Note: The demographics, histology, and sensitizing *EGFR* mutation for the two cohorts and the overall population is shown.
Abbreviation: Osi, osimertinib.

Twenty-seven patients received first-line osimertinib and 35 patients received later-line osimertinib (after prior EGFR-TKI). The clinical characteristics of the two groups are presented in **Table 1**. The cohorts had similar clinical characteristics with the exception of shorter follow-up on the first-line patients, which reflects the recent approval of osimertinib as a first-line treatment (April 2018). By the data cut-off date (May 1, 2019), over two thirds of patients treated with first-line osimertinib at MSKCC were still on therapy (32% discontinued first-line osimertinib), whereas the majority of patients treated with later-line osimertinib at MSKCC had discontinued treatment (64% discontinued later-line osimertinib). Median follow-up on for patients who received first-line osimertinib was 17.2 months (95% confidence interval, 12.0–24.9 months) and later-line osimertinib 28.5 months (95% confidence interval, 19.7–32.5 months). Among the patients reported with resistance to first-line osimertinib, the median TTD on osimertinib was 13.6 months (95% confidence interval, 12.4–25.5 months) with 19 (70%) patients alive at data cutoff. The median TTD on osimertinib for the later-line cohort was 15.2 months (95% confidence interval, 13.0–19.9 months) with 25 (71%) patients alive at data cutoff.

Concurrent genomic alterations seen with first-line osimertinib

The molecular landscape of concurrent alterations identified before and after treatment with first-line osimertinib is depicted in **Fig. 1A**. The most frequent cooccurring pretreatment mutation was *TP53* (70%, $n = 19$). Common co-occurring amplifications/deletions were *EGFR* amplifications (33%, $n = 9$) and *CDKN2A/B* deletions (15%, $n = 4$ each). There was no significant enrichment of specific alterations in either the pretreatment or posttreatment setting (**Fig. 1B**). Known mechanisms of acquired resistance to EGFR-TKIs were identified in 41% ($n = 11$) of cases (**Fig. 1C**). In the first-line setting, *EGFR* G724S was the only on-target acquired mutation identified. Off-target resistance mechanisms included *MET* amplifications (Supplementary Table S1), *MET* H1094Y mutation, *KRAS* G12A mutation, *TRIM24-BRAF* fusion, and *RUFY2-RET* fusion.

Concurrent genomic alterations seen with later-line osimertinib

In patients who received later-line osimertinib, the most frequent cooccurring mutations pre-osimertinib were *TP53* (54%, $n = 19$), and *RB1* (14%, $n = 5$); the most common cooccurring amplification was *EGFR* (31%, $n = 11$; **Fig. 2A**). Among the patients that received

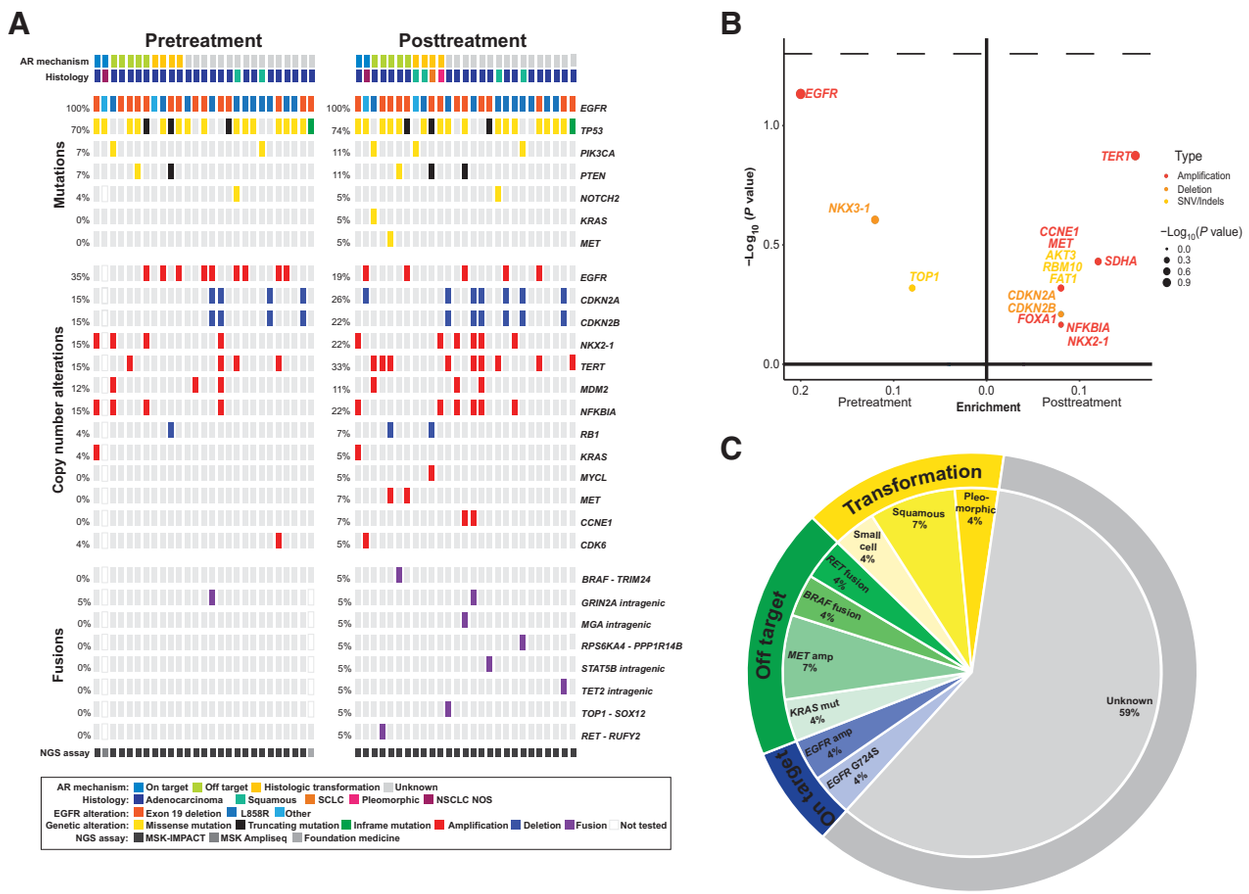


Figure 1.

Genomic alterations identified with first-line osimertinib. **A,** Frequency of alterations pre- and post-osimertinib in patients treated with first-line osimertinib. The in-figure legend specifies details on acquired resistance mechanism, histology, initial *EGFR* mutation, alteration type, and NGS assay. **B,** Enrichment of individual altered genes pre-osimertinib (left) and post-osimertinib (right). Alteration type is indicated in the in-figure legend. The dashed line represents a $P = 0.05$. The frequency difference between the two sample sets is plotted on the *x*-axis and its significance [−log10(*P* value)] on the *y*-axis. **C,** The distribution of established mechanisms of resistance by type of alteration in patients treated with first-line osimertinib. AR, acquired resistance.

later-line osimertinib, *EGFR* T790M ($P = 0.003$) was enriched in the pre-osimertinib samples and acquisition of *EGFR* C797S ($P = 0.04$) was enriched in the postprogression samples (**Fig. 2B**). Established mechanisms of resistance were identified in 71% (25 patients) of cases (**Fig. 2C**). *EGFR* C797 mutations (C797S = 9 or C797G = 1) were common, occurring in 29% of cases. Off-target resistance mechanisms, including a *AGK-BRAF* fusion, *MET* exon 14 alteration, and *KRAS* G12D mutation, occurred in cells lacking *EGFR* T790M, while *EGFR* T790M was retained with *ALK* fusions and an *ERBB2* Y772_A775dup.

Comparisons between first-line and later-line osimertinib

Overall, *EGFR*-mediated acquired resistance was associated with a longer time on osimertinib and improved OS compared with other resistance mechanisms (median TTD 18.0 months; 95% confidence interval, 13.3–33.2; vs. 13.2 months, 95% confidence interval, 10.6–16.3; $P = 0.04$; median OS not reached vs. 29 months 95% confidence interval, 24.6–not reached; $P < 0.001$). Notably, the proportion of off-target resistance in the first-line setting was higher than the later-line cohort ($P = 0.01$) suggesting that off-target resistance emerges earlier and/or treatment with first-line osimertinib may enrich for off-target resistance.

Associations with sensitizing EGFR mutation

Acquired alterations were analyzed by initial sensitizing *EGFR* mutation (exon 19 deletion vs. L858R mutation; **Fig. 3A** and **B**). *EGFR* C797S was more frequently seen with *EGFR* exon 19 deletion (**Fig. 3A** and **B**; $P = 0.03$). Posttreatment *CDKN2A/B* deletions and *TERT* amplifications were more commonly seen with *EGFR* L858R mutations ($P = 0.02$; $P = 0.03$, respectively). However, these associations were not significant when accounting for multiple comparisons.

Histologic transformation

Histologic transformation was identified in 15% of cases (9 patients), 15% in the first-line setting and 14% in the later-line setting (**Fig. 4A**). We identified five cases of squamous cell transformation (**Fig. 4B**), one case of pleomorphic transformation with squamous, sarcomatoid, and small cell features, and three cases of small cell transformation. There was no overarching genomic correlate associated with squamous

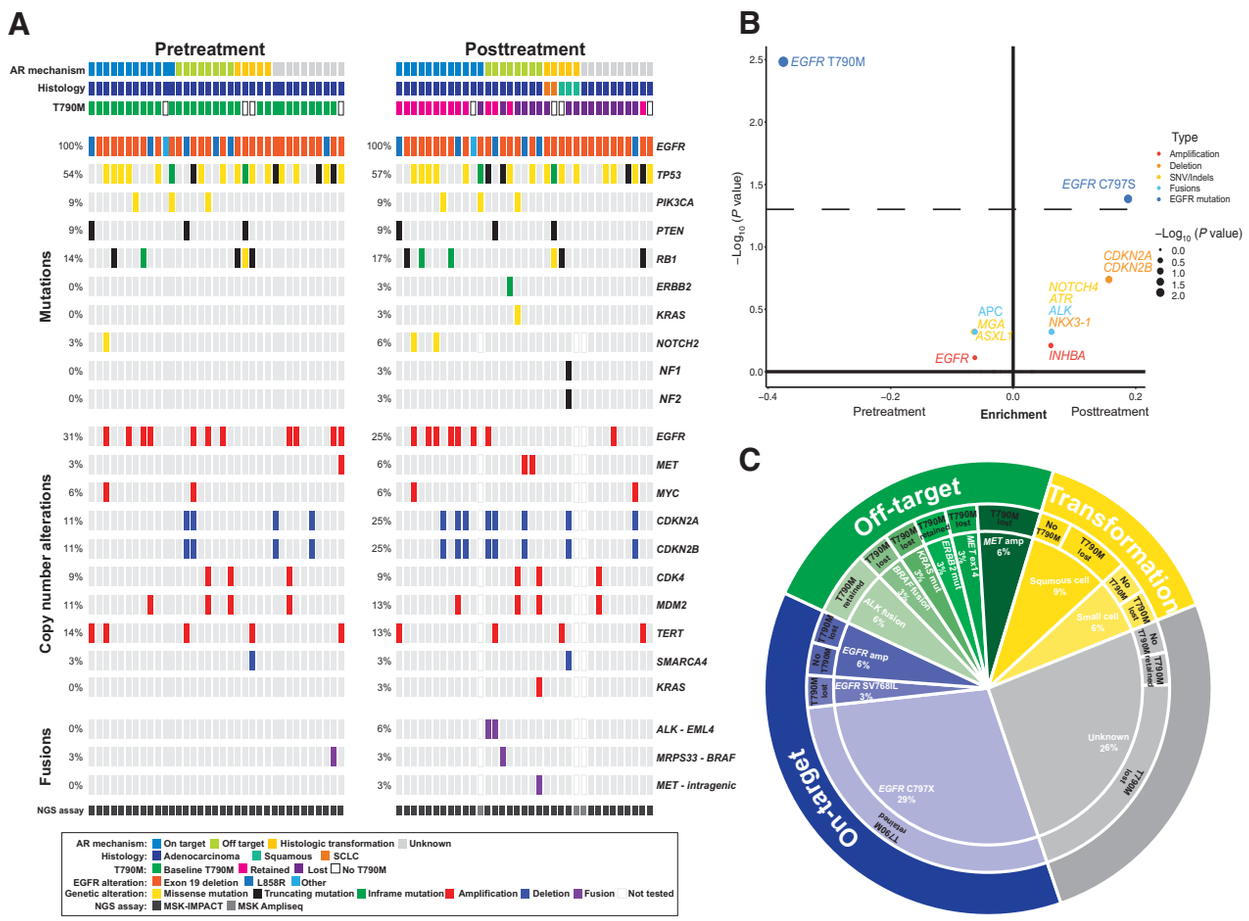


Figure 2.

Genomic alterations identified with later-line osimertinib. **A,** Frequency of alterations pre- and post-osimertinib in patients treated with later-line osimertinib. The in-figure legend specifies details on acquired resistance mechanism, histology, initial *EGFR* mutation, alteration type, and NGS assay. **B,** Enrichment of individual altered genes pre-osimertinib (left) and post-osimertinib (right). The dashed line represents a $P = 0.05$. The frequency difference between the two sample sets is plotted on the *x*-axis and its significance [−log10(*P* value)] on the *y*-axis. **C,** The distribution of established mechanisms of resistance by type of alteration in patients treated with later-line osimertinib. AR, acquired resistance.

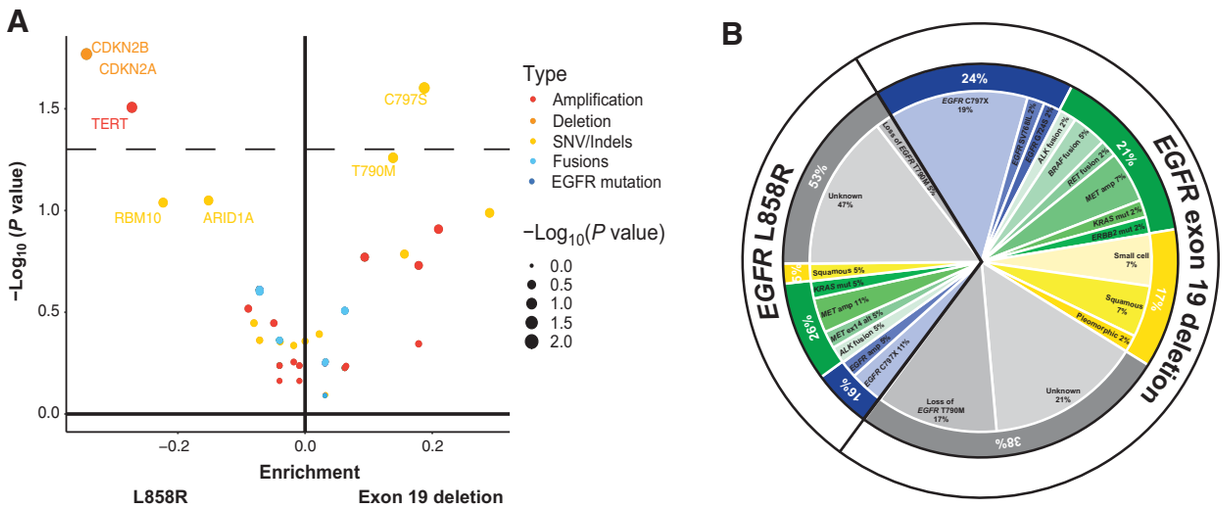


Figure 3.

Molecular alterations by sensitizing *EGFR* mutation. **A,** Enrichment of genomic alterations by sensitizing *EGFR* mutation. The dotted line represents a $P = 0.05$. Enrichment of mutations in patients with *EGFR* exon 19 deletions (right) versus *EGFR* L858R (left). For each mutation, the frequency difference between the two cohorts is plotted on the *x*-axis and its significance [−log10(*P* value)] on the *y*-axis. **B,** The distribution of mechanisms of resistance organized by sensitizing *EGFR* mutation.

transformation. One patient acquired a *PIK3CA* E726K mutation; no preexisting or acquired *SOX2* amplifications were identified, but 1 patient had low-level chromosome 3q gain and 1 had low level *PIK3CA* copy number gain in both the pretreatment and posttreatment samples (**Fig. 4A**). The patient with pleomorphic transformation acquired a very high level chromosome 3q amplification (49-fold) and *FGF3/FGF4/FGF19* amplification (15-fold; **Fig. 4A**). Consistent with prior literature, the small-cell lung cancer tumors (SCLC) all had preexisting alterations of *RB1* and *TP53* identified in their pretreatment samples (**Fig. 4A**).

Clinical course after treatment with osimertinib

Patient outcomes post-osimertinib are summarized by resistance mechanism and line of therapy (**Fig. 5**). Five of the 9 patients with histologic transformation have died (4 within 10 months post-osimertinib progression). The patients with small-cell transformation all received platinum with etoposide as a part of their subsequent therapy (**Fig. 4C**). Among the 5 patients with squamous transformation, varying treatment strategies were employed and outcomes were mixed with limited follow-up (**Fig. 4C**). In several instances, patients with acquired off-target alterations were treated with targeted therapies. For instance, 2 patients with tumors that acquired *ALK* fusions were treated with osimertinib and ALK TKIs (crizotinib, alectinib, and lorlatinib) with durable responses (**Fig. 5C** and **D**). The clonal evolution of identified alterations is illustrated over interval biopsy samples for 2 patients (**Fig. 5C** and **D**).

Functional studies of *MET* H1094 and *EGFR* SV768IL, as novel resistance mechanisms to osimertinib

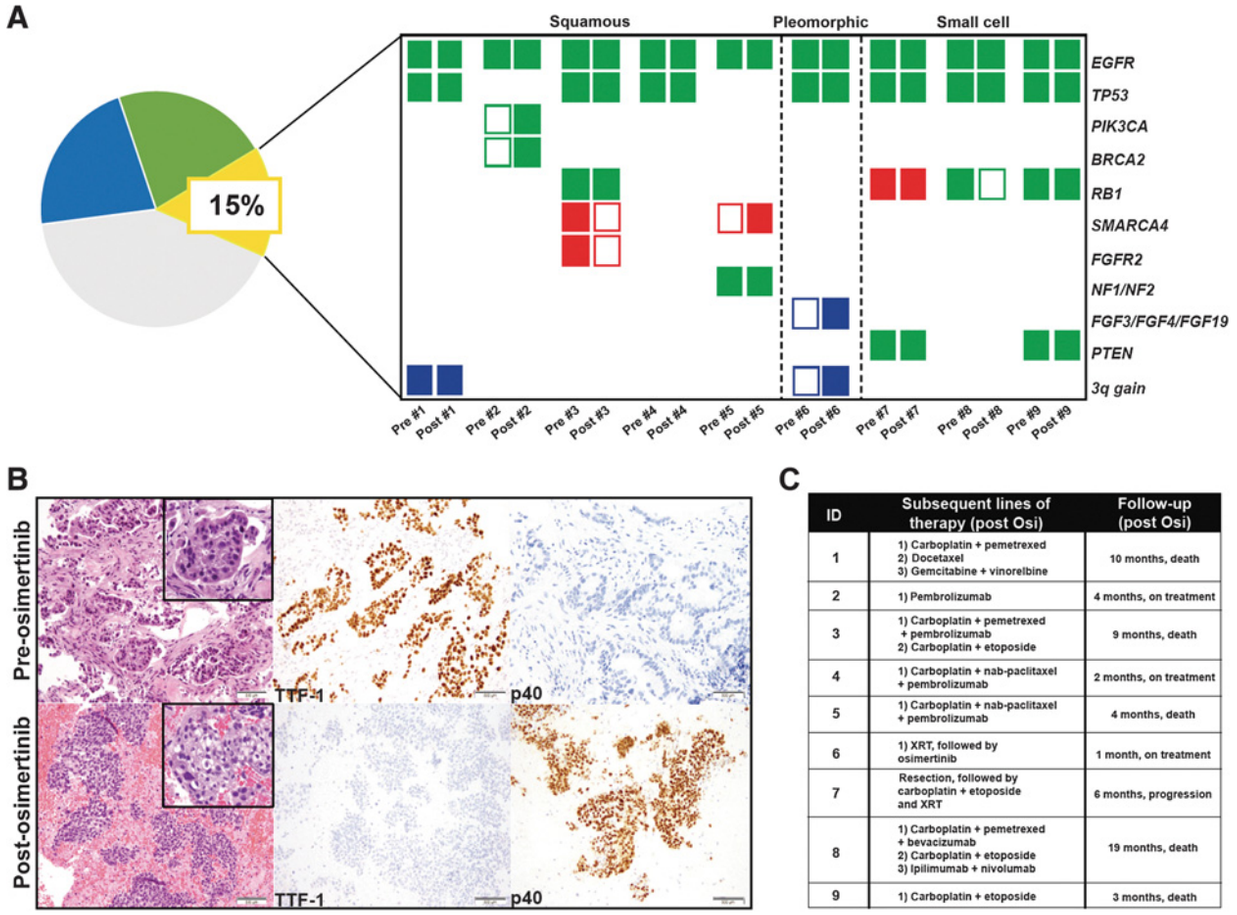
We hypothesized that acquired alterations in *MET* H1094, identified in the first-line setting and *EGFR* S768I + V769L (*EGFR* SV768IL) mutations found in the later-line setting could represent putative resistance mechanisms and explored these alterations in preclinical models.

MET H1094 mutations confer resistance to osimertinib

We expressed wild-type *MET, MET* H1094R, and *MET* H1094Y, and a kinase dead *MET* H1094Y (K1110A mutant) in the PC9, *EGFR* exon 19 deleted lung adenocarcinoma cell line. Mutations were introduced into *MET* using site-directed mutagenesis and mutations confirmed by DNA sequencing (**Fig. 6A**). Western blotting of whole-cell extracts confirmed overexpression of MET and the corresponding mutants (**Fig. 6B**). The two *MET* mutants demonstrated similar levels of MET phosphorylation versus wild-type MET. The kinase dead *MET* H1094Y mutant showed a low level of MET phosphorylation. Sensitivity to growth inhibition by osimertinib was reduced in PC9 cells expressing either *MET* H1094 mutant compared with PC9 cells expressing an empty vector (EV) plasmid (PC9-EV) or PC9 cells overexpressing wild-type MET (PC9-MET; ref. **Fig. 6C**). The *MET* H1094R mutant induced greater resistance to osimertinib compared with the *MET* H1094Y mutant (959- vs. 136-fold increase in IC_{50} value for growth inhibition, compared with PC9-EV cells). PC9 cells expressing the two *MET* H1094 mutants were slightly more sensitive to crizotinib than the PC9-EV or PC9-MET cells (**Fig. 6D**).

Combined inhibition of MET and EGFR overcomes *MET* H1094-mediated osimertinib resistance

To determine whether combination crizotinib and osimertinib would be an effective therapeutic strategy for *EGFR*-mutant cancers expressing MET-H1094, we examined whether these two drugs act synergistically to inhibit growth. We used the method of Chou–Talalay (32), where cells are treated with drug combinations and then a parameter called the combination index (CI) is determined. CI < 1 indicates synergy, CI > 1 indicates antagonism between the two drugs, and CI = 1 indicates an additive effect. Growth was inhibited in PC9-MET-H1094Y cells when the two drugs were combined, compared with either drug alone (**Fig. 6E** and **F**). The CI values were <1 for all drug concentrations tested (**Fig. 6G**). We next examined the effect of the *MET* H1094Y mutation on osimertinib-induced caspase 3/7 activity as a measure of apoptosis. Treatment of PC9-EV cells with



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ID	Subsequent lines of therapy (post Osi)	Follow-up (post Osi)
1	1) Carboplatin + pemetrexed 2) Docetaxel 3) Gemcitabine + vinorelbine	10 months, death
2	1) Pembrolizumab	4 months, on treatment
3	1) Carboplatin + pemetrexed + pembrolizumab 2) Carboplatin + etoposide	9 months, death
4	1) Carboplatin + nab-paclitaxel + pembrolizumab	2 months, on treatment
5	1) Carboplatin + nab-paclitaxel + pembrolizumab	4 months, death
6	1) XRT, followed by osimertinib	1 month, on treatment
7	Resection, followed by carboplatin + etoposide and XRT	6 months, progression
8	1) Carboplatin + pemetrexed + bevacizumab 2) Carboplatin + etoposide 3) Ipilimumab + nivolumab	19 months, death
9	1) Carboplatin + etoposide	3 months, death

Figure 4.

The genomic profile and clinical outcomes of patient with histologic transformation. **A,** Genomic patterns pre- and post-osimertinib by histologic transformation subtype. Green shaded rectangle represents mutation. Blue shaded rectangle represents copy number gain. Red shaded rectangle represents deletion. **B,** Pretreatment biopsy shows adenocarcinoma, which is positive for TTF-1 and negative for p40 by IHC. Posttreatment sample show squamous cell carcinoma, which is negative for TTF-1 and positive for p40 by IHC. **C,** Treatment regimens received by patients with histologic transformation and OS after osimertinib (Osi).

osimertinib induced activation of caspase 3/7 as expected but did not stimulate caspase 3/7 activity in PC9-MET or PC9-MET-H1049Y cells (**Fig. 6H**). In contrast, treatment of the PC9 cell lines with crizotinib alone did not stimulate caspase 3/7 activity in any of the cell lines (**Fig. 6H**). However, a combination of crizotinib and osimertinib resulted in a significant increase in caspase 3/7 activity in PC9-MET and PC9-MET-H1094Y (**Fig. 6G**). Taken together, these results endorse *MET* H1094Y to be a novel mechanism of resistance to osimertinib that can be overcome by combined inhibition of MET and EGFR.

Compound *EGFR* S768I+V769L (*EGFR* SV768IL) mutations confer resistance to osimertinib

To determine whether the observed mutations in *EGFR* exon 20 confer resistance to osimertinib, we generated cell lines with the S768I and V769L mutations (EGFR-SV768IL) by site-directed mutagenesis (Supplementary Fig. S1A). Wild-type EGFR or EGFR-SV768IL was expressed in PC9 and HCC827 cell lines and expression confirmed by Western blotting (Supplementary Fig. S1B). EGFR-SV768IL was more heavily phosphorylated than wild-type EGFR in both cell lines, indicating higher level of activity (Supplementary Fig. S1B) of the SV768IL variant. Similarly, expression of EGFR-SV768IL resulted in phosphorylation of ERK1/2 and AKT to a higher extent than that observed with expression of wild-type EGFR. Growth of PC9 (Supplementary Fig. S1C) and HCC827 (Supplementary Fig. S1C) expressing EGFR-SV768IL was resistant to the inhibitory effects of osimertinib, compared with cells expressing either an empty vector (EV) or wild-type EGFR. The IC_{50} value of osimertinib for PC9-EGFR-SV768IL cells was 166-fold higher than that for PC9-EV cells (Supplementary Fig. S1C). Similarly, the IC_{50} value of osimertinib in HCC827-EGFR SV768IL cells was 244-fold higher than that of HCC827-EV cells (Supplementary Fig. S1C). We also examined the effect of EGFR-SV768IL on osimertinib-induced caspase 3/7 activity in PC9 cells. Whereas osimertinib caused a significant increase in caspase 3/7 activity in PC9-EV and PC9-EGFR cells, no increase was observed in PC9-EGFR-SV768IL cells (Supplementary Fig. S1D).

Discussion

We identified an array of acquired resistance mechanisms to osimertinib using paired pre- and posttreatment tissue samples. In our cohort of first-line patients with limited follow-up and shorter time

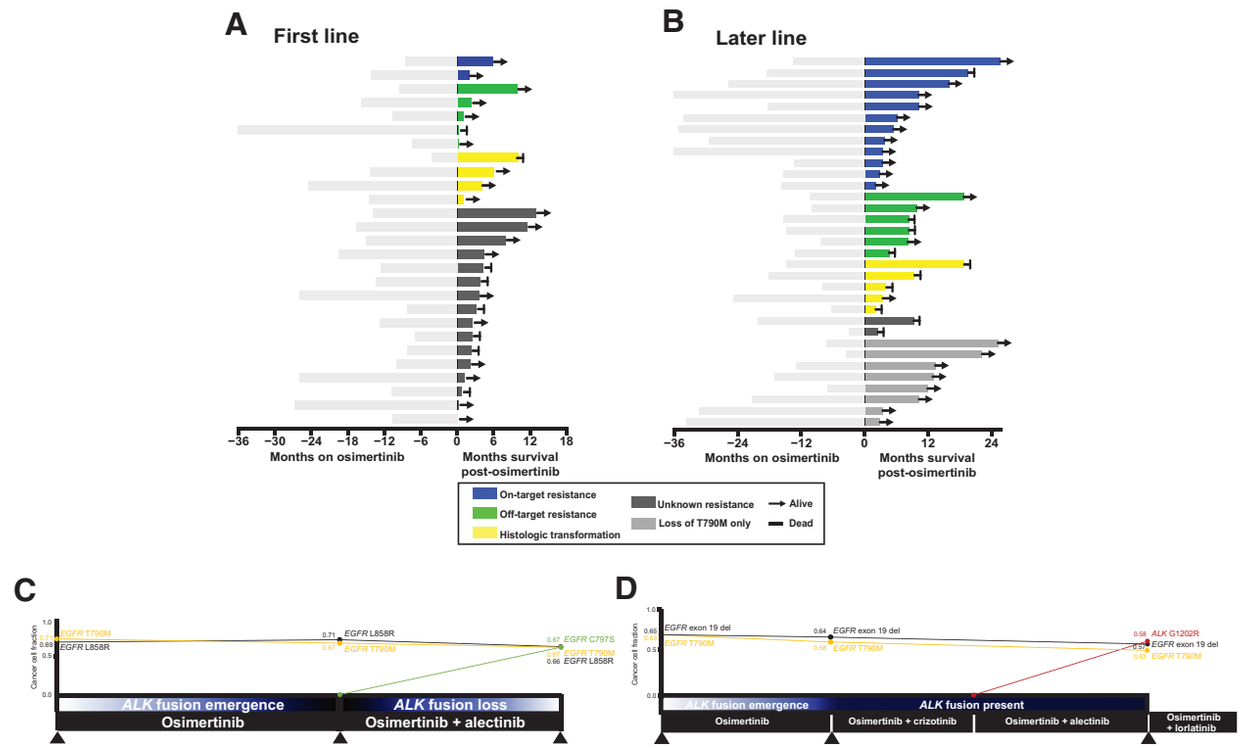

Figure 5.

Clinical outcomes pre/post-osimertinib progression by resistance mechanism. Time on osimertinib (months) is depicted to the left (light gray) and OS after osimertinib (months) is depicted on the right for patients who received first-line osimertinib (**A**), received later-line osimertinib (**B**). **C** and **D,** Longitudinal analysis of 2 patients who received later-line osimertinib. **C,** One patient acquired an *EGFR* C797S mutation and lost an *ALK* fusion after treatment with osimertinib and alectinib. **D,** The other patient acquired an *ALK* G1202R mutation after treatment with osimertinib and alectinib. Treatment summaries from osimertinib onwards are noted in black bars with time (months) along the *x*-axis. Summary of molecular alterations (MSK-IMPACT) prior to starting osimertinib and at two resistance time points are shown for each patient (colored lines). Cancer cell fractions of driver and resistance alterations based on FACETS analysis (see Supplementary Materials and Methods) at each biopsy timepoint.

on osimertinib, *EGFR*-mediated resistance was uncommon, whereas off-target resistance, including histologic transformation, was seen frequently. Notably, there appears to be a time-dependent pattern of resistance with off-target resistance emerging earlier resulting in less durable responses to osimertinib. This mirrors our earlier finding that on-target resistance mutations (i.e., *EGFR* T790M) are associated with more indolent disease and arise as after a longer time on EGFR-TKI and with better postprogression survival (33). Development of off-target resistance after a short period may result from preexisting subclones that emerge quickly on treatment. To ascertain whether resistance mechanisms to first- and later-line osimertinib truly differ will require lengthier follow-up in patients on first-line osimertinib.

EGFR C797S, the most common *EGFR* mutation acquired on later-line osimertinib, was not identified in our first-line cohort of patients. The frequency of *EGFR* C797S in the first-line FLAURA study was 8% (15); both first-line cohorts (ours and FLAURA) report lower frequencies of *EGFR* C797S compared with later-line osimertinib cohorts (15%–32%; Supplementary Fig. S2; refs. 6, 8, 10, 34, 35) again suggesting first-line and later-line osimertinib may have different resistance spectra. Later-line osimertinib is utilized only in tumors with acquired *EGFR* T790M; these tumors have demonstrated continued dependence on EGFR signaling and may be predisposed to acquire tertiary *EGFR* mutations (i.e., *EGFR* C797S) compared with *EGFR*-mutant tumors at large resulting in the disparate frequencies of *EGFR*-acquired mutations in the first-line and later-line setting. *MET* amplification was also identified at a lower rate (7%) than most reports in the later-line setting (10%–26%; Supplementary Fig. S2; refs. 6, 8, 10, 34, 35) and is on the lower-end of first-line reports (5%–15%; Supplementary Fig. S2; refs. 15, 36). Previous studies lacking pretreatment tissue or plasma may overestimate acquired *MET* amplifications, which can be seen concurrently with *EGFR* prior to treatment (6, 35, 37, 38). In addition, plasma-based platforms typically have lower sensitivity to assess copy number changes (21–25).

Tissue analysis is critical to characterizing resistance mechanisms. Histologic transformation, which cannot be detected via plasma testing, was a frequent mechanism of resistance in our study. Rates of transformation and other off-target resistance mechanisms may be higher with osimertinib compared with earlier generation TKIs due to better on-target inhibition. Prior to this report, squamous cell transformation was identified infrequently (5, 8, 16–20). This phenomenon is surely under recognized because of the increasing reliance on ctDNA for identification of resistance mechanisms. Recognition of histologic transformation is imperative as it has prognostic and therapeutic implications. Patients with squamous cell transformation in our cohort had short postprogression survival (**Figs. 4** and **5**). Similar to small-cell transformation (1, 2, 39), patients with squamous cell transformation may require treatments tailored to this cancer type. Primary squamous cell lung cancers, unlike the prerequisite *RB1* and

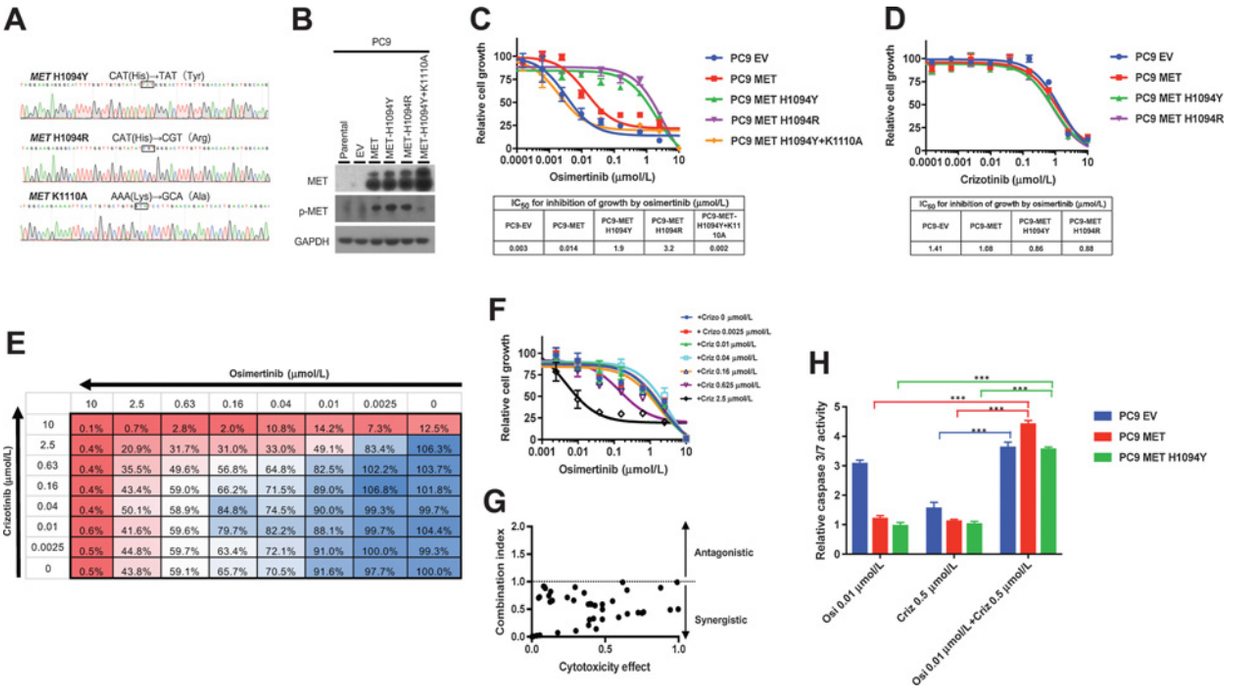

Figure 6.

Combined inhibition of MET and EGFR overcomes MET-H1094–mediated osimertinib resistance. **A,** *MET*-H1094 mutations were introduced by site-directed mutagenesis and confirmed by Sanger sequencing. **B,** Plasmids containing the *MET* mutants were stably introduced into PC9 cells via lentiviral transduction and then expression of MET confirmed by Western blotting. Isogenic PC9 cells were treated with osimertinib (Osi; **C**) or crizotinib (Criz; **D**) for 96 hours and then growth determined using alamarBlue viability dye (top). IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism (bottom). **E–G,** Cells were treated with the indicated combined concentrations of osimertinib and crizotinib for 96 hours and then growth determined. **E** and **F,** The percent inhibition of growth at each drug combination. **G,** The Chou–Talalay method was used to examine whether crizotinib and osimertinib inhibited growth in a synergistic manner. The dot plot shows the CI as a function of the fraction affected. CI < 1 indicates synergy between the two inhibitors. **H,** Caspase 3/7 activity was determined in cells treated for 48 hours with the indicated inhibitors for 48 hours and then caspase 3/7 activity determined. Results represent the fold change in caspase 3/7 enzymatic activity above the corresponding untreated cells. All experiments were repeated three times and included triplicate determinations of each condition. The K110A mutation results in an inactive kinase. Results represent the mean ± SD (***, $P < 0.0001$).

TP53 mutations identified in small-cell lung cancers, exhibit considerable genomic complexity. Understanding the etiology of squamous cell transformation will require comprehensive investigation, made more challenging by the fact that *de novo* squamous cell lung cancers do not have an overarching genomic signature. Further study will include understanding the gene expression subtype of the transformed cases and assessing nongenomic processes that may play a role in histologic transdifferentiation such as transcription factor networks and the epigenome.

Recent data suggest that the initial sensitizing *EGFR* mutation may bias the resistance mechanisms that emerge. To date, *EGFR* G724S has only been identified with *EGFR* exon 19 deletions, and structural and *in vitro* models support *EGFR* G724S as conferring resistance only when concurrent with an *EGFR* exon 19 deletion (40). We similarly demonstrate that *EGFR* C797S is preferentially coupled with *EGFR* exon 19 deletions. We also confirm the previous findings that *EGFR* C797S was only seen in tumors that retained *EGFR* T790M suggesting continued *EGFR* dependence in these tumors. Typically, off-target or unknown resistance mechanisms are seen in the absence of *EGFR* T790M suggesting a loss of *EGFR* dependence in these tumors.

We and others have described acquired chromosomal rearrangements (*ALK, RET, BRAF, ERBB2*, and *MET* exon 14) as resistance mechanisms to EGFR-TKIs (5–7, 10–15, 41–43). We again identified *BRAF, ALK*, and *RET* fusions in this series. The relatively high frequency of these otherwise extremely rare oncogene fusions in the setting of acquired resistance to osimertinib requires further exploration. Eight percent of all *RET* fusions and 50% of all *BRAF* fusions identified in lung cancers at MSKCC by MSK-IMPACT over the time period of this study were found in patients with *EGFR*-mutant lung cancer and acquired resistance to osimertinib (31, 44). This high frequency of acquired fusions supports a predisposition for genomic rearrangements driven by the selective pressure of osimertinib.

We also identified and validated the mutation *MET* H1094Y and the compound mutation *EGFR* S768 + V769L as resistance mechanisms with potential associated treatments. Prior work demonstrated increased catalytic activity and cognate autophosphorylation of MET H1094Y as compared with the wild-type MET kinase domain, confirming *MET* H1094Y to be an oncogenic and transformative mutation. From a structural perspective, this mutation resides in close proximity to the ATP-binding site, and based on molecular modeling studies, these mutations may activate MET kinase by destabilizing the inhibitory conformation of the activation loop (45, 46). Prior case reports of *de novo EGFR* S768I and V769L compound mutations have been published with mixed responses to first- and second-generation EGFR TKIs (47–49). It is not clear whether treating a

patient who acquires on-target resistance to osimertinib will respond to early generation TKIs and the appropriate trials are underway (NCT03755102; ref. 9).

Over half of our first-line cohort had unknown mechanisms of resistance. In these cases, resistance may be due to epigenetic modifications, changes in protein expression, or novel genomic alterations. Further analyses will need to integrate epigenetic, RNA, and protein expression analyses to uncover the yet undetermined mechanisms of resistance to osimertinib. In addition, clonal evolution and tumor heterogeneity also play a fundamental role in resistance to targeted therapies and should be considered in future analyses. As osimertinib has only recently been integrated as first-line treatment, our first-line cohort was biased toward resistance mechanisms that emerge earlier on treatment and makes directed comparison with the later-line cohort challenging, but provides the unique perspective of identifying early emerging mechanisms of resistance. Another limitation of our study is that histologic transformation could also represent outgrowth of a preexisting clone of tumor cells that were not previously identified. However, multiple sections throughout each sample of pathologic tissue were reexamined to confirm no evidence of preexisting squamous cell or small cell histology. This will be an overarching limitation for all future studies of lineage plasticity in this patient subset since most metastatic patients only have small core-needle biopsies done. Also, molecular data from single-lesion biopsies may not reflect the entirety of genetic alterations due to tumoral heterogeneity. Finally, although our sample size was modest, this is the largest analysis to date of osimertinib resistance utilizing paired tumor tissue.

In conclusion, our study establishes that mechanisms of resistance to osimertinib are diverse, with sensitizing *EGFR* mutation, time on osimertinib therapy, and line of therapy all influencing the resistance spectra identified. Off-target resistance arises early on first-line osimertinib after a shorter duration of osimertinib treatment. Histologic transformation appears common with first-line osimertinib and highlights the continued importance of tissue-based assays to evaluate acquired resistance. With resistance mechanisms dependent on original sensitizing *EGFR* mutation, further assessment of how pretreatment alterations forecast resistance will be important as the field amends first-line treatments to delay or prevent resistance. Identifying and overcoming these resistance mechanisms will require a multifaceted approach utilizing both plasma and tissue molecular and histopathologic analyses.

Disclosure of Potential Conflicts of Interest

P.K. Paik is an employee/paid consultant for Celgene, EMD Serono, AstraZeneca, Calithera, and Takeda. M. Offin reports receiving speakers bureau honoraria from PharmaMar, Novartis, Targeted Oncology, and OncLive. M.E. Arcila reports receiving speakers bureau honoraria from Biocartis and Invivoscribe. M.G. Kris is an employee/paid consultant for AstraZeneca, Pfizer, and Regeneron. R. Somwar reports receiving commercial research grants from Helsinn Healthcare, Loxo Oncology, and 14NER Oncology. G.J. Riely reports receiving commercial research grants from Merck, Pfizer, Novartis, Takeda, and Mirati, and is an advisory board member/unpaid consultant for Takeda and Merck. M. Ladanyi is an employee/paid consultant for National Comprehensive Cancer Network/AstraZeneca. H.A. Yu is an employee/paid consultant for Daiichi and AstraZeneca, and reports receiving other commercial research support from AstraZeneca, Daiichi, Pfizer, Novartis, and Lilly. No potential conflicts of interest were disclosed by the other authors.

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