Combining Genomic Biomarkers to Guide Immunotherapy in Non–Small Cell Lung Cancer

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ABSTRACT

Purpose: The clinical value of *STK11*, *KEAP1*, and *EGFR* alterations for guiding immune checkpoint blockade (ICB) therapy in non–small cell lung cancer (NSCLC) remains controversial, as some patients with these proposed resistance biomarkers show durable ICB responses. More specific combinatorial biomarker approaches are urgently needed for this disease.

Experimental Design: To develop a combinatorial biomarker strategy with increased specificity for ICB unresponsiveness in NSCLC, we performed a comprehensive analysis of 254 patients with NSCLC treated with ligand programmed death-ligand 1 (PD-L1) blockade monotherapy, including a discovery cohort of 75 patients subjected to whole-genome sequencing (WGS), and an independent validation cohort of 169 patients subjected to tumor-normal large panel sequencing. The specificity of *STK11*/*KEAP1*/*EGFR* alterations for ICB unresponsiveness was assessed in the contexts of a low (<10 muts/Mb) or high (≥10 muts/Mb) tumor mutational burden (TMB).

Results: In low TMB cases, *STK11*/*KEAP1*/*EGFR* alterations were highly specific biomarkers for ICB resistance, with 0/15 (0.0%) and 1/34 (2.9%) biomarker-positive patients showing treatment benefit in the discovery and validation cohorts, respectively. This contrasted with high TMB cases, where 11/13 (85%) and 15/34 (44%) patients with at least one *STK11*/*KEAP1*/*EGFR* alteration showed durable treatment benefit in the discovery and validation cohorts, respectively. These findings were supported by analyses of progression-free survival and overall survival.

Conclusions: The unexpected ICB responses in patients carrying resistance biomarkers in *STK11*, *KEAP1*, and *EGFR* were almost exclusively observed in patients with a high TMB. Considering these alterations in context, the TMB offered a highly specific combinatorial biomarker strategy for limiting overtreatment in NSCLC.

Introduction

Pharmacological blockade of the inhibitory immune receptor programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1) has transformed the treatment of non–small cell lung cancer (NSCLC). This immune checkpoint blockade (ICB) is especially well-known for the durability of the responses (5-year survival exceeding 25% in specific subgroups; ref. 1), whereas the proportion of patients experiencing treatment benefit is relatively limited. To minimize serious adverse events and costs, biomarker-based approaches to identify patients without benefit of ICB are urgently needed. In this scenario, high specificity is the key, as the small chance of a durable response has made clinicians rightfully hesitant to withhold these standard-of-care treatments with potentially large benefits from their patients.

Several biomarkers, including alterations in *STK11*/*LKB1*, *KEAP1*, and *EGFR*, are correlated with ICB outcomes in NSCLC and have potential for guiding immunotherapy in this disease. However, these individual biomarkers exhibit limitations in terms of their specificity to identify patients who are unlikely to benefit from ICB. Alterations of *STK11*, encompassing somatic mutations and bi-allelic deletions, are one of the most frequent genomic events in NSCLC, and the 5%–33% (2–6) of tumors harboring these alterations demonstrate inferior ICB response rates (2, 7–11). Yet, a large study, including 1,261 patients with NSCLC, of which 260 patients harbored *STK11* mutations, demonstrated a low but clinically relevant overall response rate (ORR) of 17.3% upon ICB treatment in the *STK11* mutant population and an ORR of 11.6% in the *STK11*+*KRAS* double mutant population (9). Another main genomic event underlying NSCLC is the alteration of *KEAP1*, which occurs in approximately 20% of cases (3). Its relationship to ICB responsiveness has been more contradictory, with most groups reporting a negative effect on outcome (8, 9, 12–15), whereas others reported no effect (16), or a positive effect (17–19). Finally, alterations in the receptor tyrosine kinase *EGFR* are key drivers of NSCLC and occur in approximately 10%–20% and approximately 50% of Caucasian and Asian populations, respectively, especially in patients with a never-smoking history and low numbers of (tobacco-induced) mutations (3, 20–22). Although the presence of *EGFR*

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Translational Relevance

Most patients with non–small cell lung cancer (NSCLC) are resistant to immune checkpoint blockade (ICB) and hence suffer from overtreatment, but proposed biomarkers for guiding treatment have limited specificity for ICB resistance. We discover and validate a combinatory biomarker strategy with markedly improved specificity for ICB unresponsiveness, with clinical potential to refine treatment selection for patients with NSCLC.

alterations is consistently associated with reduced efficacy rates of ICB, the larger studies to date have reported ORR of 10%–12% with anti–PD-(L)1 monotherapy (23–25). Thus, alterations in *STK11*, *KEAP1*, and *EGFR* as individual biomarkers fail to identify ICB-resistant patients consistently, which hampers their clinical utility in this context and emphasizes the need for a more comprehensive approach.

In line with the notion that ICB enhances endogenous T-cell–based antitumor immunity, which partly relies on the recognition of neoepitopes arising from the somatically mutated cancer genome (26), the total number of mutations in a tumor—known as the tumor mutational burden (TMB)—has been identified as a strong genomic biomarker for responsiveness to ICB therapy in NSCLC (17, 27, 28). The levels of predicted neoepitopes were found to carry similar predictive capacity as the TMB, as these variables are tightly correlated (2, 15, 17, 29). Importantly, ICB responses of NSCLC are also frequently observed in the context of a low TMB, indicating that the TMB also has insufficient specificity for identifying non-responding patients as a standalone biomarker, limiting its clinical applicability in this context.

We hypothesized that the (rare) ICB responses occurring in patients with alterations in *STK11*, *KEAP1* or *EGFR* would be largely confined within the population harboring a high TMB, where an abundance of neoepitopes could elicit powerful immune responses and tumor regression, which overwhelm mechanisms of relative resistance. To comprehensively study this, we collected whole-genome sequencing (WGS), PD-L1 IHC, and treatment outcome data of 75 patients with NSCLC treated with PD-(L)1 blockade monotherapy. The resulting combinatorial biomarker strategy for identifying patients who fail to respond to ICB was then validated in an independent cohort of 169 patients with NSCLC treated with PD-(L)1 blockade monotherapy.

Materials and Methods

Patient cohorts and study procedures

Discovery cohort. We selected patients with advanced-stage NSCLC who were included in 14 academic, teaching, and general hospitals across the Netherlands between April 2016 and July 2019, under the protocol of the Center for Personalized Cancer Treatment (CPCT) consortium (CPCT-02 Biopsy Protocol, ClinicalTrial.gov no. NCT01855477). This trial was approved by the medical ethics committee of the University Medical Center Utrecht, the Netherlands, and conducted in accordance with the Declaration of Helsinki. More details on the consortium and the full patient cohort have been published previously (30). In brief, the CPCT-02 consortium was established to collect tumor biopsies of patients with advanced-stage solid malignancies, to analyze the cancer genome by WGS and to

discover predictors for systemic treatment outcome. Patients eligible for inclusion were ≥18 years and had locally advanced or metastasized solid tumors. Condition for enrollment was the possibility to safely obtain a histological biopsy from a metastasis or primary tumor before the start of a new line of systemic treatment. All patients in the CPCT-02/Hartwig database provided written informed consent for paired tumor-normal WGS and collection of clinical characteristics. For the 19 additional patients included in the Netherlands Cancer Institute, this concerned bio-banked samples collected before April 21, 2016, for which informed consent was not required as per local legislation. Patients included in the analyses were treated with anti–PD-1/anti–PD-L1 monotherapy according to standard-of-care, where treatment choices were made by the treating oncologist, independent of trial participation. Collection and sequencing of samples was performed as described previously (30). Only biopsies with a sequencing-estimated tumor purity ≥20% were included in the study. Only evaluable patients with at least one radiological response measurement according to RECIST v1.1 were included in the analyses.

Validation cohort. For validation, we analyzed published data of a cohort of patients with NSCLC treated with ICB and subjected to MSK-IMPACT large panel sequencing (11), which are freely available via cBioPortal (https://www.cbioportal.org/study/summary?id=lung_msk_mind_2020). Mutation data, TMB measurements, gene copy-number data, tumor purity estimates, treatment information, and clinical outcome were downloaded on November 11, 2022. For optimal consistency, we applied the same inclusion criteria on the discovery and validation cohorts and included patients that (i) were treated with anti–PD-(L)1 monotherapy, (ii) had available genomics data, (iii) had a tumor purity ≥20%, and (iv) were evaluable using at least one radiological response measurement according to RECIST v1.1. This yielded a validation cohort of 169 patients.

Treatment outcome measures

Clinical responses to treatment were evaluated on the basis of radiological response assessment according to RECIST v1.1. (31) and defined as progressive disease (PD), stable disease (SD), partial response (PR), or complete response (CR). To enable a binary classification approach, the endpoint durable clinical benefit (DCB; yes vs. no) was used, where DCB was defined as a best overall response of CR, PR, or SD ≥6 months. Progression-free survival (PFS) was defined as the time between the first administration of the checkpoint inhibitor until progression or death due to any cause. Patients who did not progress yet, were censored on the date of the last CT-scan without progression. Overall survival (OS) was defined as the time between first administration until death due to any cause. Patients still alive were censored on the date of last clinical follow-up.

Whole-genome analysis

WGS and bioinformatics analysis of the discovery cohort were performed as previously described in detail (30), with an optimized pipeline based on open source tools, which is freely available on GitHub (https://github.com/hartwigmedical/pipeline5). Integrated purity, ploidy, structural variant and copy-number somatic analysis was performed using in-house tools GRIDSS, PURPLE and LINX (32). Integrated functionalities of PURPLE (v2.39) include mutation-specific calculations of the probabilities for subclonality, bi-allelic status (loss of heterozygosity, LOH), and driver mutation status (as opposed to passenger mutation status). In addition, mutations in known hotspots were flagged.

Definition of clonal mutations

Mutations (nucleotide substitutions and indels) with a subclonal probability >95% were considered to be subclonal mutations. For the discovery cohort, we calculated the subclonal probability using an integrated functionality of PURPLE v2.39. As PURPLE requires WGS data as input, we followed another previously published (33) approach to determine subclonality of panel-sequencing–based mutation calls of *STK11* and *KEAP1*. Here, subclonal mutations were identified using binomial statistics, testing if the observed number of mutant reads of a particular mutation was significantly lower than expected in the "minimal" clonal scenario, which refers to the situation where each tumor cell contained exactly one copy of the mutant allele (and all potential other copies of the same allele in the cancer cells were wild-type, WT). As *STK11* and *KEAP1* were found to be diploid in all mutant samples in the validation cohort, the expected variant allele frequency in the "minimal" clonal scenario was simply equal to tumor purity divided by 2 (the gene copy number). Thus, the probability of clonality was modeled as a Bernoulli experiment using the Python package SciPy (RRID:SCR_008058) as: SciPy.stats.binom_test($n_{successes}$, n_{trials}, $P(success_{minimal\_scenario})$, alternative = "*less*"). Here, $n_{successes}$ represented the number of mutant reads, n_{trials} represented the sequencing coverage of the genomic region (total number of reads) and $P(success_{minimal\_scenario})$ represented the expected variant allele frequency in the "minimal" clonal scenario (the tumor purity divided by 2).

TMB estimates

Using WGS (discovery cohort) or MSK-IMPACT sequencing (validation cohort), the TMB was calculated as the genome-wide number of non-synonymous somatic nucleotide substitutions and small indels per coding Mb. For the discovery cohort, the cTMB was determined on the basis of clonal mutations only.

Definition of driver mutations

As classical tumor-suppressor genes, the mutational landscape of *STK11* and *KEAP1* is not dominated by hotspot mutations and hence highly diverse. Therefore, the majority of *STK11* and *KEAP1* mutations are variants of unknown significance (VUS), representing a mixture of pathogenic and passenger mutations. To address this in the discovery cohort, we first classified all homozygous deletions and bi-allelic nonsense, splice or indel variants as pathogenic variants, whereas all other variants were classified as VUSs. Next, we leveraged our WGS data by using PURPLE (v2.39) to calculate, for all VUSs, the passenger probability, defined as the probability that this mutation had occurred by chance given the genome-wide mutational profile of the sample. To ensure the consideration of pathogenic *STK11*/*KEAP1* alterations, we excluded VUSs classified as likely passenger mutations (>20% passenger probability). For the validation cohort, the calculation of PURPLE-based passenger probabilities was not possible, and we hence considered all nonsynonymous (clonal) alterations in *STK11* and *KEAP1*. For *EGFR* alterations, we only considered clinically actionable alterations in both the discovery and validation cohorts. For alterations in *KRAS*, we considered mutations in known hotspots.

PD-L1 status

PD-L1 status was determined by IHC as part of routine diagnostics, and this information was retrospectively collected from clinical files.

Additional statistical procedures

All analyses were performed in Python 3, with the exception of Kaplan–Meier plot generation and the calculation of the median OS and PFS using the Kaplan–Meier method, which were performed using the "survminer" package (RRID:SCR_021094), version 0.4.6, in R. Hazard ratios (HR) and corresponding 95% confidence intervals (95% CI) and *P* values were estimated from Cox regression models, as implemented by the Python function "CoxPHFitter" of the package "lifelines" (RRID:SCR_024899), version 0.23.3. Wilcoxon rank-sum, χ^2, and Fisher's exact tests were, respectively, performed using the "stats.ranksums," "stats.chi2_contingency," and "stats.fisher_exact" functions of the SciPy package (RRID:SCR_008058), version 1.4.1, in Python. The "Seaborn" package (RRID:SCR_018132), version 0.11.0, in Python was used for plotting (except from the generation of Kaplan–Meier plots, see above).

Data availability

The full clinicogenomic dataset used for biomarker analyses for this discovery cohort is available in Supplementary Table S1. This table also contains the patient IDs that could be used to obtain the raw WGS data. This included 56 patients who provided written informed consent for including their sequencing data in the Hartwig dataset and their data are freely available via standardized procedures after approval of a data access request at Hartwig Medical Foundation (https://www.hartwigmedicalfoundation.nl/en/). For the 19 additional patients included in the Netherlands Cancer Institute, this concerned bio-banked samples collected before April 21, 2016, for which informed consent was not required as per local legislation. For this reason, these samples could not be added to the general Hartwig database but data are available via the corresponding author upon reasonable request.

Results

Discovery cohort description

Seventy-five patients with stage IV NSCLC were treated with ICB monotherapy (PD-1 blockade: 72 patients, PD-L1 blockade: 3 patients) and successfully analyzed by WGS (Materials and Methods; **Table 1**; Supplementary Table S1). Most patients received ICB as second-line treatment, after initial treatment with chemotherapy. Smoking status was available for 64 out of 75 (85%), of which 9 (14%) were never smokers. Adenocarcinoma was the most frequent histology, representing 47 out of 75 (62.7%) patients. Routine diagnostic tests for PD-L1 expression were performed in 57 out of 75 (57%) patients. Of these, 27 (47%) patients showed low PD-L1 expression (<1% PD-L1 positivity tumor cells), 16 (28%) patients demonstrated intermediate PD-L1 expression (1%–50% PD-L1 positivity) and 14 (25%) patients were found to express high levels of PD-L1 (>50% PD-L1 positivity of tumor cells). In total, 28 out of 75 (37%) patients obtained DCB, defined as an objective response or durable SD lasting >6 months, according to RECIST v1.1 criteria (31).

WGS-based TMB and DCB with PD-(L)1 blockade

Our WGS data of tumors and matched germline allowed us to precisely quantify the genome-wide number of non-synonymous somatic mutations in each tumor per coding megabase (Mb), known as the TMB. In line with previous findings (17, 27, 28), we found that the TMB, as a continuous parameter, was strongly associated with DCB [two-sided Wilcoxon rank-sum test *P* = 0.00029; area under the receiver operating curve (ROC) = 0.75; **Fig. 1A**]. On the basis of the TMB cutoff value of 10 muts/Mb that is broadly used worldwide (28, 34–36), we next defined subgroups with low or high TMB. We found that patients with a low TMB showed a clearly reduced DCB rate versus those with high TMB (20% vs. 66%, respectively; two-sided Fisher's exact test *P* = 0.000087; **Fig. 1B**). In line with this, patients

Table 1. Baseline characteristics of patients used for biomarker analyses.

Cohort	Discovery	Validation	
Patients (n)	75	169	
Age (mean; SD)	62.6 (9.9)	66.3 (9.9)	$P = 0.0083$
Gender (%)			
Male	39 (52.0)	71 (42.0)	$P = 0.16$
Female	36 (48.0)	98 (58.0)	
Smoking status (n; %)			
Current/former	57 (76.0)	145 (85.8)	$P = 0.83$
Never	7 (9.3)	21 (12.4)	
Unavailable	11 (14.7)	3 (1.8)	
Pack years (mean; SD)	29.1 (19.3)	31.4 (27.5)	$P = 0.93$
ECOG (%)			
0	18 (24.0)	20 (11.8)	$P = 0.0035$
1	40 (53.3)	136 (80.5)	
2	9 (12.0)	13 (7.7)	
>2	1 (1.3)	0 (0)	
Unavailable	7 (9.3)	0 (0)	
Treatment (n; %)			
Nivolumab	48 (64.0)	70 (41.4)	$P = 0.00019$
Pembrolizumab	24 (32.0)	62 (36.7)	
Atezolizumab	1 (1.3)	35 (20.7)	
Durvalumab	2 (2.7)	2 (1.2)	
Treatment line (n; %)			
1	13 (17.6)	50 (29.6)	$P = 0.24$
2	54 (73.0)	98 (58.0)	
3	5 (6.8)	15 (8.9)	
4	2 (2.7)	5 (3.0)	
5	0 (0)	1 (0.6)	
Histology (n; %)			
Adenocarcinoma	47 (62.7)	130 (76.9)	$P = 0.026$
Squamous cell carcinoma	13 (17.3)	23 (13.6)	
NOS	13 (17.3)	10 (5.9)	
Other	2 (2.7)	6 (3.6)	
PD-L1 expression status (n; %)			
<1%	27 (36.0)	63 (37.3)	$P = 0.92$
1%–50%	16 (21.3)	38 (22.5)	
>50%	14 (18.7)	38 (22.5)	
Unavailable	18 (24.0)	30 (17.8)	
DCB (n; %)			
NO	47 (62.7)	103 (60.9)	$P = 0.89$
YES	28 (37.3)	66 (39.1)	

Note: Data are shown separately for the discovery (left) and validation [right, Vanguri et al. (11)] cohorts. Two-sided P values are shown for between-cohort comparisons of available data and were calculated with the Wilcoxon rank-sum test (continuous variables), Fisher's exact test (dichotomous variables), or χ^2 test (discrete variables with >2 levels).

with a low TMB had significantly shorter PFS (HR, 3.50; 95% CI, 1.96–6.24; $P = 0.000024$; **Fig. 1C**), and OS (HR, 3.80; 95% CI, 1.86–7.75; $P = 0.00025$; **Fig. 1D**). The association of the clonal TMB (cTMB; defined as the genome-wide number of clonal non-synonymous mutations per coding Mb) with DCB was slightly more significant as compared with the "general" TMB in both the continuous analysis (two-sided Wilcoxon rank-sum test $P = 0.00018$; Supplementary Fig. S1A) and the discrete analysis (low vs. high cTMB, based on the same cutoff of 10 muts/Mb; two-sided Fisher's exact test $P = 0.000049$; Supplementary Fig. S1B). However, it should be noted that only a single patient in the cohort showed discordant grouping in the TMB versus cTMB analysis; this was a patient with high TMB but low cTMB and who failed to obtain DCB. In contrast with a recent study

using whole-exome sequencing (WES; ref. 15), we could not find evidence that tumor purity confounded the WGS-based TMB (Supplementary Fig. S2), perhaps because of our (standard) procedure to exclude samples with a tumor purity <20% from further consideration. Taken together, the TMB was strongly associated with treatment outcome in our cohort, but the 20% DCB rate in patients with a low TMB limits the clinical value of the TMB as a standalone biomarker. Therefore, additional biomarkers are needed to complement the TMB to enhance specificity for identifying patients who fail to benefit from PD-(L)1 blockade in NSCLC.

PD-L1 protein expression and DCB in patients with low TMB

We next assessed whether PD-L1 IHC could complement the TMB to achieve higher specificity of non-responsiveness. Patient stratification based on low, intermediate, or high PD-L1 tumor cell positivity only resulted in significantly different DCB rates in the population with a high TMB, whereas this was not the case in the population with low TMB (high TMB: two-sided χ^2 test $P = 0.047$, $n = 22$ patients with available data; low TMB: two-sided χ^2 test $P = 0.45$, $n = 35$ patients with available data; **Fig. 1E**). Notably, "double low" patients with both a low TMB and a low PD-L1 score still demonstrated a potentially clinically relevant 11% DCB rate (vs. 20% in the population with low TMB regardless of PD-L1 status). Thus, in our dataset, the combined consideration of these two biomarkers was insufficient to overcome the specificity issue of the TMB alone.

STK11 alterations and DCB in the discovery cohort

Building on previous work (2, 7–11), we next investigated whether clonal, pathogenic alterations in STK11 can complement the TMB in identifying patients without DCB with increased specificity. Twenty patients carried a total of 22 somatic STK11 alterations (Supplementary Table S2), which were all clonal and included 4 bi-allelic deletions and 18 nonsynonymous mutations (two patients carried two mutations). Two mutations were VUSs classified as likely passenger mutations and were excluded from further consideration (Materials and Methods). This resulted in a set of 20 clonal, pathogenic STK11 alterations occurring in 18 patients, including 9 patients with a low TMB and 9 patients with a high TMB. Of these 18 patients, 17 (94%) patients had an STK11 alteration affecting both alleles (mutation plus LOH or bi-allelic deletion).

Among patients with a clonal, pathogenic STK11 alteration occurring in the context of a low TMB, 0 out of 9 (0%) obtained DCB, versus 7 out of 9 (78%) of patients when STK11 alterations occurred in the context of a high TMB (Fisher's exact test–based $P = 0.0023$; **Fig. 1F**). These findings were supported by survival analyses, as STK11 alteration status was significantly associated with shorter PFS (HR, 2.40; 95% CI, 1.11–5.17; $P = 0.026$; **Fig. 1G**), and OS (HR, 3.49; 95% CI, 1.53–5.17; $P = 0.0031$; Supplementary Fig. S3A) in the population with low TMB, whereas this was not the case for the population with a high TMB (Supplementary Fig. S3B and S3C). Of note, the STK11 mutant patient with a low TMB that had the longest PFS ($\sim$6 months; **Fig. 1G**) lacked radiological response assessments at earlier time points. Therefore, this patient had a best overall response of PD, without confirmed clinical benefit of ICB. In our cohort, STK11 alterations were not correlated with the TMB in the populations with low or high TMB (low TMB: $P = 0.66$; high TMB: $P = 0.37$; both by two-sided Wilcoxon rank-sum test). Two out of 8 (25%) patients with co-alterations in STK11 and KRAS showed DCB and these co-alterations were neither significantly associated with DCB in the full cohort, nor in the populations with low or high TMB (full cohort: $n = 8$ patients with co-alterations, $P = 0.70$; low TMB: $n = 5$ co-alterations, $P = 0.57$; high
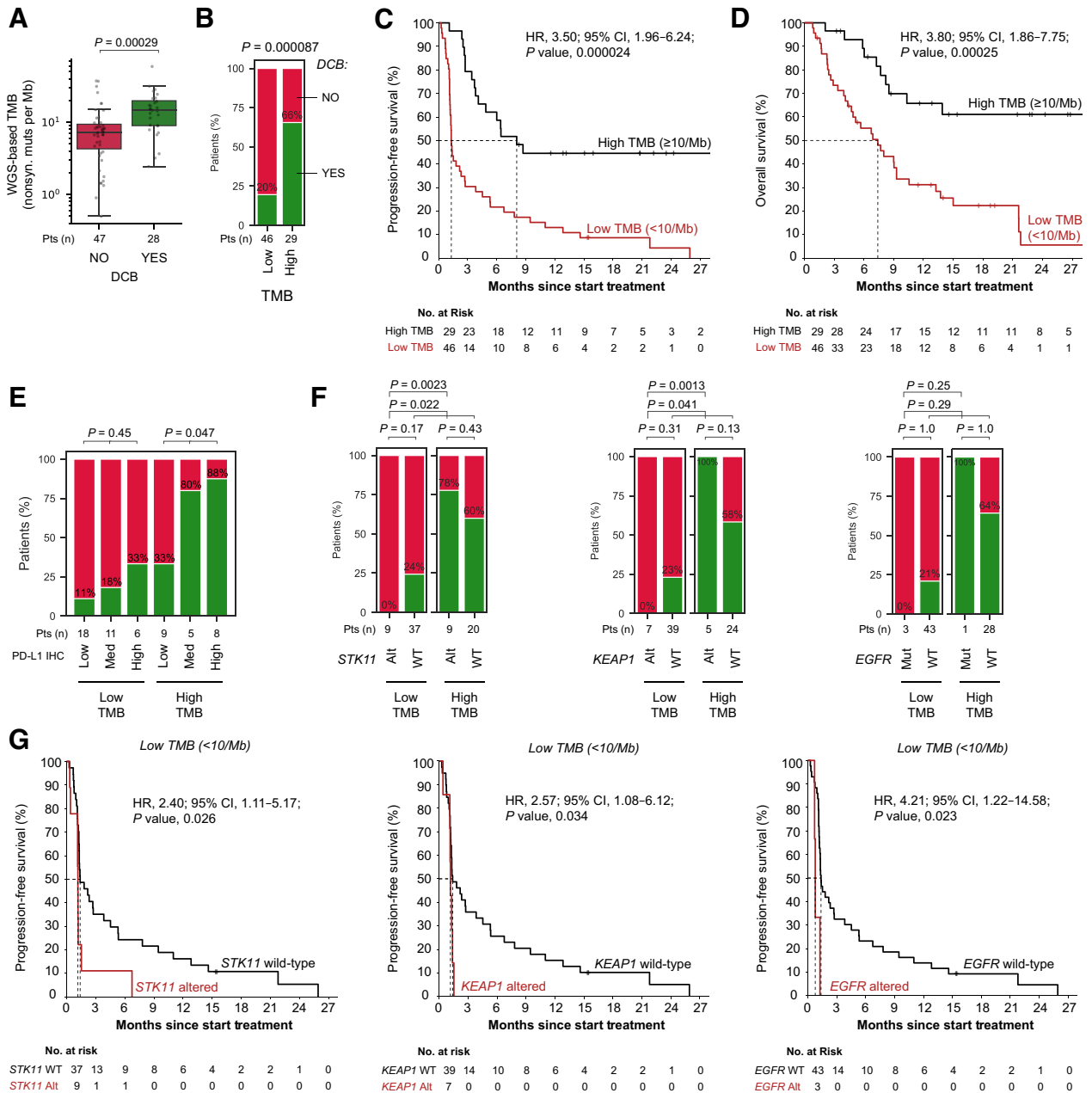
Figure 1.
Biomarkers associated with outcome of ICB monotherapy in the discovery cohort. **A,** Boxplot showing the (WGS-based) TMB (y-axis) for patients with/without DCB (x-axis). Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and individual data points, respectively. The Wilcoxon rank-sum test–based two-sided P value is shown. **B,** Stacked bar plot of the percentage of patients with (green) or without (red) DCB (y-axis), stratified for low (<10 muts/Mb) versus high (≥10 muts/Mb) WGS-based TMB (x-axis). The Fisher's exact test–based two-sided P value is shown. **C,** Kaplan–Meier curve showing the PFS of patients with a low (<10 muts/Mb; red) or high (≥10 muts/Mb; black) WGS-based TMB. Dashed lines indicate the median PFS. The numbers at risk are shown in the table below the plot. Cox regression–based univariate hazard ratio, 95% confidence intervals, and two-sided Wald's test-based P value are shown. **D,** As C, but for overall survival. **E,** Stacked bar plot of the percentage of patients with (green) or without (red) DCB (y-axis), stratified on the x-axis for the level of PD-L1 positivity of tumor cells on IHC (low, <1% positive cells; medium, 1%–50%; high, >50%) and the TMB (low vs. high). The P value is calculated by two-sided χ^2 test for the low and high TMB subgroups. **F,** Stacked bar plots of the percentage of patients with (green) or without (red) DCB (y-axis), stratified on the x-axis based on the TMB (low vs. high) and presence/absence of alterations in STK11 (left plot), KEAP1 (center plot) or EGFR (right plot). The Fisher's exact test–based two-sided P values are shown. **G,** Kaplan–Meier curves showing the PFS of patients with a low (<10 muts/Mb) TMB, stratified on the basis of the presence/absence of alterations in STK11 (left plot), KEAP1 (center plot), or EGFR (right plot). Dashed lines indicate the median survival. The numbers at risk are shown in the table below the plot. Cox regression–based univariate hazard ratios, 95% confidence intervals, and two-sided Wald's test-based P values are shown.

TMB: $n = 3$ co-alterations, $P = 1.00$; all by two-sided Fisher's exact test). Taken together, these data suggest that *STK11* alteration status and the TMB are complementary biomarkers, whose combined consideration allows the identification of non-responders with increased specificity as compared with considering either one alone.

KEAP1 alterations and DCB in the discovery cohort

We proceeded to investigate whether clonal, pathogenic alterations in *KEAP1*—that have previously been linked to ICB resistance (8, 9, 12–15)—could also enhance the specificity of identifying patients without DCB when combined with the TMB. Sixteen patients exhibited a total of 18 somatic alterations in *KEAP1* (Supplementary Table S2), consisting of three bi-allelic deletions and 15 mutations, all of which were determined to be clonal. Among these, four were VUS classified as likely passenger events and were therefore excluded from further analysis (Materials and Methods). The remaining 14 pathogenic and clonal *KEAP1* alterations occurred across 12 patients, with 10 alterations displaying genomic evidence of bi-allelic alteration (mutation plus LOH or bi-allelic deletion). One of the two patients without direct evidence of bi-allelic alteration of *KEAP1* may in fact had both alleles altered, as this patient carried two *KEAP1* mutations whose combined copy number was equal to the total gene copy number in the tumor but affected genomic locations too distant to accurately determine whether the mutations occurred in trans.

Among patients with a clonal, pathogenic *KEAP1* alteration and a low TMB, 0 out of 7 (0%) exhibited DCB upon ICB treatment, versus 5 out of 5 (100%) patients when *KEAP1* alterations occurred in the context of a high TMB (Fisher's exact test–based $P = 0.0013$; **Fig. 1F**). These findings were corroborated by the PFS analysis, as *KEAP1* alteration status showed a significant association with shorter PFS in the low TMB population (HR, 2.57; 95% CI, 1.08–6.12; $P = 0.034$; **Fig. 1G**), but not in the high TMB population (Supplementary Fig. S3B). Although the association of *KEAP1* alteration status with OS did not attain statistical significance in the low TMB population (HR, 2.24; 95% CI, 0.89–5.61; $P = 0.086$; Supplementary Fig. S3A), long-term OS of *KEAP1* altered patients was only observed in the context of a high TMB (Supplementary Fig. S3C). Of note, there was no correlation observed between *KEAP1* alterations and the TMB in both the low and high TMB populations (low TMB: $P = 0.22$; high TMB: $P = 0.11$; both by two-sided Wilcoxon rank-sum test). Furthermore, three out of 5 (60%) patients with co-alterations in *KEAP1* and *KRAS* showed DCB, all of which had a high TMB. Collectively, these findings indicated that the combined assessment of *KEAP1* alteration status and TMB served as an effective combinatorial biomarker strategy, offering increased specificity in identifying ICB-resistant patients compared with relying on either biomarker individually.

EGFR alterations and DCB in the discovery cohort

As actionable *EGFR* alterations have previously been identified as markers of ICB resistance (23–25), we next focused on this alteration class. In total, 4 patients in the discovery cohort harbored actionable *EGFR* alterations known to be targets of tyrosine kinase inhibitors (Supplementary Table S2). Three of these patients also harbored a low TMB, none of which obtained DCB from ICB treatment, whereas the only *EGFR* mutant patient with a high TMB had DCB (**Fig. 1F**). Despite the low numbers, *EGFR* mutations were significantly associated with both shorter PFS and OS in the population with a low TMB (PFS: HR, 4.21; 95% CI, 1.22–14.58; $P = 0.023$; OS: HR, 4.97; 95% CI, 1.44–17.1; $P = 0.011$; **Fig. 1G**; Supplementary Fig. S3A). In contrast, the single *EGFR*-mutant patient with a high TMB had excellent

survival, with PFS and OS both exceeding 1 year and still ongoing at the last follow-up (Supplementary Fig. S2B and S2C). Of note, other actionable drivers affecting *ALK*, *RET*, *HER2*, and *ROS1*, which have also been previously linked to inferior ICB responsiveness (24), were not observed in our cohort.

Combinatorial biomarker strategy in the discovery cohort

We next combined these insights into the design of a combinatorial biomarker strategy, where patients were predicted to obtain no DCB in case they had a clonal, pathogenic alteration in either *STK11*, *KEAP1*, or *EGFR*, which occurred within the context of a low TMB. Among 15 patients complying with these criteria in the discovery cohort, the DCB rate was 0% and significantly lower as compared with all other patients (Fisher's exact test–based two-sided $P = 0.00056$; **Fig. 2A**), or to other patients with a low TMB (Fisher's exact test–based two-sided $P = 0.021$; **Fig. 2A**). In both the low and high TMB subgroups of our cohort, the presence of *STK11*/*KEAP1*/*EGFR* alterations was neither associated with the TMB (**Fig. 2B**), nor with most clinical baseline characteristics (the only exception being a weak positive association of *STK11*/*KEAP1*/*EGFR* alterations with adenocarcinoma histology in the high TMB subgroup; Supplementary Table S3). Only in the context of a low TMB, the presence of a clonal, pathogenic alteration in either *STK11*, *KEAP1*, or *EGFR* was strongly associated with shorter PFS (low TMB population: HR, 3.27; 95% CI, 1.63–6.55; $P = 0.00083$; high TMB population: HR, 0.41; 95% CI, 0.14–1.18; $P = 0.10$; **Fig. 2C** and **D**) and OS (low TMB population: HR, 3.91; 95% CI, 1.82–8.38; $P = 0.00046$; high TMB population: HR, 0.11; 95% CI, 0.013–0.84; $P = 0.033$; **Fig. 2E** and **F**). Together, considering *STK11*/*KEAP1*/*EGFR* alterations in the context of the TMB provided a highly specific strategy for identifying non-responding patients in the discovery cohort, without misclassifying any responding patients.

Independent validation of the genomic biomarker strategy

To validate our combinatorial genomic biomarker strategy with an independent dataset, we analyzed published data of 169 patients with NSCLC treated with PD-(L)1 blockade monotherapy (11). In comparison with the discovery cohort, patients in the validation cohort showed similar response rate, PD-L1 expression, smoking history, and pretreatment history (**Table 1**). Patients in the validation cohort were, however, slightly older (66.3 vs. 62.6 years), had more often an ECOG performance status above 0 (88.2% vs. 73.5%), had more frequent adenocarcinoma histology (76.9% vs. 62.7%), and were more frequently treated with atezolizumab (20.7% vs. 1.3%; **Table 1**). Aware of potential biases that may be introduced by the fact that the genomics testing of this validation cohort was not performed using WGS but through large panel sequencing, we considered this a relatively stringent validation scenario, as technical differences may reduce the consistency between the discovery and validation datasets. Therefore, we directly compared the TMB between the validation and discovery cohorts and, reassuringly, found no evidence of a systematic technical bias as the TMB distribution was highly comparable between the cohorts (Wilcoxon rank sum–based two-sided $P = 0.57$; **Fig. 3A**). Furthermore, the TMB was again associated significantly with DCB in the validation cohort [continuous: Wilcoxon rank sum–based two-sided $P = 0.00036$, area under the ROC $= 0.66$; discrete (using the same threshold of <10 nonsynonymous mutations per coding Mb): Fisher's exact test–based two-sided $P = 0.00049$; **Fig. 3B** and **C**], whereas a clinically relevant 27% DCB-rate was observed among patients with a low TMB (**Fig. 3C**). Together, these results demonstrated the reasonable comparability between the two cohorts for moving forward with the validation.
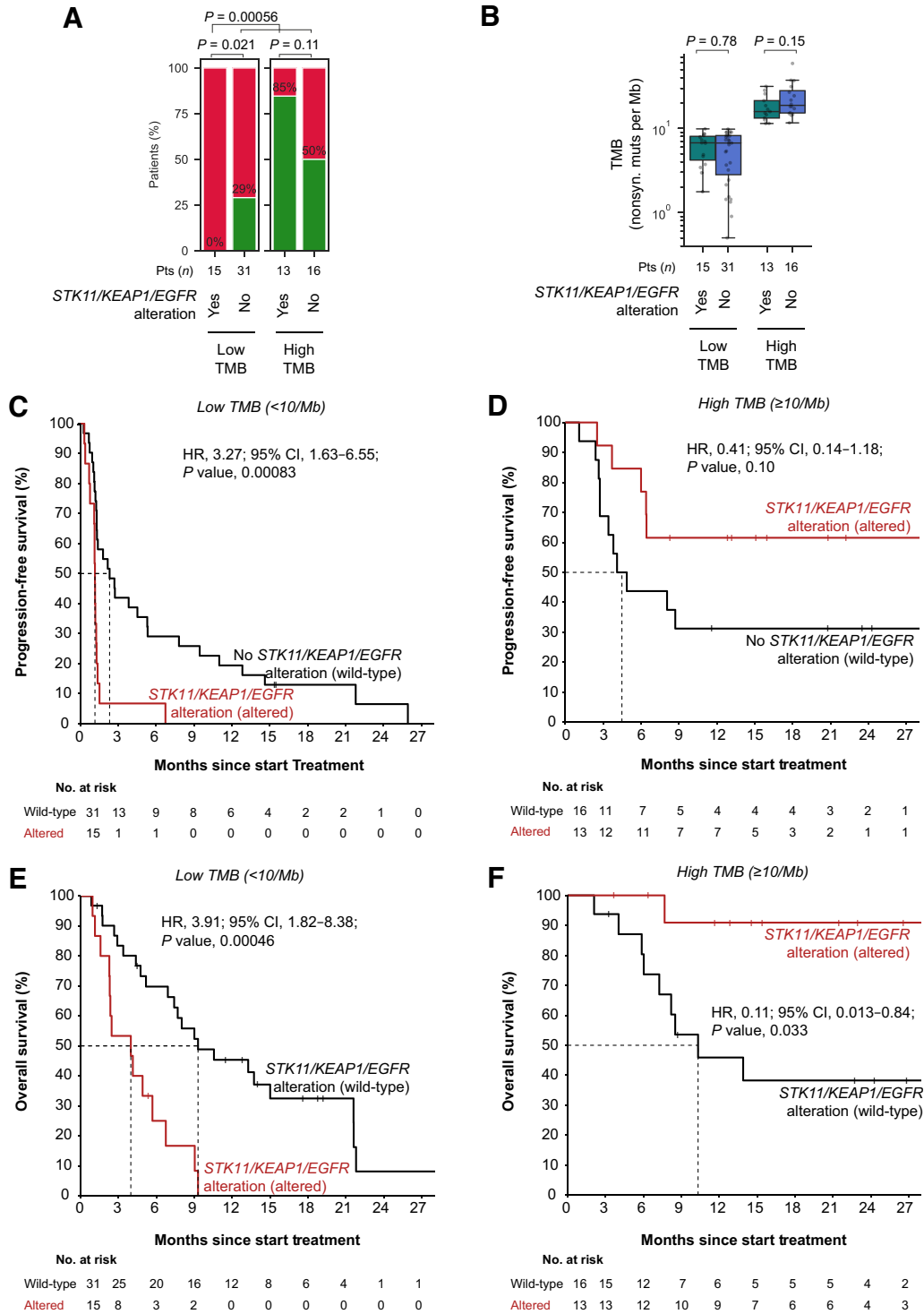
Figure 2.
Associations of *STK11/KEAP1/EGFR* alterations with outcome of ICB treatment in low/high TMB contexts in the discovery cohort. **A,** Stacked barplot of the percentage of patients with (green) or without (red) DCB (*y*-axis), stratified on the *x*-axis based on the TMB (low vs. high) and presence/absence of at least one *STK11/KEAP1/EGFR* alteration. The Fisher's exact test–based two-sided *P* value is shown. **B,** Boxplot of the TMB (*y*-axis), stratified on the *x*-axis based on the TMB (low vs. high) and presence/absence of at least one *STK11/KEAP1/EGFR* alteration. The Fisher's exact test–based two-sided *P* value is shown. The Wilcoxon rank-sum test–based two-sided *P* value is shown. **C,** Kaplan–Meier curves showing the PFS of patients with a low (<10 muts/Mb) TMB, stratified on the basis of the presence/absence at least one *STK11/KEAP1/EGFR* alteration. Dashed lines indicate the median survival. The numbers at risk are shown in the table below the plot. Cox regression–based univariate hazard ratio, 95% confidence interval, and two-sided Wald's test–based *P* value are shown. **D,** As C, but for patients with a high (≥10 muts/Mb) TMB. **E,** As C, but for OS. **F,** As D, but for OS.
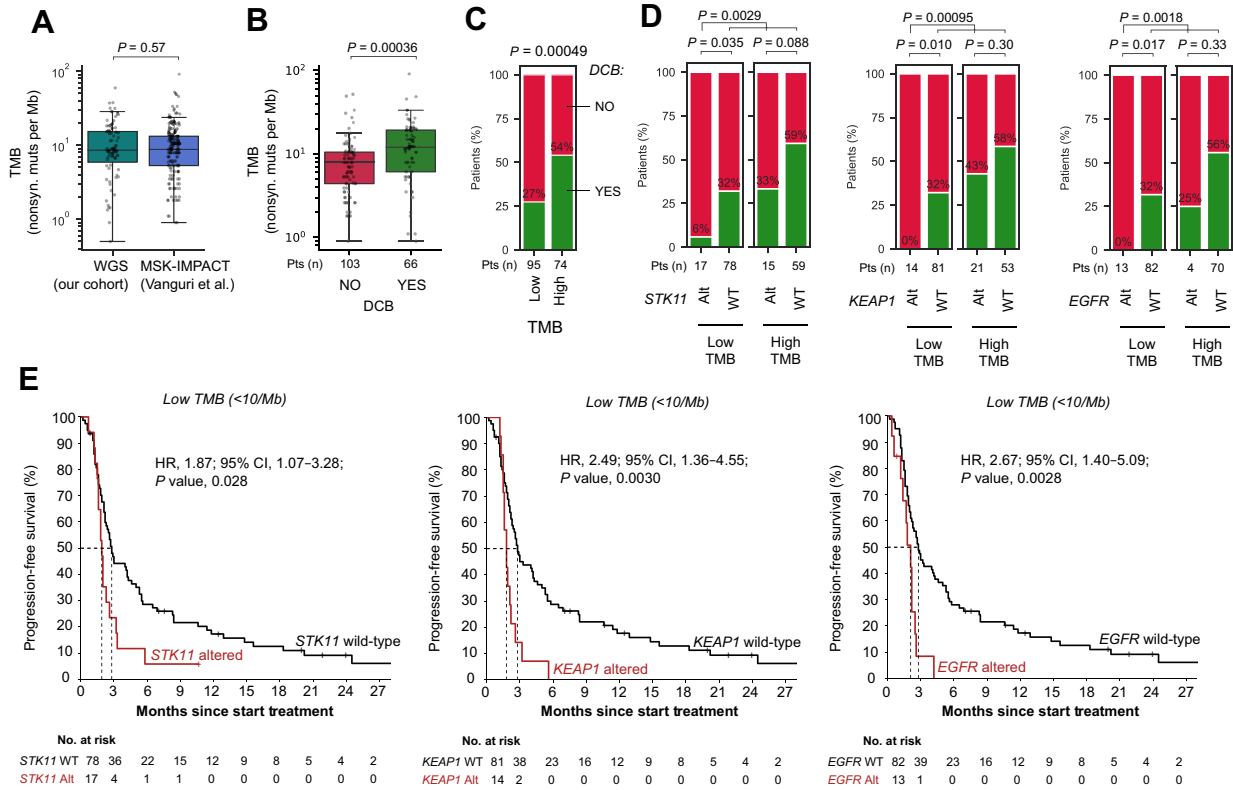
Figure 3.
The TMB and *STK11/KEAP1/EGFR* alterations and their association with outcome of ICB treatment in the validation cohort. **A,** Boxplot showing the TMB (*y* axis) for patients in the discovery and validation cohort (*x*-axis). Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and individual data points, respectively. The Wilcoxon rank-sum test–based two-sided *P* value is shown. **B,** Boxplot showing the TMB (*y*-axis) for patients with/without DCB (*x*-axis) in the validation cohort. Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and individual data points, respectively. The Wilcoxon rank-sum test–based two-sided *P* value is shown. **C,** Stacked bar plot of the percentage of patients with (green) or without (red) DCB (*y* axis) in the validation cohort, stratified for low (<10 muts/Mb) versus high (≥10 muts/Mb) TMB (*x*-axis). The Fisher's exact test–based two-sided *P* value is shown. **D,** Stacked bar plots of the percentage of patients with or without (red) DCB (*y* axis) in the validation cohort, stratified on the *x*-axis based on the TMB (low vs. high) and presence/absence of alterations in *STK11* (left plot), *KEAP1* (center plot) or *EGFR* (right plot). The Fisher's exact test–based two-sided *P* values are shown. **E,** Kaplan–Meier curves showing the PFS of patients with a low (<10 muts/Mb) TMB in the validation cohort, stratified on the basis of the presence/absence of alterations in *STK11* (left plot), *KEAP1* (center plot), or *EGFR* (right plot). Dashed lines indicate the median survival. The numbers at risk are shown in the table below the plot. Cox regression–based univariate hazard ratios, 95% confidence intervals, and two-sided the Wald's test–based *P* values are shown.

We first validated our findings on a per-gene basis by studying the associations of treatment outcome with the alteration status of individual genes in the validation cohort. Consistent with the discovery cohort, patients in the validation cohort showed very low DCB rates if their tumors harbored an alteration in *STK11*, *KEAP1* or *EGFR* plus a low TMB (*STK11*: DCB in 1 out of 17 (6%) patients; *KEAP1*: DCB in 0 out of 14 (0%) patients; *EGFR*: DCB in 0 out of 13 (0%) patients; **Fig. 3D**; Supplementary Table S4). Long-term PFS of patients with *STK11/KEAP1/EGFR* alterations was almost exclusively observed in the context of a high TMB (**Fig. 3E**; Supplementary Fig. S4A). The only exception was a patient experiencing DCB whereas having an *STK11* E119* alteration in the context of a TMB of 6.1 and 60% PD-L1 positivity on tumor cells, who experienced a clinically relevant PFS of 11 months that was still ongoing at data cutoff. Furthermore, patients with *STK11* and *KEAP1* alterations that occurred in the context of a low TMB had poor OS (Supplementary Fig. S4B and S4C). Patients with *EGFR* alterations tended to have relatively long OS regardless of their short PFS on ICB treatment and/or their TMB status (Supplementary Fig. S4B and S4C), likely reflecting a dominating effect of TKI

therapy on the OS of these patients. Of note, 3 out of 17 (17.6%) patients with *KEAP1+KRAS* co-alterations and 2 out of 19 (10.5%) patients with *STK11+KRAS* co-alterations experienced DCB, all of which occurred in the context of a high TMB.

We next moved forward with testing our combinatorial genomic biomarker strategy in the validation cohort. Among 34 patients in the validation cohort with at least one *STK11/KEAP1/EGFR* alteration and a low TMB, we observed a DCB rate of 3% (1 patient), which was significantly lower as compared with all other patients in the validation cohort (Fisher's exact test–based two-sided $P = 2.2 \times 10^{-7}$; **Fig. 4A**), or in comparison with only the other patients harboring a low TMB (Fisher's exact test–based two-sided $P = 0.000027$; **Fig. 4A**). In both the low and high TMB subgroups of the validation cohort, the presence of *STK11/KEAP1/EGFR* alterations was not associated with the TMB (**Fig. 4B**) or clinical baseline characteristics (Supplementary Table S5). In line with the negative association of *STK11/KEAP1/EGFR* alterations with DCB, we did however observe an overrepresentation of patients with PD-L1–negative tumors among patients with these alterations in the validation cohort [low TMB population: PD-L1
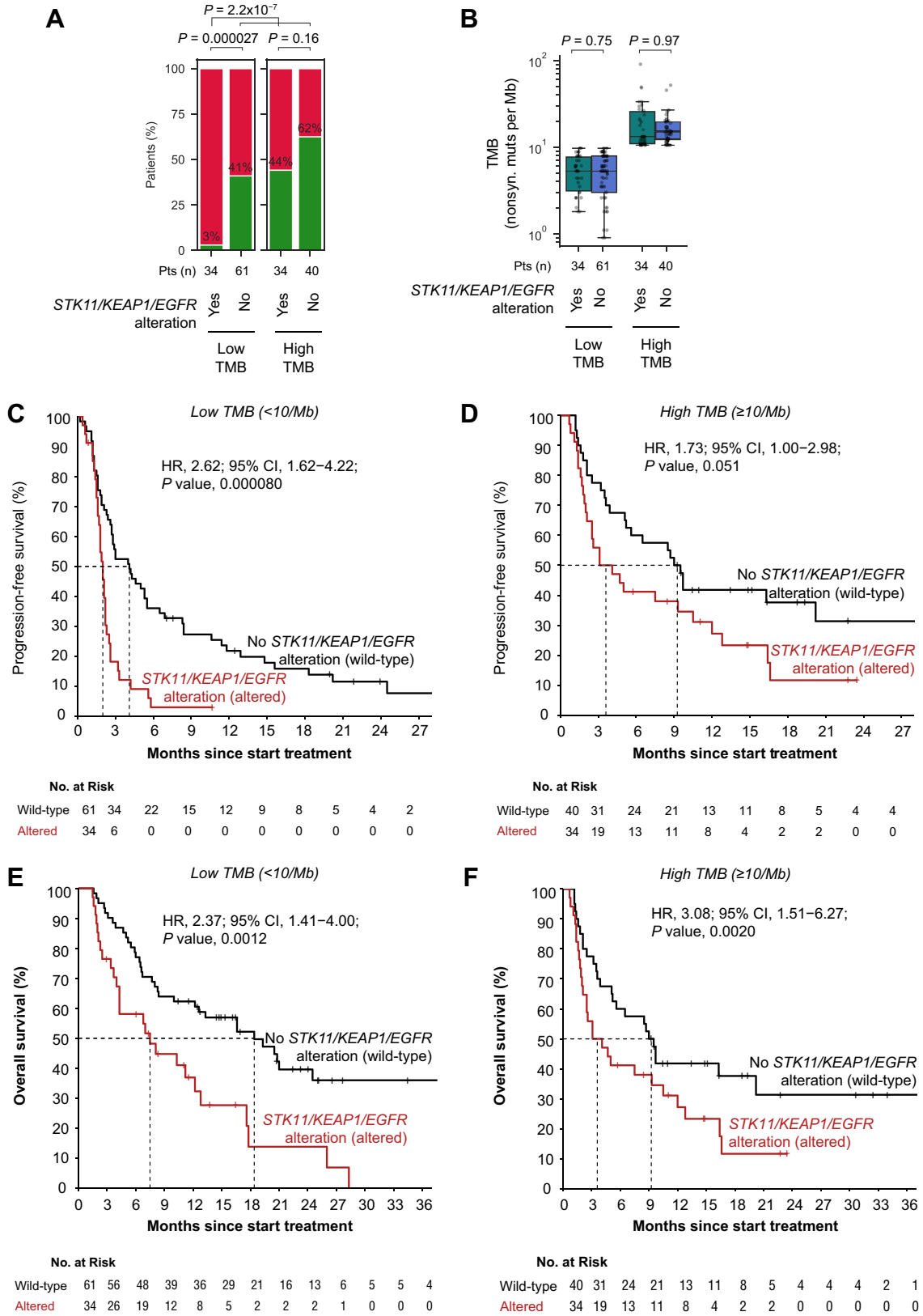
score <1% in 20 out of 34 (58.8%) patients with *STK11/KEAP1/EGFR* alterations versus 20 out of 61 (32.8%) in triple WT patients; Supplementary Table S5]. In the population with a low TMB, *STK11/KEAP1/EGFR* alterations were also strongly associated with shorter PFS (HR, 2.62; 95% CI, 1.62–4.22; $P = 0.000080$; **Fig. 4C**). In contrast, in the population with a high TMB, a clinically relevant proportion of patients with *STK11/KEAP1/EGFR* alterations obtained long-term PFS (~27% 1-year PFS probability; **Fig. 4D**). Furthermore, in terms of OS, *STK11/KEAP1/EGFR* alterations were significantly associated with poor outcome in the population with low TMB (HR, 2.37; 95% CI, 1.41–4.00; $P = 0.0012$; **Fig. 4E**), as well as in the population with high TMB (HR, 3.08; 95% CI, 1.51–6.27; $P = 0.0020$; **Fig. 4F**). Taken together, considering *STK11/KEAP1/EGFR* alterations together with the TMB also provided a highly specific strategy to identify patients with ultralow ICB responsiveness in the validation cohort.

Discussion

Despite the successful identification of a variety of genomic biomarkers for guiding ICB treatment in NSCLC, clinical implementation of precision immunotherapy for this disease is hampered by the insufficient specificity of biomarkers for identifying ICB-resistant patients. At the same time, only a minority of patients with NSCLC benefit from ICB treatment and more effective (combinatorial) biomarker strategies in this space are urgently needed. Here, we performed whole-genome analysis of a discovery cohort and the analysis of an independent validation cohort subjected to large-panel sequencing, and report the key observation that patients with NSCLC who respond to ICB despite the presence of resistance biomarkers in *STK11/KEAP1/EGFR* almost always harbor a high TMB. These signals of activity could shed new light on studies testing ICB treatment in populations carrying these resistance markers. For example, the KEYNOTE-789 study (37) found a modest benefit of ICB plus chemo versus chemo-alone in patients with TKI resistant, *EGFR* mutant NSCLC; however, this study just failed to attain the predefined efficacy boundaries of $P = 0.0117$ for PFS and $P = 0.0118$ for OS. Our results suggest that it is important to study whether this modest benefit of the ICB plus chemo combination in this setting is more pronounced in the context of a high TMB. At the same time, our results are important for the development of more specific strategies for identifying ICB-resistant NSCLC. Through a straightforward algorithm predicting ICB unresponsiveness based on the presence of at least one *STK11/KEAP1/EGFR* alteration plus a low TMB, we only misclassified 0 and 1 responding patients in the discovery and validation cohorts, respectively. As this approach allowed us to identify 32% and 29% of patients without DCB in the discovery and validation cohorts, our approach carries potential to substantially limit overtreatment in this patient population. Taken together, the specificity of established resistance biomarkers for ICB therapy in NSCLC could be markedly improved by considering them in conjunction with the TMB.

On the basis of existing literature, we hypothesized that the specificity of our combinatorial biomarker strategy relies on a mixture of prognostic and predictive effects. *STK11* inactivation has been associated with immune evasion and exclusion (7, 9, 38), but also to poor outcome on chemotherapy (14, 16, 39, 40). *KEAP1*-altered NSCLC has also been described to show poor outcome on chemotherapy, suggestive of a poor prognostic effect of these alterations (16, 39). Indeed, the results of a recently reported randomized controlled trial are in line with mixed prognostic and predictive effects of *STK11/KEAP1* alterations in the context of ICB (8). Biologically, ICB treatment may elicit relatively weak antitumor immune responses in tumors with low TMB, allowing tumors with *STK11/KEAP1/EGFR* alterations to escape the ICB-driven immune response. In the context of a high TMB, however, ICB-based immunity could be powerful enough to (sometimes) overrule the negative predictive and/or prognostic effects of *STK11/KEAP1/EGFR* alterations. More broadly, an important point of our study is that both prognostic and predictive biomarkers have the potential to be applied in the clinic for guiding ICB treatment if these biomarkers help identify patients with ultralow response rates.

Although our combinatorial biomarker strategy had great specificity for patients without DCB in both the discovery and validation cohorts, the observation that a patient with an *STK11*-mutant tumor and a low (panel sequencing–based) TMB had long-term survival in the validation cohort is important. Larger, prospective follow-up studies are needed to determine the precise frequency of such outlier events and future decision making should weigh these against the potential harmful effects of ineffective ICB treatment on health, quality of life and costs. Because we did not observe these outliers in our WGS-based analysis of the discovery cohort, it is important to assess whether these rare misclassifications could be effectively prevented by using more extensive sequencing techniques like WGS or WES. WGS yields orders of magnitude more data and hence more precise estimates of the TMB (36). Indeed, we found that the WGS-based TMB had more discriminative capacity to classify patients with/without DCB in the discovery cohort as compared with the panel sequencing–based TMB in the validation cohort (area under the ROC 0.75 vs. 0.66, respectively). Furthermore, the patient who responded despite carrying an *STK11* mutation plus a low TMB showed high PD-L1 expression (60% tumor cell positivity) and it should be investigated in larger studies if PD-L1 testing would be of value as another diagnostic layer in combination with TMB plus *STK11/KEAP1/EGFR* testing. Finally, although the fact that the patients in our study were treated with anti–PD-(L)1 monotherapy presented a major advantage for identifying ICB-specific biomarkers, it remains an open question how our results generalize to ICB plus chemotherapy combination treatment. To address the questions outlined above, a confirmatory, prospective,

Figure 4.
Associations of *STK11/KEAP1/EGFR* alterations with outcome of ICB treatment in low/high TMB contexts in the validation cohort. **A,** Stacked bar plot of the percentage of patients with (green) or without (red) DCB (*y*-axis) in the validation cohort, stratified on the *x*-axis based on the TMB (low vs. high) and presence/absence of at least one *STK11/KEAP1/EGFR* alteration. The Fisher's exact test–based two-sided *P* value is shown. **B,** Boxplot of the TMB (*y*-axis), stratified on the *x*-axis based on the TMB (low vs. high) and presence/absence of at least one *STK11/KEAP1/EGFR* alteration in the validation cohort. The Fisher's exact test–based two-sided *P* value is shown. The Wilcoxon rank-sum test–based two-sided *P* value is shown. **C,** Kaplan–Meier curves showing the PFS of patients with a low (<10 muts/Mb) TMB in the validation cohort, stratified on the basis of the presence/absence at least one *STK11/KEAP1/EGFR* alteration. Dashed lines indicate the median survival. The numbers at risk are shown in the table below the plot. Cox regression–based univariate hazard ratio, 95% confidence interval, and two-sided Wald's test–based *P* value are shown. **D,** As C, but for patients with a high (≥10 muts/Mb) TMB. **E,** As C, but for OS. **F,** As D, but for OS.

randomized-controlled trial is planned to compare the efficacy of ICB plus chemo versus chemo-alone in patients with *STK11/KEAP1/EGFR* alterations and a low WGS-based TMB.

In conclusion, in this discovery and validation study, we showed how established biomarkers for ICB treatment in NSCLC can be effectively combined into a biomarker strategy with increased specificity for identifying resistant patients. The ultralow ICB response rates of patients with alterations in *STK11/KEAP1/EGFR* plus a low TMB suggest that it may be safe to withhold ICB monotherapy from this population and prospective, randomized follow-up studies are needed to confirm this.

Authors' Disclosures

Authors' Contributions

J. van de Haar: Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **J.M. Mankor:** Conceptualization, data curation, formal analysis, investigation, methodology, writing–original draft, writing–review and editing. **K. Hummelink:** Data curation, formal analysis, writing–review and editing. **K. Monkhorst:** Conceptualization, data curation, formal analysis, supervision, methodology, writing–review and editing. **E.F. Smit:** Conceptualization, formal analysis, supervision, methodology, writing–original draft, writing–review and editing. **L.F.A. Wessels:** Resources, software, formal analysis, supervision, methodology, writing–original draft, writing–review and editing. **E. Cuppen:** Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, methodology, project administration, writing–review and editing. **J.G.J.V. Aerts:** Conceptualization, formal analysis, supervision, funding acquisition, methodology, writing–original draft, project administration, writing–review and editing. **E.E. Voest:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, methodology, writing–original draft, project administration, writing–review and editing.t

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Note

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