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Concurrent TP53 mutations facilitate resistance evolution in EGFR mutant lung adenocarcinoma

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Abstract

PURPOSE: Patients with *EGFR*-mutant non-small cell lung cancer (NSCLC) experience variable duration of benefit on EGFR tyrosine kinase inhibitors (TKI). The effect of concurrent genomic alterations on outcome has been incompletely described.

MATERIAL AND METHODS: In this retrospective study, targeted next-generation sequencing data was 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: 269 patients were identified for inclusion in the cohort. Among 185 response-evaluable patients with pre-treatment specimens, *TP53* alterations were the most common event associated with decreased first-line progression-free survival (PFS), and associated with decreased overall survival along with *DNMT3A*, *KEAP1* and *ASXL1* alterations. Reduced PFS on later-

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line osimertinib in 33 patients was associated with *MET*, *APC* and *ERBB4* alterations. Further investigation of the effect of *TP53* alterations demonstrated 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 TKIs.

Keywords

EGFR; non-small cell lung cancer; genomics

Introduction:

EGFR-directed tyrosine kinase inhibitors (TKI) have dramatically improved outcomes for patients whose tumors harbor sensitizing *EGFR* alterations¹. However, long-term disease control remains elusive for most patients due to the inevitable development of resistance, typically within 8–18 months^{2,3}. 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)^{4–6}, 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^{7–9}). These studies helped define how cancers escape inhibition by EGFR TKIs, but 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. Prior studies analyzing the impact of pre-treatment co-mutations have identified worse outcomes associated with alterations in *TP53*, *PIK3CA*, *PTEN*, and others^{2,10–13}. However, 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 non-targetable *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 months before diagnosis; current smokers: patients who quit <12 months or still smoked at diagnosis). Tumor measurements and response assessment were performed retrospectively by a thoracic radiologist (M.N.) on the baseline and follow-up scans during EGFR TKI therapy using Response Evaluation In Solid Tumors (RECIST) version 1.1, to determine best overall response and date of progression, as previously published.^{14–16} Progression-free survival 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 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 specimens¹⁷, and development of T790M mutation or small cell mutation 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^{18–21}. 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 significant if they were a loss-of-function alteration, including nonsense, frameshift, insertion/deletion or splice site alteration. Missense mutations were considered functionally significant if they were either: 1) present in the OncoKB hotspots database²², 2) present in the Catalogue of Somatic Mutations in Cancer²³ > 3 times, or 3) deleterious based on in silico prediction from the PolyPhen-2 (Polymorphism Phenotyping v2) prediction tool²⁴. Copy number events classified as ‘high amplification’ or homozygous deletions were considered functionally significant.

Tumor mutational burden (TMB) was calculated as the number of nonsynonymous alterations per megabase (Mb) of genome examined, specific to the OncoPanel version

employed²⁵. 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 copy number altered genes over the number of genes included in each panel version. Mutational signature analysis was performed using SigMA²⁶, a validated method for mutational signature analysis from targeted panels that utilizes likelihood-based measures and machine-learning to account for low mutation counts. We ran SigMA using pre-calculated OncoPanel weights and previously identified lung adenocarcinoma signatures (COSMIC signatures 1, 2, 3, 4, 5, 13, 17a, 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 (APOBEC) due to low frequency of mutations in the other signatures. Mutual exclusivity testing was performed using WexT²⁷. Genes were assigned to pathways based on previous annotations^{10,28,29}.

TCGA Analysis:

Previously published lung adenocarcinoma sequencing and clinical metadata from The Cancer Genome Atlas (TCGA)³⁰ was obtained from cBioPortal³¹. TMB was calculated as the sum of nonsynonymous mutations. Genome doubling as previously calculated using ABSOLUTE was obtained³². Proportion copy number altered was calculated as the sum of length of segments with |copy ratio| > 0.2 over the length of all segments. DeconstructSigs³³ 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-Rank 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. Pre- and post-treatment enrichment was performed via logistic regression with adjustment for TMB. Progression-free and overall survival 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 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 are at the 95% level. Statistical significance is defined as P<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 were identified who had both received targeted therapy for an actionable *EGFR* alteration and who had at least one tumor specimen that had undergone genomic profiling (Table 1; Supp Fig 1A; Supp Table 1). Due 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). Pre-treatment specimens were available in 189 patients (*pre-treatment 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*) (Supp Fig 1B; Supp Table 2). Patients treated with first-line osimertinib (n=2) are included in the TKI1 cohort. Thirty patients had a paired sample before and after a single TKI, 5 of whom had biopsies at each time point (Supp Fig 1C). Radiographic progression on first TKI therapy was evaluable in n=264 patients, and on later-line osimertinib in n=82. Median PFS was 10 months on first-line TKI (PFS1), and 6 months on later-line osimertinib (PFS-Osi). Median overall survival was 31.2 months (Supp Fig 2A-C). There was no statistically significant difference in outcome by *EGFR* driver alteration (Supp 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% (62/113). 5 patients developed small cell (SCLC) transformation after first-line therapy, and another 5 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 shown in Supp Fig 3. *TP53* alterations were the most common, followed by alterations in *PRKDC*, *RB1*, *KMT2D*, and *NKX2-1*. TMB and CNA load increased with line of therapy (Supp Fig 4A-B), though in paired samples only CNA load trended toward statistical significance, suggesting that the change in TMB with therapy may be variable (Supp Fig 4C-D).

Focusing on putatively functional alterations (Methods), no gene was enriched in post-treatment tumors after correcting for TMB (Supp Fig 4E-F), though in these exploratory analyses there were non-significant trends toward increases in *MET*, *BRD4*, *CDKN2A/B*, and *KEAP1* alterations. Post-osimertinib specimens showed non-significant trends toward *PTEN*, *KMT2A*, *KRAS*, *NOTCH2*, and *MYC* alterations compared to post-TKI1 specimens. As expected, *EGFR* T790M mutations were enriched in post-TKI1 specimens, and *EGFR* C797S was enriched in post-osimertinib specimens (Supp Fig 4 G-H). Aggregation of genes into pathways and all post-treatment samples into one group demonstrated increased post-treatment alterations in splicing genes (p=0.011), and 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) (Supp Fig 4I).

To identify genes whose concurrent alteration affects *EGFR* TKI outcomes, we performed cox-proportional hazards estimation of the effect of pre-treatment 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 hazard ratio (Fig 1A). Focusing on patients with primary progressive disease, we observed that *MET* high amplifications were the only alteration enriched in these patients (Supp Fig 5A-B), though numbers were small. Other *MET* alterations, including splice site or low amplification, were not associated with PD (Supp Fig 5C). Analysis of post-progression, pre-osimertinib alterations associated with

PFS-Osi (n=37) demonstrated 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 event associated with OS, but *KEAPI* and *DNMT3A* alterations also corresponded with worse OS (Fig 1C). Forest plots for these analyses are shown in Supp Fig 6A-C. Analysis of clinicogenomic variables revealed no association between PFS1 and pre-treatment 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 associated with small cell transformation (HR 4.9, 95% CI 2–12, p=0.0007), smoking (Former vs 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 pre-treatment *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-L3 region (Supp Fig 7A-C).³⁴ Given prior reports associating *TP53* exon 8 alterations and outcome¹³, we also investigated specific *TP53* variants and exons; while analyses of specific mutational events are likely underpowered and confounded by differential event frequency, *TP53* D281N mutations did associate with shorter PFS and OS (Supp Fig 7D-F). Exon-level events were not consistent across PFS1, PFS-Osi, and OS (Supp Fig 7G-I), and events in exon 8 were not statistically significant. While 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 5–8 (Supp Fig 7J-L).

We next asked whether *TP53* alterations associate with worse outcomes by causing decreased therapeutic efficacy, manifest by higher rates of stable (SD) or progressive disease (PD). However, while almost all primary progressors harbored pre-treatment *TP53* alterations (Supp Fig 5B), *TP53* altered (MT) and wild type (WT) patients had similar RECIST distributions (Fig 2D; Supp Fig 8A). Even in patients with CR/PR as their best response, *TP53*-mutated patients had earlier progression (Supp Fig 8B-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* co-mutations was even more pronounced in the subset of patients that developed T790M (Supp Fig 8D-E), suggesting that *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 pre-treatment *Rb1* loss and *TP53* alteration had the worst outcomes, though numbers were small (Fig 2G-H). Among 13

patients who had a resistance biopsy and had *TP53* and *Rb1* alterations identified at any time point, 46% (6/13) had SCLC transformation, as did 2/4 (50%) patients with pre-treatment *TP53* and *Rb1* alterations.

While *TP53* alterations were equally likely in samples harboring L858R vs 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 (Supp Fig 9). These findings were largely recapitulated when *TP53* mutations and *Rb1* loss from any time point were considered (Supp Fig 10). 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 examining 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 v 6.84, p=0.00051)(Fig 3A) and CNA load (median 0.136 v 0.077, p=0.0014)(Fig 3B). Because copy number measurement from panel data is imprecise, we validated these findings using published WES data from the TCGA^{30,32}, which showed higher TMB, copy number load, and aneuploidy in *TP53*-mutated patients in general (Supp Fig 11A-C), and in *EGFR*-mutated samples more specifically (Supp Fig 11D-F). *TP53* alterations associated with a more pronounced increase in TMB in post- vs pre-treatment samples (median 9.68 v 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 SigMA²⁶. 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 dataset confirmed higher proportions of signature 4 alterations in *TP53* MT samples, along with fewer signature 1 and more APOBEC-associated mutations (Supp Fig 11G). Stratification by disruptive vs non-disruptive *TP53* mutations demonstrated highest mutation and copy number burden in tumors with disruptive *TP53* mutations, and intermediate phenotypes in samples with non-disruptive *TP53* mutations (Supp Fig 12).

To examine 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 (Supp 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 former/current smokers (Fig 4A; Table 2). To examine 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/former smokers with *TP53* alterations had a higher proportion of signature-4 associated mutations (Fig 4C). However, 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 demonstrated worse PFS1 in *TP53* mutated patients both with and without signature 4 mutations. There was a non-significant trend toward worse outcomes in *TP53* WT patients with signature 4 alterations, limited by the number of patients in this subgroup (Supp 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 demonstrated strong mutual exclusivity between *TP53* alterations and *MDM2* amplification (weighted exclusivity, $p=4.79E-06$) (Supp Fig 3; Supp Fig 15A). However, *MDM2* amplification had no association with PFS or OS (Supp 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^{8,10}, we also did not observe an independent effect of *CDK4/6* amplifications specifically or cell cycle alterations more broadly on outcome (Supp Fig 15E-F). However, when considered in conjunction with *TP53* alteration status, *TP53*/cell cycle co-mutated patients had worse outcomes (Fig 5A-D; Supp Fig 16A-B).

Cox proportional hazards analysis of genes associated with outcome in *TP53* WT tumors demonstrated shorter PFS in *PIK3CA* and *ASXL1*-mutated tumors, and shorter OS tumors harboring mutations in DNA epigenetic modifiers including *ASXL1*, *DNMT3A*, and *KMT2D* (Supp Fig 17A-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 pre-treatment 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 appear to be the genomic event most consistently associated with poor outcomes across different studies. However, the mechanism for why *TP53* alterations associate with worse outcomes remains underexplored. While we cannot exclude an underlying prognostic effect, here we utilize our in-depth annotations to describe in more detail the clinical effects of *TP53* alterations, demonstrating that *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.

Based on previous studies³⁹, we hypothesized that *TP53* alterations would facilitate more rapid resistance evolution by promoting cellular tolerance for genetic alterations. Consistent with this hypothesis, we found that *TP53*-altered tumors compared to wild type tumors had higher mutation burdens that became even more pronounced with TKI therapy, suggesting that *TP53* alterations promote the acquisition of somatic mutations with therapy. While pre-treatment TMB did not associate with worse outcome, as had been previously suggested⁴⁰, there was a statistically significant though small association between post-Osi TMB and PFS-Osi, suggesting that 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 *TP53* MT compared to WT tumors^{41,42}. Rather, it appeared that *TP53* alterations facilitate the acquisition of 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 pre-treatment 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 appeared independent of the location within the *TP53* gene. While non-disruptive *TP53* mutations³⁴ 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* SNVs or exons; however, these subgroup analyses may be underpowered, and multiple exons demonstrated trends toward worse hazard ratios 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^{8,10,11}, 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 wild type patients, suggesting that 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 demonstrated, and these findings together provide a strong rationale for investigating upfront therapy with dual EGFR and MET inhibition in these patients. The association between *KEAPI* alterations and reduced OS validates recent experimental and limited patient data demonstrating decreased duration of EGFR TKI therapy in *KEAPI/TP53* co-mutated patients⁴³. Additionally, while 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 studies³⁵, we did not adjust our analyses for multiple comparisons in order to facilitate hypothesis generation, and consequently the findings reported here need additional validation in other cohorts and functional studies. Additionally, 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 due to limited WES cohorts of treated tumors, but 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 that the same trends and overall biological pathways may be implicated^{8,44–46}. 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; while compounds targeting or restoring *TP53* function are of obvious interest⁴⁷, these remain under investigation. However, 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 VEGF inhibitors, which are currently under active investigation (NCT04695925)⁴⁸. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict-of-interest statement:

Dr. Van Allen reports personal fees from Tango Therapeutics, personal fees from Genome Medical, personal fees from Invitae, personal fees from Enara Bio, personal fees from Janssen, personal fees from Manifold Bio, personal fees from Monte Rosa Therapeutics, grants from Novartis, grants from BMS IION, grants from Sanofi, personal fees from Foaley & Hoag, outside the submitted work; In addition, Dr. Van Allen has a patent Institutional patents filed on chromatin mutations and immunotherapy response, and methods for clinical interpretation pending. Dr. Heymach reports grants and personal fees from AstraZeneca, grants and personal fees from GlaxoSmithKline, personal fees from Boehringer Ingelheim, personal fees from Bristol Myers Squibb, personal fees from Merck, personal fees from Catalyst, personal fees from Guardant Health, personal fees from Foundation Medicine, personal fees from Hengrui, personal fees from Lilly, personal fees from Novartis, personal fees from EMD Serono, personal fees from Sanofi, personal fees from Biotree, personal fees from Takeda, outside the submitted work; In addition, Dr. Heymach has a patent PCT/US2019/022067 pending, and a patent PCT/US2017/062326 and U.S. Provisional Patent Application Nos. 62/423,732; 62/427,692 and 62/572,716, with royalties paid to The University of Texas System Board of Regents. L.M.S. reports consulting fees from EMD Serono, scientific advisory board roles for Loxo Oncology and Foghorn Therapeutics, and honorarium from Astra Zeneca. M.N. serves as a consultant to Daiichi Sankyo, AstraZeneca, and received institutional research grant from Merck, Canon Medical Systems, AstraZeneca, Daiichi Sankyo. Dr. Vokes reports consulting/advisory fees from Sanofi/Genzyme, Oncocyte, and Lilly. Dr Janne has received consulting fees from AstraZeneca, Boehringer-Ingelheim, Pfizer, Roche/Genentech, Takeda Oncology, ACEA Biosciences, Eli Lilly and Company, Araxes Pharma, Ignyta, Mirati Therapeutics, Novartis, LOXO Oncology, Daiichi Sankyo, Sanofi Oncology, Voronoi, SFJ Pharmaceuticals, Takeda Oncology, Transcenta, Silicon Therapeutics, Syndax, Nuvalent, Bayer, Esai, Biocartis, Allorion Therapeutics, Accutar Biotech and Abbvie; receives post-marketing royalties from DFCI owned intellectual property on EGFR mutations licensed to Lab Corp; has sponsored research agreements with AstraZeneca, Daichi-Sankyo, PUMA, Boehringer Ingelheim, Eli Lilly and Company, Revolution Medicines and Astellas Pharmaceuticals; and has stock ownership in LOXO Oncology and Gatekeeper Pharmaceuticals. Dr. Le reports grants and personal fees from Eli Lilly, personal fees from AstraZeneca, grants and personal fees from Boehringer Ingelheim, personal fees from Spectrum Pharmaceuticals, personal fees from Bristol Myers Squibb, personal fees from Celgene, personal fees from EMD Serono, personal fees from Janssen, personal fees from Hengrui Therapeutics, personal fees from Novartis, personal fees from Daiichi Sankyo, outside the submitted work. Emily Chambers reports personal fees from The Broad Institute of Harvard and MIT, personal fees from Takeda Pharmaceuticals, outside the submitted work.

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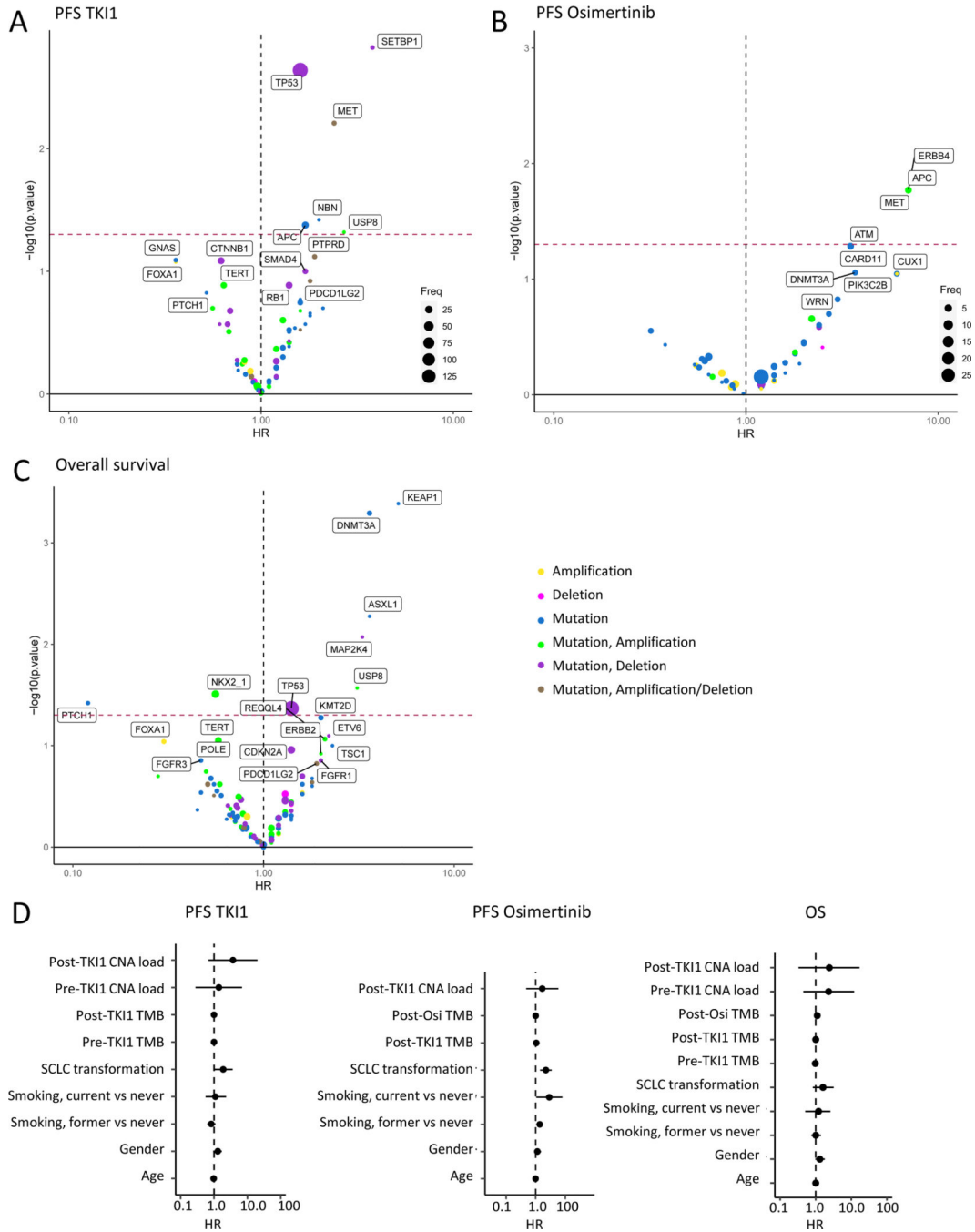


Figure 1. Genomic and clinical predictors of outcome to EGFR TKI therapy. Association between co-occurring alterations and A. Progression-free survival (PFS) on first-line EGFR TKI (PFS1)(n=184), B. PFS on subsequent osimertinib (PFS-Osi)(n=37), and C. Overall survival (OS)(n=269). Hazard ratio is shown on the x-axis, $-\log_{10}(p\text{-value})$ from univariate cox-proportional hazards model is shown on the y-axis. Points are colored by the observed genetic events as indicated. Only genes altered in 5 or more samples are included in A & C, and in 2 or more samples in B. D. Forest plots of clinicogenomic variables and PFS1 (left), PFS-Osi (middle), and OS (right). CNA: copy number alteration;

SCLC: small cell lung cancer; TKI: tyrosine kinase inhibitor; TMB: tumor mutational burden.

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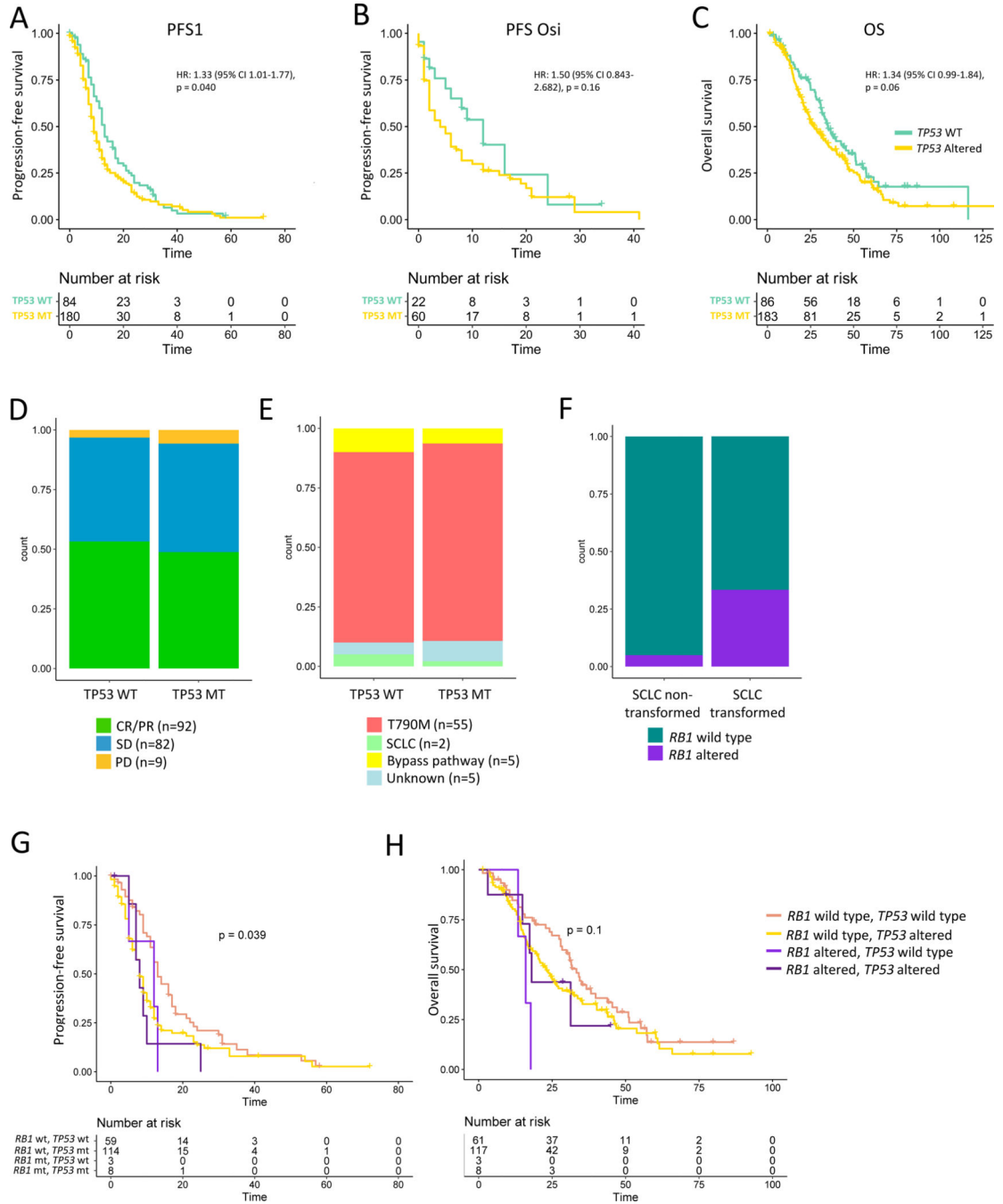


Figure 2. Association between TP53 alteration, outcome, and resistance mechanism. Association between TP53 alteration detected at any time point and A. First TKI progression-free survival (PFS1), B. PFS on subsequent osimertinib (PFS-Osi), and C. Overall survival (OS). D. Distribution of radiographic responses in patients with (MT) and without (WT) pre-treatment TP53 alteration (Fisher’s p-value=0.7541). E. Distribution of resistance mechanisms in pre-treatment TP53 MT vs WT patients (Fisher’s p-value=0.6483). F. Proportion of patients with pre-treatment Rb1 alterations in patients with and without

small cell transformation at any time point. G. PFS1 and H. OS stratified by pre-treatment *Rb1* and *TP53* mutation status. TKI: tyrosine kinase inhibitor

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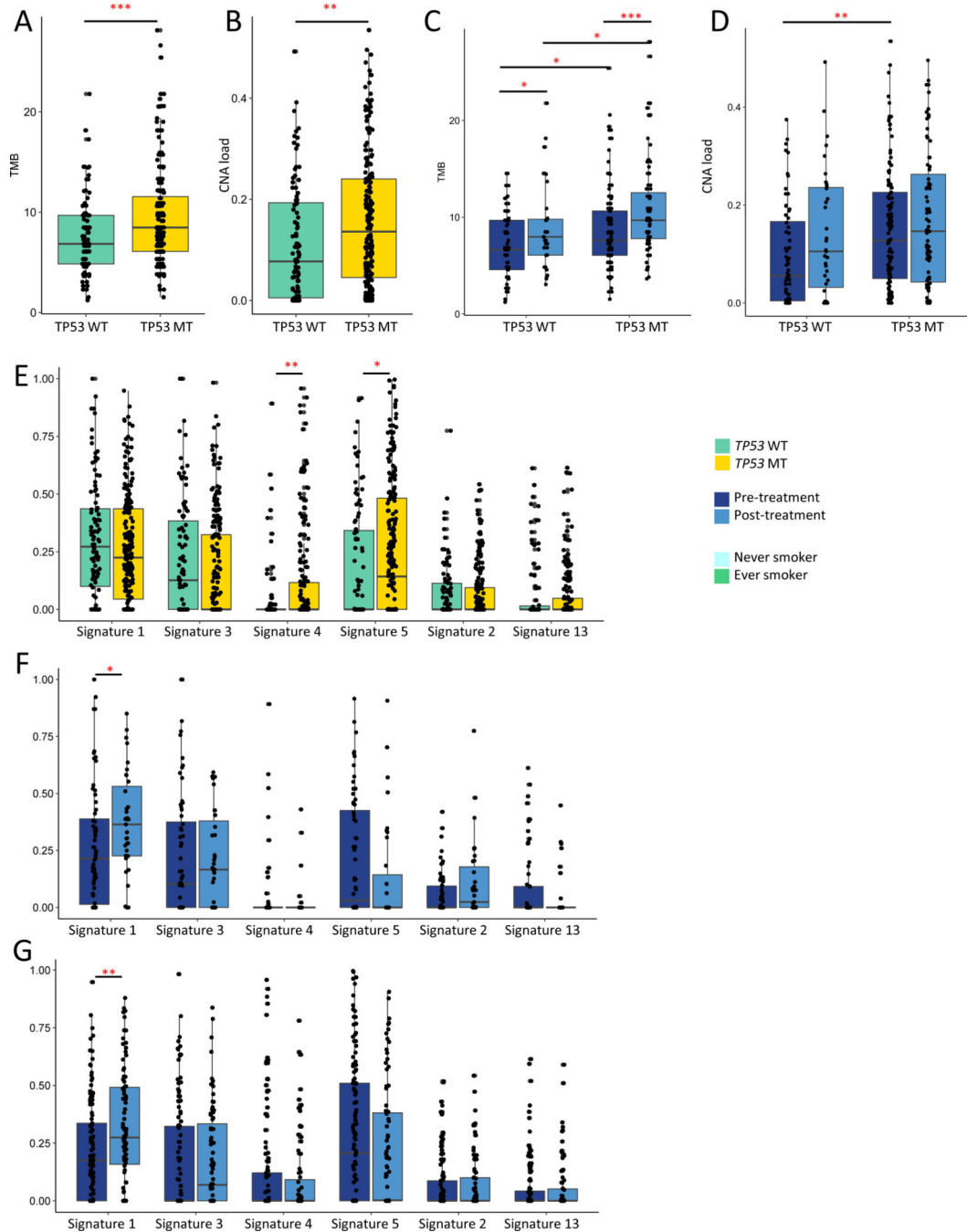


Figure 3. *TP53* alterations, mutation load, chromosomal instability and mutagenesis.

A. Tumor mutational burden (TMB) in *TP53* wild type (WT) vs altered (MT) tumors (Wilcoxon p-value=0.00051). B. Proportion copy number altered (CNA) in *TP53* WT vs MT (Wilcoxon p-value=0.0014). C. TMB in *TP53* WT vs MT tumors stratified by treatment context (*TP53* WT, pre vs post-treatment, p-value=0.042; *TP53* MT, pre vs post-treatment, p-value=5.228e-05; pre-treatment, *TP53* MT vs WT, p=0.02239; post-treatment, *TP53* MT vs WT, p=0.01113). D. CNA load in *TP53* WT vs MT tumors stratified by treatment context (*TP53* WT, pre vs post-treatment, p-value=0.11; *TP53* MT, pre vs post-treatment, p-

value=0.45; pre-treatment, *TP53* MT vs WT, p-value=0.00126). E. Proportion of mutations attributable to each signature in *TP53* MT vs WT (Signature 4, p=0.0012; Signature 5, p=0.0282). F. Proportion of mutations attributable to each signature in pre vs post-treatment samples, *TP53* WT samples (Signature 1, p=0.020. G. Proportion of mutations attributable each signature in pre vs 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.

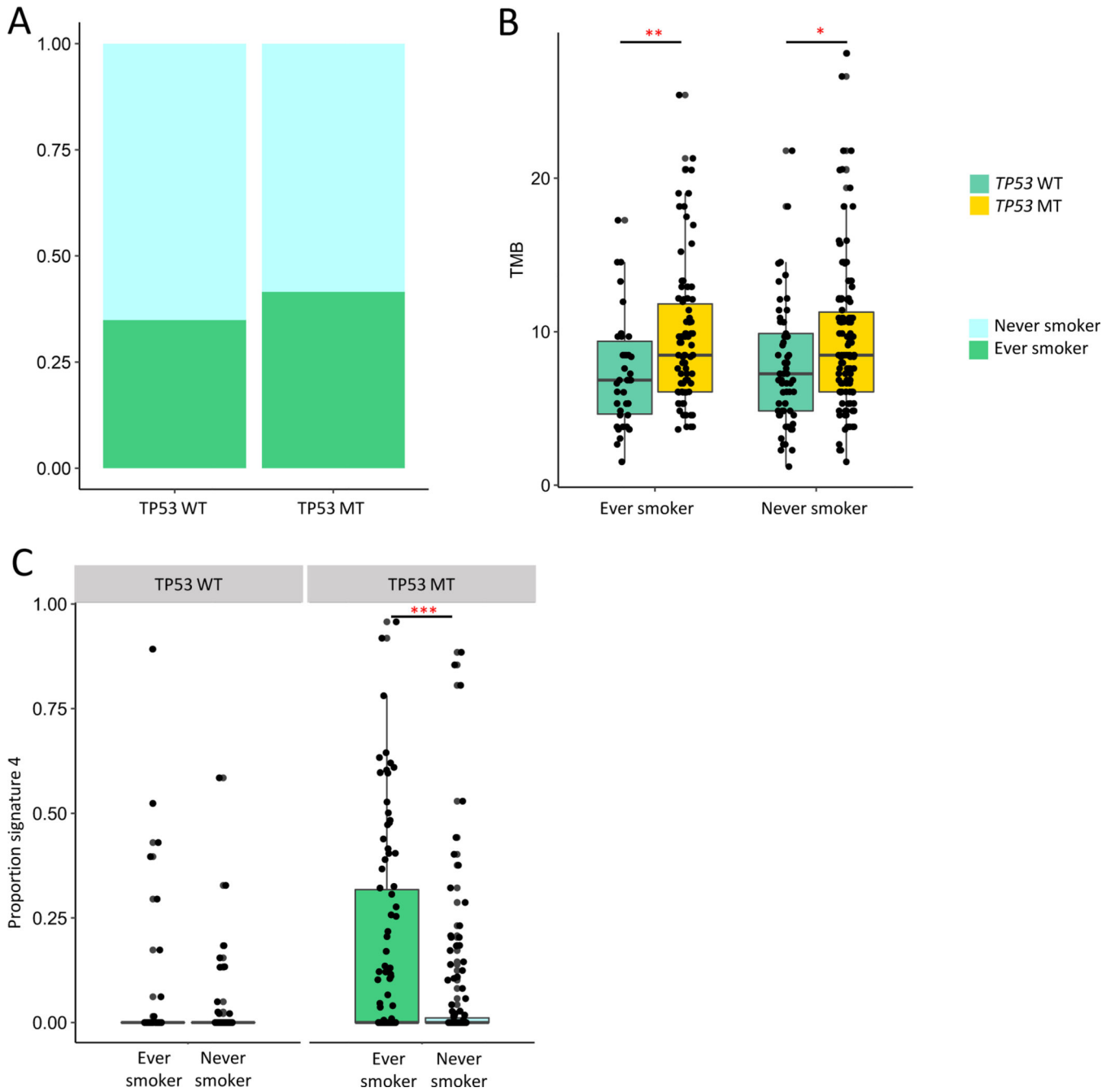


Figure 4. TP53 alterations and smoking-associated mutagenesis.

A. Proportion of ever vs never smokers in *TP53* WT vs MT patients (Chi-square p-value=0.3646). B. TMB in *TP53* WT vs MT patients in ever (left) and never (right) smokers (Ever smoker, Wilcoxon p-value=0.006424; Never smoker, Wilcoxon p-value=0.0216). C. Proportion signature 4 mutations in ever vs never smokers, stratified by *TP53* WT vs MT; *TP53* WT, Wilcoxon p-value=0.46; *TP53* MT, Wilcoxon p-value=0.0001. *p<0.05; **p<0.01, ***p<0.001. MT: altered; WT: wild type. N=269; 106 ever smokers, 163 never smokers.

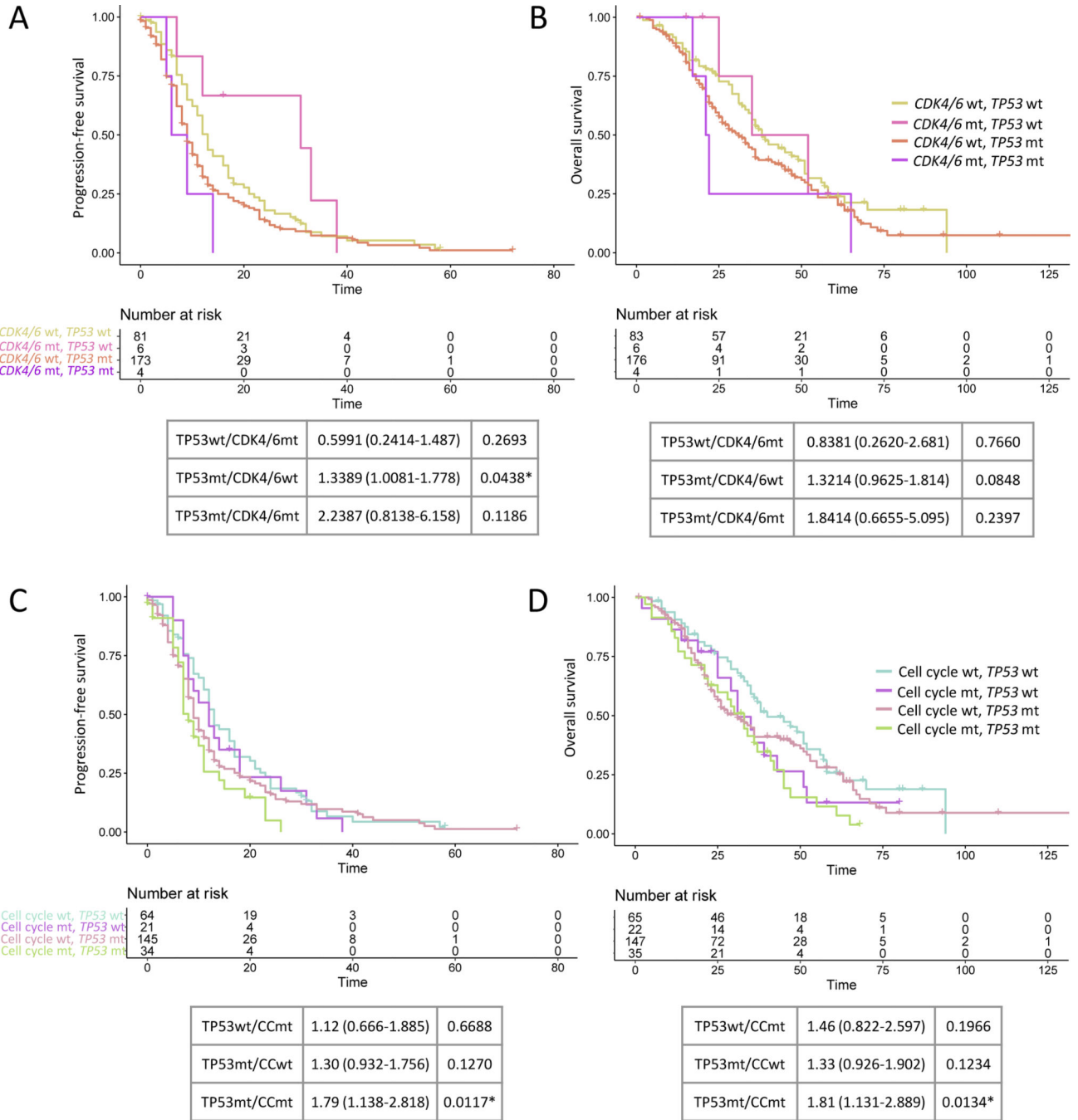


Figure 5. Cell cycle alterations and outcome.

A. First TKI progression-free survival (PFS1) and B. overall survival (OS) stratified by *CDK4/6* and *TP53* co-alteration status. Cox-proportional hazards for outcome relative to *TP53* wild type/*CDK4/6* wild type shown below. C. PFS1 and D. OS stratified by cell cycle and *TP53* co-alteration status. Cox-proportional hazards for outcome relative to *TP53* wild type/cell cycle wild type shown below. The most frequently altered cell cycle genes were

considered and were: *MDM2*, *CDK4*, *CDK6*, *CCND1*, *CCNE1*, *CDKN2A*, *CDKN2B*, and *EP300*. MT: altered; TKI: tyrosine kinase inhibitor; WT: wild type.

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Table 1.

Clinical characteristics of patients in DFCI EGFR cohort.

Cohort Characteristics	Pretreatment Cohort	
Characteristic	No. (%)	No. (%)
No of patients	269	189
Med age at diagnosis	62 (29–93)	64 (29–93)
Sex		
Male	79 (30)	57 (30)
Female	190 (70)	132 (70)
Smoking status		
Ever	106 (40)	80 (42)
Never	163 (60)	109 (58)
EGFR mutation		
Exon 19 deletion	137 (51)	92 (49)
L858R	103 (38)	74 (39)
Other	29 (11)	23 (12)
Stage at diagnosis		
I, II	37 (14)	29 (15)
III	22 (8)	15 (8)
IVa	69 (26)	52 (28)
IVb	141 (52)	93 (49)
Line of therapy, first TKI		
First	226 (84)	162 (86)
Second	39 (14)	24 (13)
Third or higher	4 (1)	3 (1)
1 st TKI		
Erlotinib	255 (94)	178 (94)
Afatinib	9 (3)	7 (4)
Gefitinib, Icotinib	3 (1)	2 (1)
Osimertinib	2 (1)	2 (1)
1 st -line Resistance mechanism assessed	124 (46)	67 (35)
T790M mutation	98	55
Bypass pathway	7	5
Small cell transformation	5	2
Other/not detected	14	5
Received osimertinib after TKI resistance	94 (35)	50 (26)
Osimertinib Resistance mechanism assessed	8	3
C797S mutation	4	
Small cell transformation	4	3
Other/not detected	1	

Table 2.

Univariate logistic regression of clinical features associated with TP53 altered vs wild type status.

Variable	Comparison	Beta	P-value
Gender	Male vs Female	0.1888	0.517
Smoking	Never vs ever	-0.2821	0.299
Age		-0.03714	0.00189**
Stage	Stage III v I,II	0.3971	0.4723
	Stage IVA v I,II	0.5306	0.2034
	Stage IVB v I,II	0.8346	0.0283*

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