Concurrent TP53 Mutations Facilitate Resistance Evolution in EGFR-Mutant Lung Adenocarcinoma

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Methods: In this retrospective study, targeted next-generation sequencing data were collected from patients with EGFR-mutant lung cancer treated at the Dana-Farber Cancer Institute. Clinical data were collected and correlated with somatic mutation data. Associations between TP53 mutation status, genomic features, and mutational processes were analyzed.

Results: A total of 269 patients were identified for inclusion in the cohort. Among 185 response-assessable patients with pretreatment specimens, TP53 alterations were the most...
common event associated with decreased first-line progression-free survival and decreased overall survival, along with *DNMT3A*, *KEAP1*, and *ASXL1* alterations. Reduced progression-free survival on later-line osimertinib in 33 patients was associated with *MET, APC, and ERBB4* alterations. Further investigation of the effect of *TP53* alterations revealed an association with worse outcomes even in patients with good initial radiographic response, and faster acquisition of T790M and other resistance mechanisms. *TP53*-mutated tumors had higher mutational burdens and increased mutagenesis with exposure to therapy and tobacco. Cell cycle alterations were not independently predictive, but portended worse OS in conjunction with *TP53* alterations.

**Conclusions:** *TP53* alterations associate with faster resistance evolution independent of mechanism in EGFR-mutant NSCLC and may cooperate with other genomic events to mediate acquisition of resistance mutations to EGFR tyrosine kinase inhibitors.

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**Keywords:** EGFR; Non-small cell lung cancer; Genomics; Resistance

**Introduction**

EGFR-directed tyrosine kinase inhibitors (TKIs) have dramatically improved outcomes for patients whose tumors harbor sensitizing EGFR alterations. Nevertheless, long-term disease control remains elusive for most patients owing to the inevitable development of resistance, typically within 8 to 18 months. Genetic analyses to date have largely focused on identifying acquired mechanisms of resistance by profiling specimens at time of progression; these analyses have identified EGFR-dependent mechanisms of resistance (e.g., *EGFR T790M* and *C797S* mutations) and EGFR-independent mechanisms that allow tumors to bypass EGFR pathway inhibition (e.g., small cell transformation or acquisition of other driver alterations, such as *MET* amplification). These studies helped define how cancers escape inhibition by EGFR TKIs, but they do not explain what determines when and how a tumor will develop resistance. Understanding the biological basis of differential time to progression could help explain why some tumors evolve resistance within weeks and others remain suppressed for years, and further inform novel therapeutic strategies to delay resistance evolution.

Our study builds on previous work examining the hypothesis that the genomic context of the driver *EGFR* mutation plays a role in when and how resistance develops. Previous studies analyzing the impact of pretreatment comutations have identified worse outcomes associated with alterations in *TP53, PIK3CA*, *PTEN*, and others. Nevertheless, these analyses have been limited by small sequencing panels, incomplete clinical annotations, or small cohort size, and it is likely that the diversity of co-occurring interactions remains underexplored. Here, we have assembled a cohort of patients treated with EGFR TKIs assessed by targeted next-generation sequencing (NGS) with comprehensive clinical annotations to further explore the role of concurrent alterations in mediating differential outcomes to EGFR TKI therapy.

**Materials and Methods**

**Study Population**

We retrospectively identified all patients with targetable *EGFR*-mutated metastatic NSCLC who had been treated at the Dana-Farber Cancer Institute (DFCI) between 2005 and 2019, had tumors assessed by targeted hybrid capture NGS, and had been treated with an EGFR TKI for metastatic or recurrent disease. For uniformity of outcome assessment, patients with historically nontargetable *EGFR* alterations, including exon 20 insertions, were excluded, as were patients with baseline T790M alterations. All patients included in this study had consented to institutional review board-approved protocols, and the study was conducted in accordance with the Declaration of Helsinki.

We collected clinical characteristics and detailed treatment histories for all patients, including smoking status (never smokers: patients who smoked <100 cigarettes; former smokers: patients who quit >12 mo before diagnosis; current smokers: patients who quit <12 mo or still smoked at diagnosis). Tumor measurements and response assessment were performed retrospectively by a thoracic radiologist (MN) on the baseline and follow-up scans during EGFR TKI therapy using Response Evaluation Criteria In Solid Tumors version 1.1, to determine best overall response and date of progression, as previously published. Progression-free survival (PFS) was defined as time from the start of either first TKI therapy (PFS1) or start of later-line osimertinib (PFS-Osi) to the date of disease progression or death. Patients alive without disease progression were censored on the date of their last contact. Overall survival (OS) was defined as time from start of first-line EGFR TKI to death from any cause, with censoring also defined at the date of last contact. Resistance mechanisms were classified as *EGFR* mutation (*T790M, C797S*), small cell transformation, bypass pathway, or other, which includes cases with no identified mechanism. Mechanism was assigned based on clinical record or direct assessment of post-treatment specimens.
development of T790M mutation or small cell transformation at any point after first TKI was annotated.

**Mutational Analysis**

Genetic sequencing and mutation calling were performed as previously described using the DFCI OncoPanel platform, which has been extensively validated for both mutation and copy number calling. Only tissue-derived sequencing was included. Version 1 of OncoPanel captures 287 genes; version 2 captures 323 genes; and version 3 captures 462 genes. Mutations were considered functionally impactful if they were a loss-of-function alteration, including nonsense, frameshift, insertion-deletion, or splice site alteration. Missense mutations were considered functionally impactful if they were either of the following: (1) present in the OncokB hotspot database; (2) present in the Catalogue of Somatic Mutations in Cancer more than 3 times; or (3) deleterious based on in silico prediction from the PolyPhen-2 (Polymorphism Phenotyping version 2) prediction tool. Copy number events classified as “high amplification” or homozygous deletions were considered functionally impactful.

Tumor mutational burden (TMB) was calculated as the number of nonsynonymous alterations per megabase (Mb) of genome examined, specific to the OncoPanel version used. Because segment length and copy ratio were only available for a subset of samples, proportion copy number altered (CNA) load was estimated as the number of CNA genes over the number of genes included in each panel version. Mutational signature analysis was performed using SigMA, a validated method for mutational signature analysis from targeted panels that uses likelihood-based measures and machine-learning to account for low mutation counts. We ran SigMA using precalculated OncoPanel weights and previously identified lung adenocarcinoma signatures (COSMIC signatures 1, 2, 3, 4, 5, 13, 17, 17b, 18, and 28). We report results for signatures 1 (5’-methylcytosine deamination), 3 (homologous recombination defect), 4 (tobacco mutagenesis), 5 (T>C substitution), 2 and 13 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) owing to low frequency of mutations in the other signatures. Mutual exclusivity testing was performed using WexT. Genes were assigned to pathways based on previous annotations.

**TCGA Analysis**

Previously published lung adenocarcinoma sequencing and clinical metadata from The Cancer Genome Atlas (TCGA) were obtained from cBioPortal. TMB was calculated as the sum of nonsynonymous mutations. Genome doubling as previously calculated using ABSSOLUTE was obtained. Proportion CNA was calculated as the sum of length of segments with |copy ratio| greater than 0.2 over the length of all segments. DeconstructSigs was used for mutational signature identification in the TCGA cohort.

**Statistical Analysis**

Categorical and continuous variables were summarized descriptively using percentages and medians. The Wilcoxon ranked sum test and Kruskal–Wallis test were used to test for differences between continuous variables, and Fisher’s exact test was used to test for associations between categorical variables. Pretreatment and post-treatment enrichment was performed by means of logistic regression with adjustment for TMB. PFS and OS were estimated using Kaplan–Meier methodology. Log-rank tests were used to test for differences in event-time distributions, and Cox proportional hazards models were fitted to obtain estimates of hazard ratios (HRs) in univariate and multivariate models. Analyses were performed in sample subsets according to treatment time point as indicated in the text; for comutation or cohort-wide analyses, the earliest sample available for each patient was identified to ensure that patients with multiple biopsies did not bias the results (single-sample cohort). All p values are two sided and confidence intervals (CIs) are at the 95% level. Statistical significance is defined as p value less than 0.05. Multiple hypothesis test correction was not performed on these exploratory analyses. All analyses were performed using R version 4.0.3.

**Results**

**Patient Population**

A total of 269 patients who had received targeted therapy for an actionable EGFR alteration and who had at least one tumor specimen that had undergone genomic profiling were identified (Table 1, Supplementary Fig. 1A, and Supplementary Table 1). Owing to the historical nature of this cohort, most patients were initially treated with first-generation EGFR TKIs; 94 patients were treated with osimertinib after progression (Table 1). Pretreatment specimens were available in 189 patients (pretreatment cohort); 91 patients had sequencing on or after treatment with first TKI therapy (post-TKI1 cohort), of whom 37 were later treated with osimertinib (pre-Osi cohort); 31 were sequenced after later-line osimertinib (post-Osi cohort) (Supplementary Fig. 1B and Supplementary Table 2). Patients treated with first-line osimertinib (n = 2) were included in the TKI1 cohort. Furthermore, 30 patients had a paired sample before and after a single TKI, five of whom had biopsies at each time point (Supplementary Fig. 1C). Radiographic progression on first TKI therapy was assessable in 264
patients and on later-line osimertinib in 82 patients. Median PFS was 10 months on first-line TKI (PFS1) and 6 months on later-line osimertinib (PFS-Osi). Median OS was 31.2 months (Supplementary Fig. 2A–C). There was no statistically significant difference in outcome by EGFR driver alteration (Supplementary Fig. 2D–F). Of 124 patients with post-TKI1 resistance annotations, 98 (79%) had a detectable T790M mutation, though direct detection rates of T790M mutation in the post-treatment specimens were only 55% (62 of 113). Furthermore, five patients developed SCLC transformation after first-line therapy, and another five developed SCLC transformation later in their treatment course.

### Concurrent Genomic Alterations and Predictors of Outcome

The most frequently co-occurring mutations and copy number events in pre- and post-TKI samples are found in Supplementary Figure 3. TP53 alterations were the most common, followed by alterations in PRKDC, RB1, KMT2D, and NXX2-1. TMB and CNA load increased with line of therapy (Supplementary Fig. 4A and B), though in paired samples only CNA load trended toward statistical significance, suggesting the change in TMB with therapy may be variable (Supplementary Fig. 4C and D).

Focusing on putatively functional alterations (Methods), no gene was enriched in post-treatment tumors after correcting for TMB (Supplementary Fig. 4E and F), though in these exploratory analyses there were non-significant trends toward increases in MET, BRD4, CDKN2A/B, and KEAP1 alterations. Post-osimertinib specimens were found to have non-significant trends toward PTEN, KMT2A, KRAS, NOTCH2, and MYC alterations compared with post-TKI1 specimens. As expected, EGFR T790M mutations were enriched in post-TKI1 specimens, and EGFR C797S was enriched in post-osimertinib specimens (Supplementary Fig. 4G and H). Aggregation of genes into the pathways and all post-treatment samples into one group demonstrated increased post-treatment alterations in splicing genes ($p = 0.011$), but there were non-significant increases in structural proteins ($p = 0.057$), cell cycle ($p = 0.11$), insulin signaling ($p = 0.110$), and PI3K/AKT pathway genes ($p = 0.126$) (Supplementary Fig. 4I).

To identify genes whose concurrent alteration affects EGFR TKI outcomes, we performed Cox proportional hazards estimation of the effect of pretreatment genetic changes on PFS1 ($n = 184$). TP53 alterations were the most common event associated with reduced PFS1, but alterations in SETBP1 and MET also had a worse HR (Fig. 1A). Focusing on patients with primary progressive disease (PD), we observed that MET-high amplifications were the only alteration enriched in these patients (Supplementary Fig. 5A and B), though the numbers were small. Other MET alterations, including splice site or low amplification, were not associated with PD (Supplementary Fig. 5C). Analysis of post-progression, pre-osimertinib alterations associated with PFS-Osi ($n = 37$) revealed worse outcomes in patients with MET, APC, and ERBB4 alterations (Fig. 1B). Considering samples across all treatment time points (single-sample cohort, $n = 269$), alterations in TP53 were again the most frequent

### Table 1. Clinical Characteristics of Patients in DFCI EGFR Cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pretreatment Cohort</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>269 (100)</td>
</tr>
<tr>
<td>Median age at diagnosis, y</td>
<td>62 (29–93)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 79 (30), Female 190 (70)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Ever 106 (40), Never 163 (60)</td>
</tr>
<tr>
<td>EGFR mutation</td>
<td>Exon 19 deletion 137 (51), L858R 103 (38), Other 29 (11)</td>
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<tr>
<td>Stage at diagnosis</td>
<td>I, II 37 (14), III 22 (8), IVa 69 (26), IVb 141 (52)</td>
</tr>
<tr>
<td>Line of therapy, first TKI</td>
<td>First 226 (84), Second 39 (14), Third or higher 4 (1)</td>
</tr>
<tr>
<td>First TKI</td>
<td>Erlotinib 255 (94), Afatinib 9 (3), Gefitinib, icotinib 3 (1), Osimertinib 2 (1)</td>
</tr>
<tr>
<td>First-line resistance mechanism assessed</td>
<td>T790M mutation 98 (55), Bypass pathway 7 (5), Small cell transformation 5 (2)</td>
</tr>
<tr>
<td>Osimertinib resistance mechanism assessed</td>
<td>C797S mutation 4 (3), Small cell transformation 4 (3), Other/not detected 1 (1)</td>
</tr>
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DFCI, Dana-Farber Cancer Institute; No., number; TKI, tyrosine kinase inhibitor.
Figure 1. Genomic and clinical predictors of outcome to EGFR TKI therapy. Association between co-occurring alterations and (A) PFS1 ($n = 184$), (B) PFS-Osi ($n = 37$), and (C) OS ($n = 269$). HR is illustrated on the x axis, and $-\log_{10}(p\text{ value})$ from univariate Cox proportional hazards model is illustrated on the y axis. Points are colored by the observed genetic events as indicated. Only genes altered in five or more samples are included in A and C and in two or more samples in B. (D) Forest plots of clinicogenomic variables and PFS1 (left), PFS-Osi (middle), and OS (right). CNA, copy number altered; Freq, frequency; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; PFS1, PFS on first-line EGFR TKI; PFS-Osi, PFS on subsequent osimertinib; TKI, tyrosine kinase inhibitor; TMB, tumor mutational burden.
event associated with OS, but KEAP1 and DNMT3A alterations also corresponded with worse OS (Fig. 1C). Forest plots for these analyses are found in Supplementary Figure 6A to C. Analysis of clinicogenomic variables revealed no association between PFS1 and pretreatment TMB or copy number burden and a weak association with age (HR = 0.98, 95% CI: 0.97–0.99, p = 0.0029). PFS-Osi was associated with small cell transformation (HR = 4.9, 95% CI: 2–12, p = 0.0007), smoking (former versus never, HR = 1.9, 95% CI: 1.1–3.1, p = 0.013), and post-Osi TMB (HR = 1.1, 95% CI: 1–1.2, p = 0.023). No features were associated with OS (Fig. 1D).

**TP53 Alterations Associate With Worse Prognosis Independent of Resistance Mechanism**

As TP53 alterations were present in significant numbers to allow further analysis, we decided to investigate this association further to better understand why TP53 associates with worse outcomes. We confirmed that, in addition to pretreatment TP53 alterations (Fig. 1A), patients with TP53 alterations detected at any time point had shorter PFS1 and trends toward reduced PFS-Osi and OS (Fig. 2A–C). The same trends were present but less pronounced when analysis was restricted to disruptive TP53 mutations, defined as truncating mutations or nonsynonymous alterations affecting the L2 to L3 region (Supplementary Fig. 7A–C). Given prior reports associating TP53 exon 8 alterations and outcome, we also investigated specific TP53 variants and exons; although analyses of specific mutational events are likely underpowered and confounded by differential event frequency, TP53 D281N mutations did associate with shorter PFS and OS (Supplementary Fig. 7D–F). Exon-level events were not consistent across PFS1, PFS-Osi, and OS (Supplementary Fig. 7G–I), and events in exon 8 were not statistically significant. Furthermore, although alterations in DNA-binding domain exons (exons 5–8) trended toward worse outcomes, aggregated events in these regions did not associate with worse outcomes than those with TP53 mutations outside exons 5 to 8 (Supplementary Fig. 7J–L).

We next asked whether TP53 alterations associate with worse outcomes by causing decreased therapeutic efficacy, manifested by higher rates of stable disease or PD. However, although almost all primary progressors harbored pretreatment TP53 alterations (Supplementary Fig. 5B), TP53 altered (mutant [MT]), and wild-type (WT) patients had similar Response Evaluation Criteria In Solid Tumors distributions (Fig. 2D and Supplementary Fig. 8A). Even in patients with complete or partial response as their best response, TP53-altered patients had earlier progression (Supplementary Fig. 8B and C), suggesting TP53 alterations affect the rate of resistance evolution rather than the likelihood or depth of initial response.

We next asked whether TP53 alterations associate with worse outcome by predisposing to small cell transformation. However, in both TP53 MT and WT patients, the dominant mechanism of resistance was EGFR T790M (Fig. 2E), and the negative effect of TP53 mutations was even more pronounced in the subset of patients that developed T790M (Supplementary Fig. 8D and E), suggesting TP53 alterations act independently of resistance mechanism. In contrast, pretreatment RB1 loss significantly associated with SCLC transformation (Fig. 2F) (Fisher’s p = 0.04076), and patients with concurrent pretreatment RB1 loss and TP53 alteration had the worst outcomes, though numbers were small (Fig. 2G and H). Among 13 patients who had a resistance biopsy and had TP53 and RB1 alterations identified at any time point, 46% (6 of 13) had SCLC transformation, as did two of four patients (50%) with pretreatment TP53 and RB1 alterations.

Although TP53 alterations were equally likely in samples harboring L858R versus exon 19 deletion, RB1 loss was more likely to co-occur with exon 19 deletion, and, accordingly, these patients were more likely to develop small cell transformation (Supplementary Fig. 9A and B). These findings were largely recapitulated when TP53 mutations and RB1 loss from any time point were considered (Supplementary Fig. 10A–C). Taken together, these analyses suggest that TP53 loss does not affect the initial efficacy of EGFR TKIs but does allow for the more rapid acquisition of resistance, independent of mechanism.

**TP53 Alterations Associate With Increased Mutagenesis**

We next sought to understand how TP53 alterations promote resistance by evaluating whether TP53 alterations associate with increased genomic instability or specific mutagenesis patterns, as these processes might act as mechanism-independent engines for resistance evolution. Including all samples in the cohort to increase power (n = 311), we observed that TP53 MT samples had a higher TMB (median 8.47 versus 6.84, p = 0.00051) (Fig. 3A) and CNA load (median 0.136 version 0.077, p = 0.0014) (Fig. 3B). Because copy number measurement from panel data is imprecise, we validated these findings using published whole-exome sequencing (WES) data from the TCGA, which revealed higher TMB, copy number load, and aneuploidy in TP53-mutated patients in general (Supplementary Fig. 11A–C), and in EGFR-mutated samples more specifically (Supplementary Fig. 11D–F). TP53 alterations were
Figure 2. Association between TP53 alteration, outcome, and resistance mechanism. Association between TP53 alteration detected at any time point and (A) PFS1, (B) PFS-Osi, and (C) OS. (D) Distribution of radiographic responses in patients with (MT) and without (WT) pretreatment TP53 alteration (Fisher’s p value = 0.7541). (E) Distribution of resistance mechanisms in pretreatment TP53 MT versus WT patients (Fisher’s p value = 0.6483). (F) Proportion of patients with pretreatment RB1 alterations in patients with and without small cell transformation at any time point. (G) PFS1 and (H) OS stratified by pretreatment RB1 and TP53 mutation status. CI, confidence interval; CR, complete response; HR, hazard ratio; MT, mutant; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PFS1, PFS on first-line EGFR TKI; PFS-Osi, PFS on subsequent osimertinib; PR, partial response; SD, stable disease; TKI, tyrosine kinase inhibitor; WT, wild type.
Table 3. TP53 alterations, mutation load, chromosomal instability, and mutagenesis. (A) TMB in TP53 WT versus MT tumors (Wilcoxon p = 0.00051). (B) Proportion CNA in TP53 WT versus MT (Wilcoxon p = 0.0014). (C) TMB in TP53 WT versus MT tumors stratified by treatment context (TP53 WT, pretreatment versus post-treatment, p = 0.042; TP53 MT, pretreatment versus post-treatment, p = 5.228e–05; pretreatment, TP53 MT versus WT, p = 0.02239; post-treatment, TP53 MT versus WT, p = 0.01113). (D) CNA load in TP53 WT versus MT tumors stratified by treatment context (TP53 WT, pretreatment versus post-treatment, p = 0.11; TP53 MT, pretreatment versus post-treatment, p = 0.45; pre-treatment, TP53 MT versus WT, p = 0.00126). (E) Proportion of mutations attributable to each signature in TP53 MT versus WT (signature 1, p = 0.0012; signature 5, p = 0.0282). (F) Proportion of mutations attributable to each signature in pre- versus post-treatment samples, TP53 WT samples (signature 1, p = 0.020). (G) Proportion of mutations attributable each signature in pre- versus post-treatment samples, TP53 MT samples (signature 1, p = 0.0024). All other comparisons, p > 0.05. *p < 0.05; **p < 0.01; ***p < 0.001. N = 311 samples, 212 with TP53 alterations, 99 without. CNA, copy number altered; MT, mutant; TMB, tumor mutational burden; WT, wild type.
associated with a more pronounced increase in TMB in post- versus pretreatment samples (median 9.68 versus 7.60, \( p = 5.228E−05 \); Fig. 3C); TKI treatment did not affect CNA load (Fig. 3D).

To further characterize the process by which TP53 MT tumors accumulate mutations, we performed mutational signature analysis of our DFCI cohort using SigMA. \(^{25}\) Focusing on the most common signatures in this cohort, we observed that TP53 MT samples had a higher proportion of signature 4 (smoking) \( (p = 0.0012) \) and signature 5 mutations \( (p = 0.028) \) (Fig. 3E). Validation in the TCGA data set confirmed higher proportions of signature 4 alterations in TP53 MT samples, along with fewer signature 1 and more apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like-associated mutations (Supplementary Fig. 11G). Stratification by disruptive versus nondisruptive TP53 mutations revealed highest mutation and copy number burden in tumors with disruptive TP53 mutations, and intermediate phenotypes in samples with nondisruptive TP53 mutations (Supplementary Fig 12A–E).

To evaluate whether TP53 mutations promote resistance evolution through specific mutational processes, we stratified mutational signature proportions by treatment status. In both TP53 WT (Fig. 3F) and mutated tumors (Fig. 3G), the only significant change with treatment was an increased proportion of signature 1-related mutations (clock-like signature), with a non-significant trend toward decreased signature 5 mutations. These trends were present but not statistically significant in the subset of paired samples (Supplementary Fig 13A–C). Taken together, these findings suggest that TP53 alterations associate with increased genomic instability and mutagenic potential, but do not associate with distinct mutational processes.

Focusing on the association with signature 4 alterations, despite more smoking-associated mutations, TP53 alterations were not more common in current or former smokers (Fig. 4A and Table 2). To evaluate the joint effects of TP53 and smoking, we stratified patients by clinical smoking status. We observed that TP53 alterations associated with higher TMB regardless of smoking history (Fig. 4B). As expected, current or former smokers with TP53 alterations had a higher proportion of signature 4-associated mutations (Fig. 4C). Nevertheless, among TP53 WT patients, even those patients with a clinical smoking history did not have a higher proportion of tobacco-attributable mutations than never smokers (Fig. 4C). Joint analysis of signature 4, TP53 alteration status, and outcome revealed worse PFS1 in TP53-mutated patients both with and without signature 4 mutations. There was no significant trend toward worse outcomes in TP53 WT patients with signature 4 alterations, limited by the number of patients in this subgroup (Supplementary Fig. 14).

**TP53 Alterations Define Context Specificity for Effect of Cell Cycle Alterations on Outcome**

Finally, we sought to understand how TP53 interacts with other genes and pathways to situate these events in the context of other genomic events previously associated with outcome. Weighted mutual exclusivity assessment revealed strong mutual exclusivity between TP53 alterations and MDM2 amplification (weighted exclusivity, \( p = 4.79E−06 \)) (Supplementary Fig. 3 and Supplementary Fig. 15A). Nevertheless, MDM2 amplification had no association with PFS or OS (Supplementary Fig. 15B–D), suggesting MDM2 amplification alone does not recapitulate the effects of TP53 loss. Interestingly, despite multiple prior reports implicating cell cycle alterations in reduced PFS, \(^{7,10}\) we also did not observe an independent effect of CDK4/6 amplifications specifically or cell cycle alterations more broadly on outcome (Supplementary Fig. 15E–G). However, when considered in conjunction with TP53 alteration status, TP53/cell cycle co-mutated patients had worse outcomes (Fig. 5A–D and Supplementary Fig. 16A and B).

Cox proportional hazards analysis of genes associated with outcome in TP53 WT tumors revealed shorter PFS in PIK3CA- and ASXL1-mutated tumors, and shorter OS in tumors harboring mutations in DNA epigenetic modifiers, including ASXL1, DNMT3A, and KMT2D (Supplementary Fig. 17A and B).

**Discussion**

In this study, we analyzed targeted sequencing data from a cohort of 269 patients with advanced, EGFR-mutated NSCLC treated with EGFR-directed TKIs. We found that pretreatment alterations in TP53 were the most common concurrent genomic event associated with decreased first-line PFS and OS, and with a non-significant trend toward decreased PFS on later-line osimertinib. This finding is consistent with reports from several prior cohorts, \(^{11–13,35–38}\) and indeed, TP53 alterations seem to be the genomic event most consistently associated with poor outcomes across different studies. Nevertheless, the mechanism for why TP53 alterations associate with worse outcomes remains underexplored. Although we cannot exclude an underlying prognostic effect, here we use our in-depth annotations to describe in more detail the clinical effects of TP53 alterations, revealing TP53 alterations do not associate with decreased radiographic response or specific resistance mechanisms; rather, TP53 alterations associate with more rapid time to progression.
by any mechanism, even in those patients with the most favorable radiographic response.

On the basis of previous studies,\textsuperscript{39} we hypothesized that \textit{TP53} alterations would facilitate more rapid resistance evolution by promoting cellular tolerance for genetic alterations. Consistent with this hypothesis, we found that \textit{TP53}-altered tumors compared with WT tumors had higher mutation burdens that became even more pronounced with TKI therapy, suggesting \textit{TP53} alterations promote the acquisition of somatic mutations with therapy. Although pretreatment TMB did not associate with worse outcome, as had been previously suggested,\textsuperscript{40} there was a statistically significant though small association between post-Osi TMB and PFS-Osi, suggesting if TMB associates with outcome, it may be by facilitating a greater diversity of resistance mechanisms. Importantly, in our analysis of mutational signatures, we did not observe distinct mutational processes in \textit{TP53} MT compared with WT tumors.\textsuperscript{41,42} Rather, it seemed that \textit{TP53} alterations facilitate the acquisition of

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**Figure 4.** \textit{TP53} alterations and smoking-associated mutagenesis. (A) Proportion of ever versus never smokers in \textit{TP53} WT versus MT patients (chi-squared $p = 0.3646$). (B) TMB in \textit{TP53} WT versus MT patients in ever (left) and never (right) smokers (ever smoker, Wilcoxon $p = 0.006424$; never smoker, Wilcoxon $p = 0.0216$). (C) Proportion signature 4 mutations in ever versus never smokers, stratified by \textit{TP53} WT versus MT; \textit{TP53} WT, Wilcoxon $p = 0.46$; \textit{TP53} MT, Wilcoxon $p = 0.0001$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. $N = 269$; 106 ever smokers, 163 never smokers. MT, mutant; TMB, tumor mutational burden; WT, wild type.
mutations through those processes already underway, including tobacco-mediated mutagenesis in smokers, and spontaneous deamination of 5-methylcytosine over time. These trends were less apparent in the copy number space, where we observed higher pretreatment copy number burden in TP53 MT tumors but more modest increases with therapy, suggesting acquisition of mutations rather than copy number events may be more important in this context. However, copy number calling from targeted NGS panels is less precise, and these analyses would benefit from validation in paired samples assessed by WES.

Notably, the association between TP53 and outcome seemed independent of the location within the TP53 gene. Although non-disruptive TP53 mutations associated with an intermediate TMB and CNA burden, suggestive of an intermediate phenotype or a more heterogeneous group, these patients had outcomes closer to patients with disruptive TP53 mutations than WT. Further studies will help determine whether this represents a thresholding effect of the TP53 phenotype on outcome, or distinct mechanisms driven by distinct TP53 genotypes. In contrast to prior reports implicating exon 8,13,38 we did not observe any robust associations between outcome and specific TP53 single-nucleotide variants or exons; however, these subgroup analyses may be underpowered and multiple exons were found to have trends toward worse HRs that might be significant in larger cohorts. Further exploration of the effect of specific TP53 alterations on chromosomal instability and treatment response to EGFR TKIs will help better characterize these associations.

We also note that TP53 alterations may interact with other genomic events to contribute to resistance evolution. In contrast to prior studies,7,10,11 we did not identify an independent association between cell cycle events and worse outcomes, despite having an equal or greater number of events in our cohort. We did note, however, that patients with compound cell cycle and TP53 alterations had worse outcomes than dual WT patients, suggesting loss of cell cycle checkpoints in the context of increased tolerance of genetic changes may further accelerate cell turnover and resistance acquisition. These findings suggest that ongoing studies combining osimertinib with CDK4/6 inhibition (NCT03455829) may be particularly effective in TP53-mutated patients.

Our study also confirms several previous findings and identifies potential novel associations. The association between baseline MET amplification and reduced PFS has been previously revealed, and these findings together provide a strong rationale for investigating upfront therapy with dual EGFR and MET inhibition in these patients. The association between KEAP1 alterations and reduced OS validates recent experimental and limited patient data revealing decreased duration of EGFR TKI therapy in KEAP1/TP53 co-mutated patients.43 In addition, although most concurrent genomic events associated with reduced outcome occurred in the context of TP53 loss, our data also suggest that alterations in PIK3CA and epigenetic modifiers may be TP53-independent mediators of differential benefit. More work in larger cohorts will be necessary to validate and further explore these findings.

Our analysis has several limitations. Despite the large size of the cohort, many of the single-gene observations occurred in a limited number of samples. Consistent with prior studies,35 we did not adjust our analyses for multiple comparisons to facilitate hypothesis generation, and consequently, the findings reported here need additional validation in other cohorts and functional studies. In addition, mutational signature analysis in panel-based data can be imprecise; we accounted for this limitation by using a validated algorithm that incorporates a panel-specific error model, and reassuringly, observed similar trends in WES from TCGA. Validation of treatment effects on copy number and mutational signature is not currently possible owing to limited WES cohorts of treated tumors, but it will be important as such cohorts become available. Finally, as a retrospective analysis, this study has multiple intrinsic limitations, including variable response and resistance assessment and historic treatment patterns with first- or second-generation TKIs as first TKI followed by osimertinib after resistance. We note that many of the trends we observed for PFS1 were present but underpowered in the pre-osimertinib specimens, and published studies of resistance to first-line osimertinib have implicated similar mechanisms in different distributions, suggesting the same trends and overall biological pathways may be implicated.44-46 Nonetheless, as cohorts of patients treated with first-line osimertinib mature, it will be important to validate that the same patterns hold.

In conclusion, our analysis further defines the effects of concurrent mutations on outcomes in EGFR-mutated NSCLC, suggesting an important role for TP53 mutations.
in facilitating the acquisition of resistance. Our analyses further suggest that the deleterious effects of other concurrent mutations may be contingent on TP53 mutation status and should be studied in this context. These findings also have clear therapeutic implications; although compounds targeting or restoring TP53 function are of obvious interest, these remain under investigation. Nevertheless, in the short-term, these findings provide a clear rationale for trialing treatment intensification in TP53-mutant patients with chemotherapy or other therapies, such as vascular epithelial growth factor inhibitors, which are currently under active investigation.

**Figure 5.** Cell cycle alterations and outcome. (A) PFS and (B) OS stratified by CDK4/6 and TP53 co-alteration status. Cox proportional hazards for outcome relative to TP53 WT/CDK4/6 WT shown below. (C) PFS and (D) OS stratified by cell cycle and TP53 co-alteration status. Cox proportional hazards for outcome relative to TP53 WT/cell cycle WT shown below. The most frequently altered cell cycle genes were considered and were MDM2, CDK4, CDK6, CCND1, CCNE1, CDKN2A, CDKN2B, and EP300. CCmt, cell cycle mutant; CCwt, cell cycle wild type; MT, mutant; PFS, progression-free survival; PFS1, PFS on first-line EGFR TKI; TKI, tyrosine kinase inhibitor; WT, wild type.
Further exploration of these and other concurrent mutations may provide important prognostic information for patients and clinicians and may facilitate combination therapeutic strategies to forestall resistance and prolong the duration of initial benefit from EGFR-targeted therapy.

**CRediT Authorship Contribution Statement**

Natalie I. Vokes, Eliezer M. Van Allen, and Pasi A. Jänne: Designed and conceived the study.

Natalie I. Vokes, Emily Chambers, Tom Nguyen, Christine A. Lydon: Assembled the clinical cohort, Performed chart abstraction.

Mizuki Nishino: Performed radiographic response assessment.


Lynette Sholl, Alexis Coolidge, Xiuning Le, John V. Heymach, Eliezer M. Van Allen, Pasi A. Jänne: Performed data interpretation.

Natalie I. Vokes, Pasi A. Jänne, and Eliezer M. Van Allen: Wrote the manuscript.

Natalie I. Vokes, Emily Chambers, Tom Nguyen, Alexis Coolidge, Christine A. Lydon, Xiuning Le, Lynette Sholl, John V. Heymach, Mizuki Nishino, Eliezer M. Van Allen, Pasi A. Jänne: Edited the manuscript.

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**Supplementary Data**

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at https://doi.org/10.1016/j.jtho.2022.02.011.

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**References**
