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# Potential Predictive Value of *TP53* and *KRAS* Mutation Status for Response to PD-1 Blockade Immunotherapy in Lung Adenocarcinoma

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## Abstract

**Purpose:** Although clinical studies have shown promise for targeting programmed cell death protein-1 (PD-1) and ligand (PD-L1) signaling in non–small cell lung cancer (NSCLC), the factors that predict which subtype patients will be responsive to checkpoint blockade are not fully understood.

**Experimental Design:** We performed an integrated analysis on the multiple-dimensional data types including genomic, transcriptomic, proteomic, and clinical data from cohorts of lung adenocarcinoma public (discovery set) and internal (validation set) database and immunotherapeutic patients. Gene set enrichment analysis (GSEA) was used to determine potentially relevant gene expression signatures between specific subgroups.

**Results:** We observed that *TP53* mutation significantly increased expression of immune checkpoints and activated T-effector and interferon-γ signature. More importantly, the *TP53*/*KRAS* comutated subgroup manifested exclusive increased expression of PD-L1 and a highest proportion of *PD-L1*$^+$/*CD8A*$^+$. Meanwhile, *TP53*- or *KRAS*-mutated tumors showed prominently increased mutation burden and specifically enriched in the transversion-high (TH) cohort. Further analysis focused on the potential molecular mechanism revealed that *TP53* or *KRAS* mutation altered a group of genes involved in cell-cycle regulating, DNA replication and damage repair. Finally, immunotherapeutic analysis from public clinical trial and prospective observation in our center were further confirmed that *TP53* or *KRAS* mutation patients, especially those with co-occurring *TP53*/*KRAS* mutations, showed remarkable clinical benefit to PD-1 inhibitors.

**Conclusions:** This work provides evidence that *TP53* and *KRAS* mutation in lung adenocarcinoma may be served as a pair of potential predictive factors in guiding anti–PD-1/PD-L1 immunotherapy. *Clin Cancer Res; 23(12); 3012–24. ©2016 AACR.*

## Introduction

Recent clinical trials with anti-programmed cell death 1 (PD-1) and its ligand PD-1 ligand (PD-L1) therapies have shown unprecedented durable responses in patients with non–small cell lung cancer (NSCLC; refs. 1, 2). Unfortunately, only a minority of the total of treated patients respond to the current immunotherapy (3). The factors that determine which patients will be drug sensitive or resistant are not fully understood. Therefore, it has become a primary priority to identify the biomarkers that determine the responsiveness to checkpoint blockade, and to develop strategies that could potentially increase the patient response rates. Encouragingly, recent studies had demonstrated that tumor mutational load (4–6), DNA mismatch repair (MMR) deficiency (7), the intensity of CD8$^+$ T cell infiltrates (8, 9) and intratumoral PD-L1 expression (10, 11) have each been proposed as distinct biomarkers of response to anti–PD-1/PD-L1 therapies. Meanwhile, these factors are functionally interrelated and are often found coordinately in individual tumor specimens (12). This raises the question of whether there exist some other variables simultaneously affect two or more of these above factors so as to provide stronger predictive value for therapeutic outcomes.

The identification of subsets of lung adenocarcinoma with oncogenic drivers has transformed the treatment of NSCLC, particularly for patients whose tumors harbor activating mutations in *EGFR*. However, the goal of developing specific therapeutic strategies for those bearing activating mutations in *KRAS* has thus far proven elusive. Meanwhile, mutations in tumor suppressor genes *TP53* and *STK11* are also common in lung adenocarcinoma and frequently co-occur with *KRAS* mutations (13–15). Given that activation of specific oncogenic pathways can have broad effects on gene expression, it is reasonable to imagine that the genetic make-up of cancer cells could have major effects on the immune tumor microenvironment (TME), by driving specific immune-related pathways. This could be through induction of immune checkpoints, secretion

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

Programmed cell death ligand 1 (PD-L1) expression, tumor mutational load, and the intensity of $CD8^+$ T-cell infiltrates have recently been proposed as predictive biomarkers for response to PD-1 blockade immunotherapy. However, there are still many treatment responses beyond the explanation of these factors. It is increased need for more effective biomarkers for PD-1 blockade. We demonstrated *TP53* and *KRAS* mutation had remarkable effects on increasing PD-L1 expression, facilitating T-cell infiltration and augmenting tumor immunogenicity. More important, we confirmed that patients with *TP53* and/or *KRAS* mutation showed sensitivity to PD-1 blockade. These findings represent the first demonstration of potential predictive value of *TP53* and *KRAS* mutation for response to PD-1 blockade immunotherapy in lung adenocarcinoma.

of specific cytokines or production of chemokines that recruit specific cell types (12). Recent studies had shown *TP53* or *KRAS* mutant NSCLC expressed higher levels of PD-L1 protein compared with corresponding wild-type tumors (16, 17). Meanwhile, it has been demonstrated that loss of *TP53* function decreased genomic stability and was associated with defects in DNA damage repair, indicating a higher mutational burden might occur in *TP53* mutational tumor (18). Therefore, we speculate that common mutations as *TP53* and *KRAS* in lung adenocarcinoma may be served as effective predictive factors in guiding anti–PD-1/PD-L1 immunotherapy.

Here, in order to systematically address the potential mechanism that *TP53/KRAS* mutation mediate immune response to lung adenocarcinoma, we describe an integrative analysis that incorporates tumor mutational load, DNA MMR deficiency, intratumoral PD-L1 expression, and content of $CD8^+$ T-cell infiltrates from cohorts of both lung adenocarcinoma repository database analysis and clinical immunotherapeutic patients. Significantly, we uncover *TP53/KRAS* mutation as a superiority group to anti–PD-1/PD-L1 therapies and highlight a new insight into common mutations in guiding immunotherapy.

## Materials and Methods

### Clinical cohorts

The Cancer Genome Atlas (TCGA), GSE72094 and Broad cohorts were retrieved from online data repository. A total of 462 patients were included in the TCGA cohort with mRNA expression profiling and gene mutation data. The GSE72094 cohort recruited 442 patients with detailed mRNA expression data and *EGFR/KRAS/TP53/STK11*sanger sequencing analysis (19). The Broad cohort contained 183 lung adenocarcinomas and matched normal tissues with detail information about mutation load and mutation spectrum (20). Most of the patients enrolled in the three cohorts were early-stage lung adenocarcinomas. A total of 85 lung adenocarcinomas from the Guangdong Lung Cancer Institute (GLCI), Guangdong General Hospital (GGH) were underwent whole genome sequencing (WGS). Key variables including demographic and clinical information are provided in Supplementary Table S1.

### Immunotherapeutic patients

Clinical and mutation data for 34 NSCLC [29 adenocarcinoma (ADC)] patients were retrieved from cbioPortal (http://www.cbioportal.org/study.do?cancer_study_id=luad_mskcc_2015). All patients treated with pembrolizumab (anti–PD-1) from 2012 to 2013 followed the protocol NCT01295827 (KEYNOTE-001). Objective response to pembrolizumab was assessed by investigator-assessed immune-related response criteria (irRC) by a study radiologist (5).

Another group consisted of 20 NSCLC (15 ADC) patients were collected prospectively in the GLCI from August 2015 to August 2016. Eleven of them were treated with pembrolizumab and nine patients were treated with nivolumab. Tumor specimens were obtained for Sanger sequencing and IHC analysis. This study was approved by the Institutional Review Board of GLCI of GGH, and all patients provided specimens with written informed consent. Clinicopathologic and molecular information are provided in Supplementary Table S2.

### mRNA expression profiling and reverse phase protein array (RPPA) analysis

For lung adenocarcinomas included in the TCGA cohort, experimental procedures regarding RNA extraction from tumors, mRNA library preparation, sequencing (on the Illumina HiSeq platform), quality control, and subsequent data processing for quantification of gene expression have been previously reported (21). Gene expression data for the GSE72094 lung adenocarcinomas have been deposited in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72094). Tumors from the GSE72094 cohort were profiled using a custom Affymetrix GeneChip. The gene expression cutoff value was chosen as median over the entire dataset (TCGA and GSE72094) to ensure all analyses of each gene were based on the same cutoff value (22, 23).

Proteomic analysis was based on RPPA from the TCGA database. The RPPA methodology and data analysis pipeline have been previously described (21). For TCGA, level 3 data were downloaded directly from the TCGA portal and utilized in subsequent analyses.

### Mutation data analysis

For the discovery set, somatic mutation data (level 2) of the 462 lung adenocarcinomas were retrieved from the TCGA data portal (https://gdc.cancer.gov/). To assess the mutational load, the number of mutated genes carrying at least one nonsynonymous mutation in the coding region was computed for each tumor. Somatic mutation data of 183 lung adenocarcinomas in Broad cohort was retrieved from cbioPortal (http://www.cbioportal.org/study.do?cancer_study_id=luad_broad). Somatic substitutions and covered bases within their trinucleotide sequence context were analyzed to characterize the mutation spectrum of 183 lung adenocarcinoma. Mutation spectrum for each sample was calculated as the percentage of each of six possible single nucleotide changes (AT>CG, AT>GC, AT>TA, GC>AT, GC>CG, GC>TA) among all single-nucleotide substitutions. The most frequent mutation signatures were C→T transitions and C→A transversions.

For the validation set (GLCI), we conducted whole-exome sequencing of DNA from tumors and matched normal blood from 85 lung adenocarcinoma patients. Enriched exome libraries were sequenced on the HiSeq 2000 platform (Illumina) to >100× coverage. Alignment, base-quality score recalibration and duplicate-read removal were performed, germline variants were

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excluded, mutations annotated and indels evaluated as previously described (4, 5, 24). Mutations between clinical groups were compared using the Mann–Whitney test.

### Gene set enrichment analysis (GSEA)

For GSEA (25), the javaGSEA Desktop Application was downloaded from http://software.broadinstitute.org/gsea/index.jsp. GSEA was used to associate the gene signature with the *TP53* or *KRAS* mutation status (*TP53*-mut vs. *TP53*-wt; *KRAS*-mut vs. *KRAS*-wt). The genes identified to be on the leading edge of the enrichment profile were subject to pathway analysis. Fold-change values were exported for all genes and analyzed with version 2.2.0 of GSEA, using the GseaPreranked module. The normalized enrichment score (NES) is the primary statistic for examining gene set enrichment results. The nominal *P* value estimates the statistical significance of the enrichment score. A gene set with nominal $P \leq 0.05$ was considered to be significantly enriched in genes.

### Immunohistochemistry

Tumor sections were assessed immunohistochemically using PD-L1 (clone: SP142, Spring Bioscience, Inc) and CD8 (clone: C8/144B, Gene Tech (Shanghai) Co. Ltd). The IHC-stained tissue sections were scored separately by two pathologists blinded to the clinical parameters.

PD-L1 expression on tumor cells and immune cell was evaluated using a three-tiered grading system. Strong: ≥50% for tumor cell (TC) or ≥10% for immune cell (IC); weak: 5%–49% for TC or 5%–9% for IC; negative: <5% for TC or IC.

The percentages of $CD8^+$ lymphocytes compared with the total amount of nucleated cells in the stromal compartments were assessed. Scoring cutoff points at 25% or 50% for each core according to the degree of cell densities: low density: <25%; intermediate density: 25% to 49%; high density: ≥50%.

### Sanger sequencing

Genomic DNA from each sample was used for sequence analysis of *EGFR* exons 18–21, *KRAS* exons 2–3 and *TP53* exons 2–11. These exons were amplified by the polymerase chain reaction (PCR) as previously described (19, 26), and the resulting PCR products were purified and labeled for sequencing using the Big Dye 3.1 Kit (Applied Biosystems) according to the manufacturer's protocol.

### Statistical analyses

Statistical analyses were conducted using GraphPad Prism (version 7.01) and SPSS version 22.0 (SPSS, Inc.). Scatter dot plot and Box and whisker plots indicate median and 95% confidence interval (CI). Statistical tests were used to analyze the clinical and genomic data, including the Mann–Whitney *U*, $\chi^2$, Fisher exact, and Kruskal–Wallis. Kaplan–Meier curves analysis of progression-free survival (PFS) were compared using the log-rank test. All reported *P* values are two-tailed, and for all analyses, $P \leq 0.05$ is considered statistically significant, unless otherwise specified.

## Results

### Correlation between *TP53* and *KRAS* mutation and PD-L1 expression in lung adenocarcinoma

To investigate the correlation between common mutations (*TP53*, *KRAS*, *EGFR*, and *STK11*) and immune checkpoint status in lung adenocarcinoma, we thus initially interrogated RNA sequencing (RNA-Seq) expression data from a repository database including 462 lung adenocarcinomas from The Cancer Genome Atlas (TCGA) and 442 lung adenocarcinomas from GEO repository (GSE72094). Both the TCGA and GEO databases showed significantly increased *PD-L1* mRNA expression in the *TP53* mutation subgroup than in other gene mutation. Specifically, the *TP53* and *KRAS* comutated group manifested prominent higher *PD-L1* expression than other comutation types (Fig. 1A).

We next sought to explore the impact of *TP53* and *KRAS* mutation on PD-L1 expression in both PD-L1 mRNA expression profiling and RPPA analysis based on the TCGA database. The results demonstrated that it was *TP53* mutation but not *KRAS* mutation that boosted PD-L1 expression (Supplementary Fig. S1A and S1B). Significantly, those with co-occurring mutations in *TP53* and *KRAS* revealed the highest PD-L1 expression (both mRNA and protein level) than single gene mutation or wild-type tumors, indicating potential synergistic effect on activating PD-L1 expression (Fig. 1B). To confirm the association between *TP53*/*KRAS* mutation and PD-L1 expression as repository data demonstrated, we detected 93 lung adenocarcinoma surgical specimens using an IHC analysis (Fig. 1C; Supplementary Fig. S1C and Table S3) and immunostaining shows *TP53*/*KRAS* comutated specimens the strongest staining for the PD-L1 protein (Fig. 1D).

Next, we further analyzed the association between *TP53* or *KRAS* mutation and other non–PD-L1 immune checkpoints. A heatmap depicted the expression level of key immune checkpoints to three groups (*TP53*, *KRAS*, and *TP53*/*KRAS*; Fig. 1E). The results displayed remarkable increased expression of most checkpoints in the *TP53* mutation group while decreased expression in the *KRAS* mutation group. More interestingly, the *TP53*/*KRAS*comutated subgroup manifested exclusive increased expression of PD-L1; however, it showed decreased expression of some other non–PD-L1 immune inhibitory checkpoints, such as Lymphocyte Activating 3 (LAG3) and V-Set Domain Containing T Cell Activation Inhibitor 1 (VTCN1; ref. 27), implying a potential candidate population for anti–PD-1/PD-L1 immunotherapy (Fig. 1F).

### *TP53* mutation facilitates $CD8^+$ T-cell infiltration and activates T-effector and interferon-γ (IFNγ) associated gene signature

The presence of tumor-infiltrating lymphocytes (TIL) is an important biomarker for predicting responses to PD-L1 blockade therapy. We continue to analyze the correlation between these above common mutations and $CD8^+$ TIL contents in lung adenocarcinoma based on the TCGA database. Our results revealed significantly increased expression of *CD8A* in *TP53* mutation and *TP53*/*KRAS* comutated than other groups (Fig. 2A). It has been proposed that four different types of immune tumor microenvironments (TME) exist based on the presence or absence of TIL and PD-L1 expression. To further explore whether *TP53* or *KRAS* mutation would influence the TME, we analyzed the correlation between *TP53* or *KRAS* mutation and TME immune types classified based on *PD-L1* and *CD8A* expression as previously described (28, 29). Positive *PD-L1* and *CD8A* were defined as above-median expression. We identified that the *TP53* mutation group displayed a higher proportion of dual positive *PD-L1* and *CD8A* ($PD\text{-}L1^+/CD8A^+$) than the *TP53* wild-type group, while there was no difference between *KRAS* mutation and wild-type (Fig. 2B and C),

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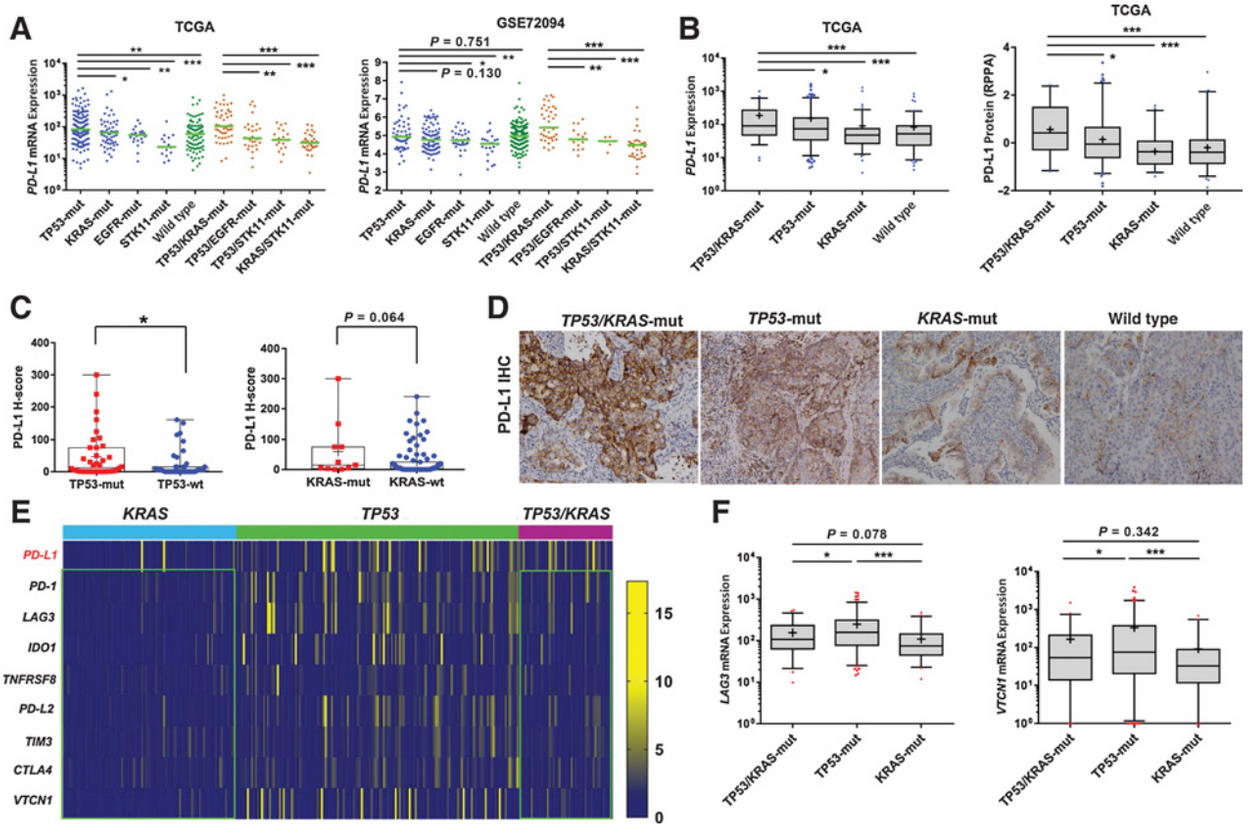


**Figure 1.**

Correlation of *TP53* and *KRAS* mutation with PD-L1 expression in patients with lung adenocarcinoma. **A,** Correlations between common mutations (*TP53/KRAS/EGFR/STK11*) and *PD-L1* mRNA expression in lung adenocarcinoma patients based on the analysis of the TCGA and GEO repository (GSE72094) database. **B,** Quantitative analysis of PD-L1 mRNA and protein expression based on *TP53* and *KRAS* mutation status. **C,** Comparison of PD-L1 IHC H-score between *TP53* or *KRAS* mutation and corresponding wild-type tumors in a cohort of 93 lung adenocarcinomas. **D,** Representative images of PD-L1 immunostaining in lung adenocarcinoma tissues with indicated gene mutation. Scale bar, 200 μm. **E,** Heatmap representation of relative mRNA expression levels of selected immune inhibitory checkpoints. **F,** Quantitative analysis of two typical inhibitory checkpoints (*LAG3* and *VTCN1*) on the base of *TP53* and *KRAS* mutation status. Mut, mutation; wt, wild-type; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

suggesting an adaptive immune resistance TME existed in the *TP53* mutation population. More importantly, the *TP53/KRAS* comutated subgroup showed the highest proportion of *PD-L1*$^{+}$/*CD8A*$^{+}$ than the *TP53* or *KRAS* single mutation and wild-type group (Fig. 2D). These observations were further confirmed by our IHC analysis that *TP53/KRAS* comutated patients manifested a strong staining of PD-L1 and high intensity of CD8$^{+}$TILs (Fig. 2E).

Given that *TP53* mutation had effects on the TME in lung adenocarcinoma, we subsequently sought to assess the relationship between *TP53* mutation and T-effector and IFNγ-associated gene signature, which have previously been associated with activated T cells, immune cytolytic activity, and IFNγ release (30, 31). An integrated heatmap depicting expression levels of T-effector and IFN-γ associated gene signature in tumors with *TP53* mutation compared with *TP53* wild-type. We identified significant increased expression of both T-effector and IFNγ-associated genes in the *TP53* mutation group, while there were no differences between *KRAS* mutation and wild-type, indicating preexisting immunity within *TP53* mutation tumor tissue (Fig. 2F and G).

### *TP53* and *KRAS* mutation shows increased mutation burden and distinct mutation spectrum

Recent studies have highlighted the relevance of tumor mutational loads and response to PD-1 blockade (5). We next speculate whether there are some common mutations in lung adenocarcinoma that affect the whole tumor mutational profile and change the tumor antigenicity. We first analyzed the TCGA and Broad databases as discovery set. The TCGA analysis showed significantly increased mutational loads in the *TP53* mutation group (median, 325), followed by *KRAS* (median, 179) and *STK11* (median, 132) mutation, *EGFR* (median, 60) mutation tumor had the lowest mutational loads. Meanwhile, the *TP53/KRAS* comutated subgroup showed significantly higher mutational loads (median, 358) than other comutated subgroup (Fig. 3A). We then tested these findings using another dataset (Broad), which consisted of 183 lung adenocarcinomas with detailed somatic mutation data, and confirmed that *TP53* and *KRAS* mutation and the *TP53/KRAS* comutated group had higher mutational loads than other groups (Fig. 3A). To further verify these findings, a total of 85 lung adenocarcinomas from GLCI detected by whole genome sequencing were defined as the validation set. GLCI data manifested the

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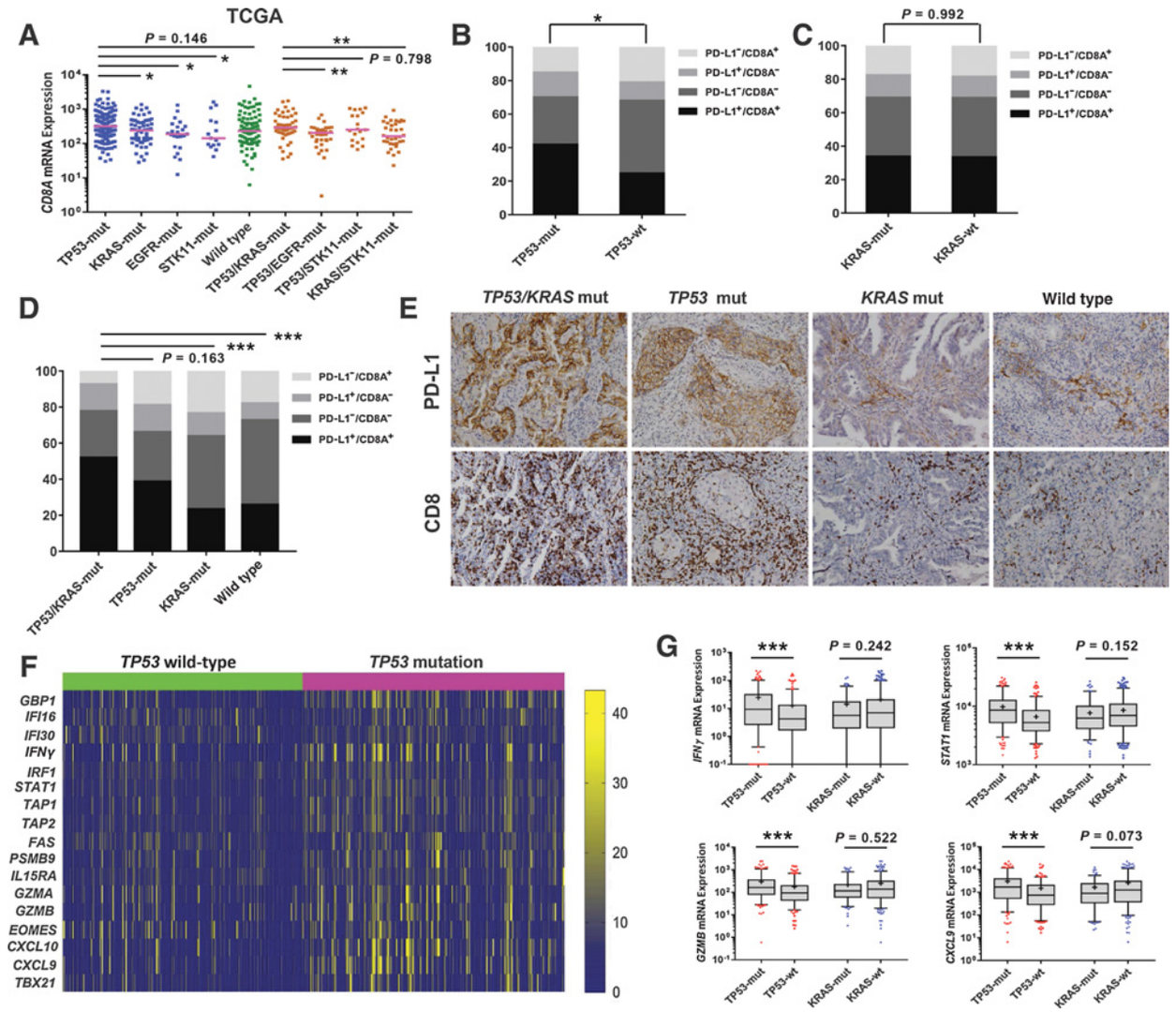


**Figure 2.**

*TP53* mutation facilitates CD8$^+$ T-cell infiltration and activates T-effector and IFNγ-associated gene signature. **A,** Association between common mutations (*TP53/KRAS/EGFR/STK11*) and *CD8A* mRNA expression in lung adenocarcinoma patients based on analysis of the TCGA dataset. **B–D,** The correlation between *TP53* or *KRAS* mutation status and TME immune types classified based on *PD-L1* and *CD8A* expression. Positive *PD-L1* and *CD8A* were defined as above-median expression. **E,** Representative images of PD-L1 and CD8 immunostaining in different subgroups according to *TP53* and *KR AS* mutation status. **F,** Heatmap depicting mRNA expression levels of T-effector and IFNγ-associated gene signature. **G,** Quantitative analysis of four key genes (*GZMB, CXCL9, STAT1*, and *IFNγ*) in T-effector and IFNγ gene signature on the base of *TP53* and *KRAS* mutation status. Mut, mutation; wt, wild-type; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

similar results with the discovery set that *TP53* mutation and the *TP53/KRAS* comutated group had higher mutational loads than other groups (Fig. 3B). It is well known that tobacco exposure was responsible for much of the mutagenesis in NSCLC. Multivariate linear regression analysis of mutation count in patients stratified by smoking status manifested that *TP53* mutation was an independent factor responsible for increased mutation burden regardless of smoking status, while *KRAS* mutation showed increased mutation burden only in nonsmokers (Supplementary Table S4).

Previous studies have established the notion that somatic mutations are primarily GC>TA transversions (32). We next investigated whether these above common mutations could affect tumor mutation spectrum by using a TCGA cohort. Transversion-high (TH) and transversion-low (TL) was based on smoking history and GC>AT, GC>TA frequency as previously described (5, 21). We can identify *KRAS* mutations were significantly enriched in the TH cohort, while *EGFR* mutations were significantly enriched in the TL group (Supplementary Fig. S2). Consistent with TCGA results, the Broad dataset showed a high rate of transversion/transition (Tv/Ti) in *KRAS* mutation and the *TP53/KRAS* comutated group while the lowest rate Tv/Ti in *EGFR* mutation (Fig. 3C). Notably, *TP53* and *KRAS* mutation was significantly correlated with high somatic mutations, high rate of Tv/Ti and C>A transversion and high smoking index (pack-years; Fig. 3D).

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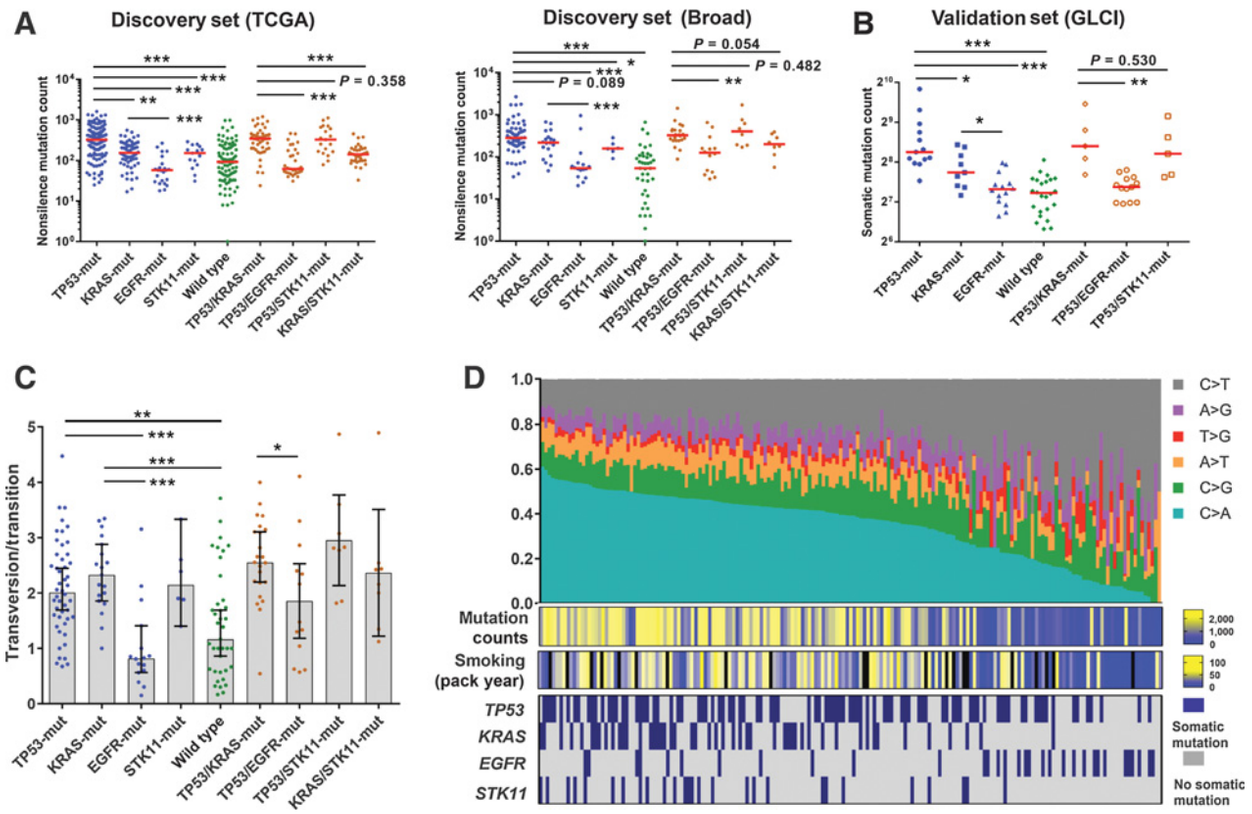


**Figure 3.**

*TP53* and *KRAS* mutation augments tumor antigenicity by transforming the mutational profile. **A,** Different tumor mutational burden driving by a specific mutation gene analyzed on the base of the discovery set (TCGA and Broad database). **B,** Different tumor mutational burden driving by specific mutation gene analyzed on the base of the validation set (GLCI data). **C,** Box plot represents the proportion of Tv/Ti according to indicated mutation subgroups. **D,** Heatmap displays integrated relationship between mutation burden, mutation spectrum, smoking, and 4 common mutations status based on analysis of the Broad database.***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

### Impact of *TP53* and *KRAS* mutation on the cell cycle, DNA replication, and damage repair–related genes

We sought to determine whether alterations in DNA replication and damage repair–related genes resulted from *TP53* or *KRAS* mutation could account for differential mutation burden and mutation spectrum. GSEA reveals prominent enrichment of signatures relating to cell cycle, DNA replication and DNA repair in both the *TP53* and *KRAS* mutation groups. However, there were distinct differences between these two groups. *TP53* mutation predominantly led to acceleration of cell-cycle and DNA replication, which potentially increased mutation probability, for unrepaired DNA damages that do not kill the cell by blocking replication would tend to cause replication errors and thus mutation. *KRAS* mutation manifested various defects of DNA repair including MMR, nucleotide excision repair (NER), and base excision repair (BER) that greatly enhanced point mutation (Fig. 4A).

Recent studies showed that *POLE* mutation is associated with disruption of the exonuclease activity required for DNA proofreading and results in a high mutational burden or an "ultra-mutator" phenotype (33, 34). We identified significantly increased mutation frequencies of *POLE* in the *TP53* mutation group ($P = 0.002$) while decreased mutation frequencies of *POLE* in the *EGFR* and *STK11* mutation groups compare with their corresponding wild-type group, indicating *TP53* mutation tends to cause DNA replication errors (Fig. 4B).

We next determined the correlation between these common mutations and DNA damage repair–related genes. DNA double-strand breaks (DSB) elicit that DNA damage response largely relies on the activity of ataxia telangiectasia mutated (ATM), which have been found to be mutated in human disorders associated with genome instability (35, 36). The results had revealed that a high frequency of *ATM* mutation was found predominantly in the *KRAS* and *STK11* mutation groups, and ATM protein analysis further confirmed that waning expression of ATM protein was specifically found in the *KRAS* and *STK11* mutation groups (Fig. 4B and C).

MMR-deficient tumors were recently shown susceptibility to checkpoint blockade immunotherapy (7). Our former GSEA identified that *KRAS* mutation was negatively correlated with MMR-related gene expression, and we next verified whether *KRAS* or other genes mutation affected the mutation status and protein expression of MMR-related genes. Four primary MMR-related genes, including *MSH2*, *MSH6*, *MLH1*, and *PMS2*, were coanalyzed. Consistent with GSEA, high mutation frequency of MMR-related genes was exclusively identified in the *KRAS* mutation group. Furthermore, the protein of MSH2 and MSH6 was significantly decreased in tumors with *KRAS* mutation; however, it was

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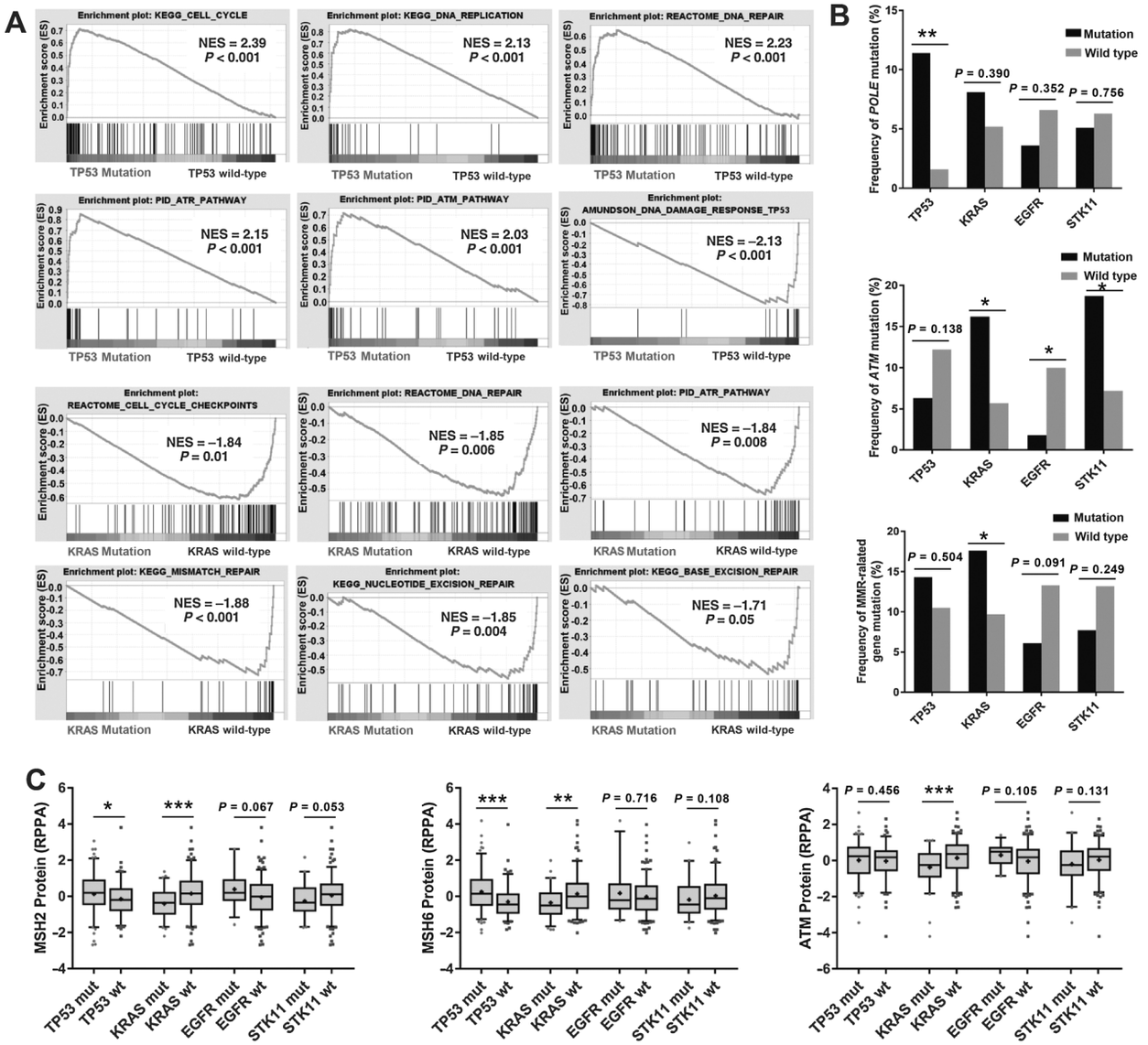


**Figure 4.**

Impact of *TP53* and *KRAS* mutation on the cell cycle, DNA replication and damage repair–related gene signatures. **A,** GSEA reveals acceleration of cell-cycle and DNA replication–related gene signatures as prominent modules in the *TP53* mutation group and impaired of DNA damage repair–related gene signatures, including MMR, NER, and BER in the *KRAS* mutation group compared with wild-type. **B,** Estimated proportion representation of *POLE, ATM* and MMR-related gene mutations in four groups according to indicated gene mutational status. **C,** Box plot representation of MMR-related proteins (MSH2 and MSH6) and ATM protein in four groups according to indicated genes' mutational status. NSE, normalized enrichment score; mut, mutation; wt, wild-type; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

increased in tumors with *TP53* mutation, suggesting that *KRAS* mutation might be a potential driver agent to induce MMR deficiency and in consequence produce more neoantigens (Fig. 4B and C).

### Patients with *TP53* or *KRAS* mutation, especially co-occurring *TP53*/*KRAS* mutations, show favorable clinical benefit to anti–PD-1 treatment

*TP53* and *KRAS* mutation showed remarkable effects on regulating PD-L1 expression, facilitating T-cell infiltration and augmenting tumor immunogenicity. We presumed that patients with these two mutations probably had increased sensitivity to PD-1 blockade immunotherapy. In support of this hypothesis, publicly available trial data (MSKCC, KEYNOTE-001) were reanalyzed. A total of 34 advanced NSCLC (29 ADC) patients were prescribed pembrolizumab from 2012 to 2013 following the NCT01295827 protocol. All tumor tissues underwent whole-exome sequencing. We observed significantly increased nonsynonymous mutation and candidate neoantigen burden in the *TP53* or *KRAS* mutation group compared with the wild-type

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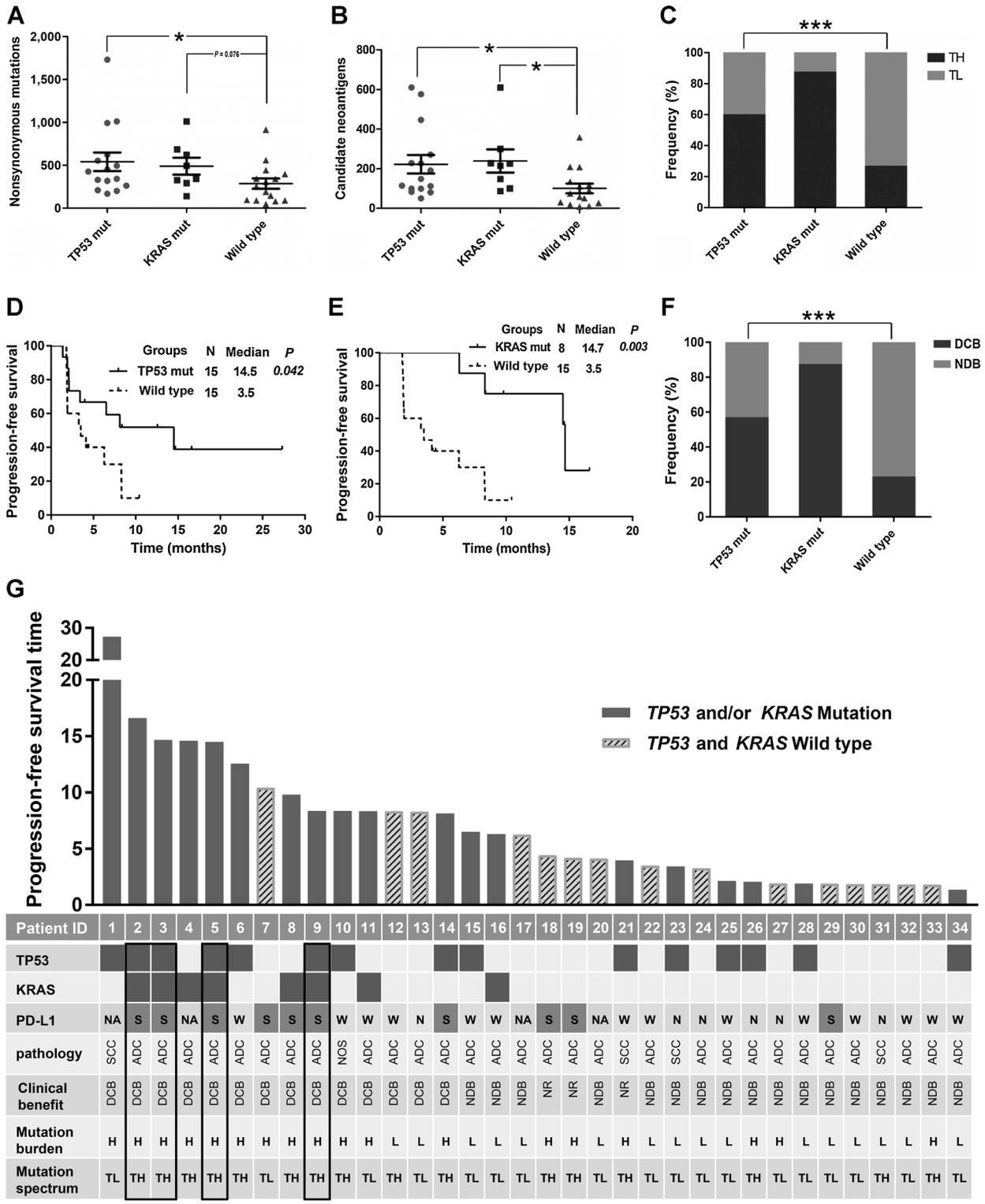


**Figure 5.**

The correlation between *TP53*/*KRAS* mutation and clinical response to PD-1 blockade. Comparison of nonsynonymous mutation (**A**) and candidate neoantigen (**B**) burden in *TP53* or *KRAS* mutation and wild-type group. **C,** Proportion representation of transversion dominant mutation in the indicated group based on *TP53* or *KRAS* mutation. **D** and **E,** Kaplan–Meier survival curves estimates of PFS compared *TP53* or *KRAS* mutation with the wild-type group in patients treated with pembrolizumab. **F,** Proportional representation of clinical benefit of pembrolizumab in the indicated group based on *TP53* or *KRAS* mutation. **G,** Individual PFS of 34 NSCLC patients coupled with their mutational status of *TP53* and *KRAS*, pathology, PD-L1 expression, mutation burden, mutation spectrum and clinical benefit to pembrolizumab in each patient. TH, transversion high; TL, transversion low; DCB, durable clinical benefit; NDB, no durable benefit; ADC, adenocarcinoma; mut, mutation; H, High nonsynonymous burden (mutation $\geq$ 209); L, low nonsynonymous burden (mutation < 209); PD-L1 expression: S, strong ($\geq$50%); W, weak (1~49%); N, negative (<1%); NA, not available; ***, $P < 0.001$; *, $P < 0.05$.

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group (Fig. 5A and B). Consistent with the analysis from mutation burden, there was a strikingly high proportion of TH in the *TP53* and *KRAS* mutation group (Fig. 5C).

Notably, *TP53* or *KRAS* mutation patients obtained a significantly prolonged progression-free survival (PFS) compared with wild-type patients who underwent pembrolizumab treatment (median PFS, *TP53*-mut vs. *KRAS*-mut vs. wild-type: 14.5 vs. 14.7 vs. 3.5 months, $P = 0.012$; Fig. 5D and E). Most of *TP53* or *KRAS* mutation patients enjoyed a durable clinical benefit during treatment, while most of wild-type patients showed no durable benefit (Fig. 5F). More importantly, 4 lung adenocarcinoma patients who concomitantly harbored *TP53* and *KRAS* mutation manifested superior PFS, and all of them had a durable clinical benefit. Meanwhile, these patients displayed a high mutation burden, high rate of transversion, and strong staining of PD-L1 (Fig. 5G).

To further confirm these observations from a public database, we prospectively collected 20 NSCLC (15 ADC) patients who were treated with pembrolizumab ($n = 11$) or nivolumab ($n = 9$) from August 2015 to August 2016 in our center (GLCI). All of the patients underwent at least one assessment after baseline. Patients' tissues were used for DNA sequencing for *EGFR*, *KRAS*, and *TP53*, and paraffin-embedded specimens were detected for IHC analysis of PD-L1 and CD8 (Table 1). Eight of the patients showed *TP53* mutation and 3 patients showed *KRAS* mutation. Up to August 25, 2016, 6 patients experienced partial response (PR). Two of them concomitantly harbored *TP53* and *KRAS* mutation, 3 patients with single *KRAS* or *TP53* mutation, and 1 patient without common mutation. Besides, 6 patients were evaluated as progression disease (PD) after 2 or 3 cycles of immunotherapy and 2 of them harbored *EGFR* mutation (Fig. 6A and Table 1). Patients with *TP53* and/or *KRAS* mutation showed prolonged PFS than both genes negative patients treated with PD-1 inhibitors. Six patients were assessed as PR and five of them had an ongoing response (Fig. 6B).

Next, we focused on 1 patient who had a durable clinical benefit (DCB) with *TP53* and *KRAS* comutation (Fig. 6C). IHC was used for analysis of PD-L1, CD8, and MMR-related genes, including MSH2, MSH6, MLH1, and PMS2. Consistent with our expectation, the patient showed strong staining for both PD-L1 and CD8. Meanwhile, four MMR-related genes displayed different intensity of immunostaining: weak positive for MLH1, moderate positive for MSH6 and PMS2, and strong positive for MSH2, suggesting that a potential possibility of MMR deficiency existed in this tumor (Fig. 6D).

## Discussion

Although the expression of PD-L1 on the surface of tumor cells, as measured by IHC, is recommended as a predictive factor to identify patients who would benefit from PD-1 blockade, not all PD-L1–positive patients respond well (10, 37). The underlying biology of such limitations has not been clearly understood until recent studies, which showed that the presence of TILs and mutational burden correlated with T-effector signature and immunogenic features that supported the response to anti–PD-1/PD-L1 therapy (5, 8, 12, 38, 39). Here, we first identified a group of oncogenic driver (*EGFR* and *KRAS*) and tumor suppressor (*TP53* and *STK11*) mutations of lung adenocarcinoma that distinctively affected immune checkpoints expression, T-cell infiltration, and tumor immunogenicity. Specifically, our findings revealed that *TP53* mutation remarkably increased PD-L1

**Table 1.** Molecular and demographic characteristics and efficacy of PD-1 inhibitors in 20 non–small cell lung cancer

| Characteristic | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 | P11 | P12 | P13 | P14 | P15 | P16 | P17 | P18 | P19 | P20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | 66 | 54 | 52 | 53 | 56 | 58 | 58 | 58 | 51 | 53 | 62 | 50 | 57 | 64 | 58 | 75 | 59 | 70 | 50 | 53 |
| Gender | Male | Male | Male | Male | Male | Male | Female | Male | Male | Male | Male | Male | Female | Male | Female | Male | Male | Male | Male | Male |
| Smoking history (page years) | 40 | 60 | 30 | 40 | 70 | 0 | 0 | 60 | 30 | 30 | 120 | 48 | 0 | 60 | 0 | 10 | 0 | 75 | 20 | 35 |
| ECOG PS | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 |
| Pathology | ADC | ADC | ADC | SCC | ADC | ADC | ADC | LELC | ADC | ADC | SCC | NEC | ADC | ADC | ADC | ADC | SCC | ADC | ADC | ADC |
| Clinical staging | IV | IIIA | IV | IIIA | IV | IV | IIIB | IIIB | IV | IV | IV | IV | IIIB | IV | IV | IV | IV | IV | IV | IV |
| EGFR | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | L858R | WT | WT | WT | L858R | WT |
| KRAS | G12D | WT | G12V | WT | G12C | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT |
| TP53 | WT | WT | Q167fs | Y234H | Y126_Splice | NA | G187_splice | WT | V151F | P87L | NA | WT | NA | H214R | WT | WT | M160I | WT | WT | NA |
| PD-L1 | Weak | NA | NA | Weak | Strong | NA | Negative | Negative | NA | Strong | Negative | NA | NA | Strong | NA | Weak | Strong | Negative | Negative | Negative |
| CD8+TILs | Negative | NA | NA | Weak | Strong | NA | Negative | Weak | NA | Weak | Negative | NA | NA | Strong | NA | Weak | Negative | Negative | Negative | Negative |
| Immunotherapy | Pembro 14 CY | Pembro 7 CY | Nivo 17 CY | Pembro 9 CY | Pembro 9 CY | Nivo 8 CY | Nivo 9 CY | Nivo 6 CY | Nivo 8 CY | Nivo 4 CY | Nivo 8 CY | Pembro 5 CY | Pembro 6 CY | Pembro 4 CY | Pembro 2 CY | Pembro 3 CY | Pembro 3 CY | Nivo 3 CY | Pembro 2 CY | Nivo 2 CY |
| Best response | PR | PR | PR | PR | PR | SD | PR | SD | SD | SD | SD | SD | SD | SD | PD | PD | PD | PD | PD | PD |
| PFS (months) | 11 m | 9.5 m | 9.5 m | 8 m | 7 m | 7.5 m | 6.0 m | 5.5 m | 5.0 m | 4.5 m | 4.5 m | 3.5 m | 3.5 m | 3.5 m | 2 m | 2 m | 2.5 m | 2.0 m | 1.5 m | 1.5 m |

Abbreviations: ADC, adenocarcinoma; SCC, squamous carcinoma; CY, cycles; LELC, lymphoepithelioma-like carcinoma; NA not available; NEC, neuroendocrine carcinoma; Nivo, nivolumab; PD, progression disease; Pembro, pembrolizumab; PFS, progression-free survival; PR, partial response; SD, stable disease; WT, wild-type.

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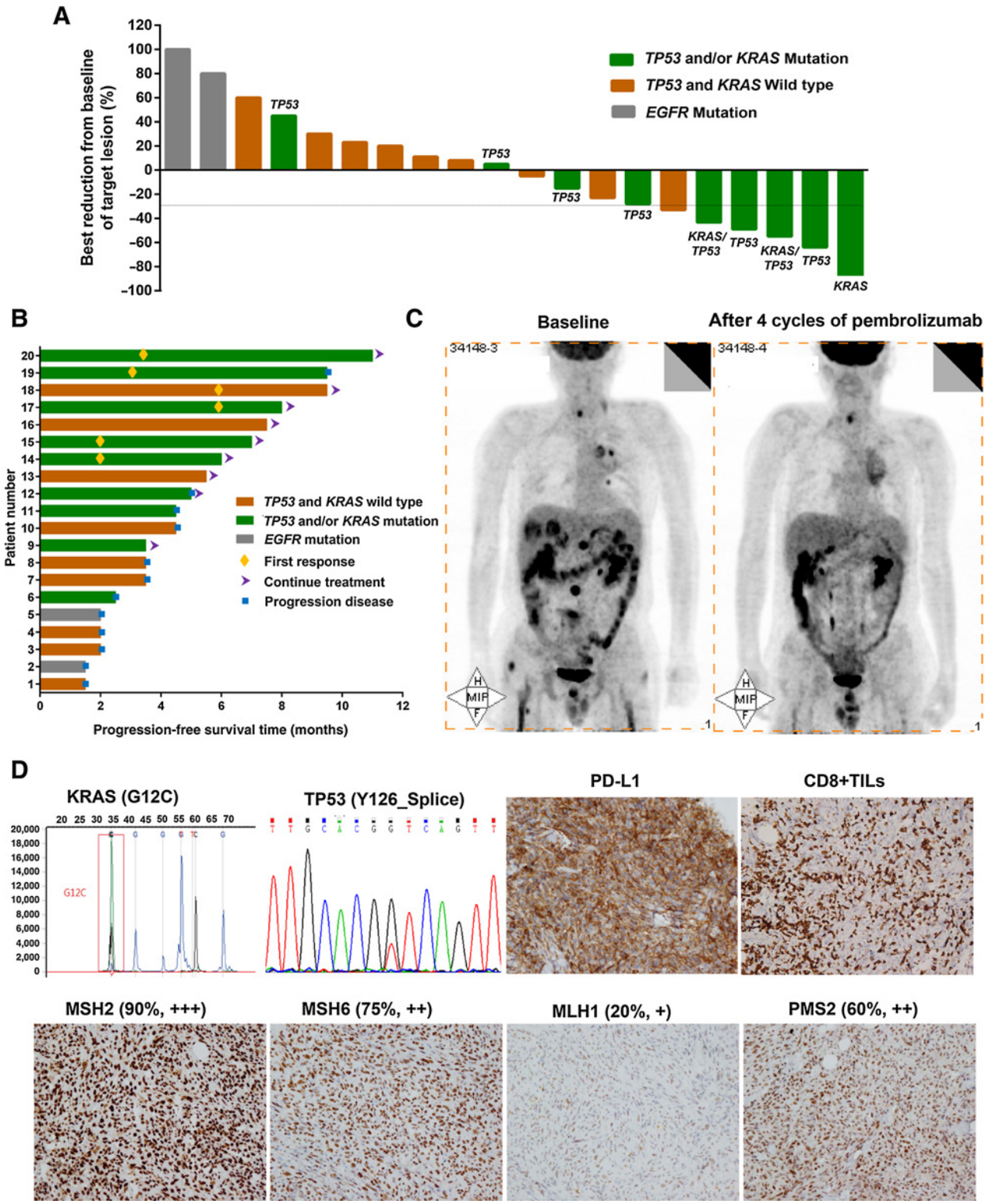


**Figure 6.**

Antitumor activity and biomarkers analysis of PD-1 blockade in patients with NSCLC. **A,** Best tumor burden change from baseline in target lesions in 20 NSCLC (15 ADC) patients who received nivolumab or pembrolizumab. The presence of mutation genes in each patient was indicated. **B,** Time to progression and duration of response in individual patients, as defined by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. **C,** PET/CT scan shows typical imaging alternation in a patient after 4 cycles of pembrolizumab treatment. **D,** Biomarker analysis of *TP53* and *KRAS* mutation status and protein expression of PD-L1, CD8, MSH2, MSH6, MLH1, and PMS2 detected by DNA sequencing and IHC.

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expression and facilitated $CD8^+$ T-cell infiltration, and accompanied with a higher proportion of dual positive *PD-L1* and *CD8A* than other mutation groups. Furthermore, *TP53*-mutated tumors showed prominently increased somatic mutation burden and specifically enriched in the TH subset. Previous studies have classified the TME into four groups on the basis of PD-L1 expression and TIL recruitment. These include type I (PD-L1 positive with TILs driving adaptive immune resistance), type II (PD-L1 negative with no TIL indicating immune ignorance), type III (PD-L1 positive with no TIL indicating intrinsic induction), and type IV (PD-L1 negative with TIL indicating the role of other suppressors in promoting immune tolerance; refs. 28, 29). Significantly, type I is lately thought to be associated with a high mutational burden, *PD-L1* amplification, and oncogenic viral infection, which defines a subtype sensitivity to PD-1 blockade (29). These notions, to some extent, support our findings that *TP53* mutation represents a state of adaptive immune resistance and a high immunogenicity, which contributes to a probable sensitivity to PD-1 blockade (40). Nevertheless, we could also discover a fact that *TP53* mutation equally enhanced some other non–PD-L1 immune–inhibitory checkpoints expression, such as *LAG3* and *VTCN1*, which might serve as potential primary resistance to *TP53* mutation patients treated with anti–PD-1/PD-L1 (41).

Recent studies based on a phase III clinical trial have identified that patients who harbored an *EGFR* mutation displayed unfavorable response to PD-1 blockade than those with a wild-type *EGFR* (42–44). This may be the first finding in which driver mutation of NSCLC was involved in altering sensitivity to immunotherapy. The most likely explanation is that patients with *EGFR* mutation were prone to produce a weak immunogenic tumor and an immunosuppressive TME. These perspectives were also confirmed in our study that *EGFR* mutation showed the lowest mutation burden and lowest rate of Tv/Ti than other mutations. Besides, *EGFR* mutation did not increase the expression of PD-L1, like others reported, but with a relatively lower expression than *TP53* and *KRAS* mutation (45), which further supported their hyposensitivity to PD-1 blockade. *KRAS* mutation was the second important oncogenic driver mutation in lung adenocarcinoma. The development of more effective treatment strategies for patients with *KRAS* mutation is hampered by the biologic and phenotypic heterogeneity of *KRAS*-mutant tumors. More recently, some studies suggested that patients with activating mutations in *KRAS* may probably benefit from PD-1 blockade, but the underlying mechanisms remained elusive and most of the researchers attributed this predilection to the association between smoking and the presence of *KRAS* mutations (5, 42). In this study, we uncovered potential mechanisms that account for this correlation. We discovered a significant increase of mutation load in *KRAS*-mutant tumors. Particularly, a predominant higher proportion of Tv/Ti was also found in this subgroup. Furthermore, we observed that *KRAS* mutations defected DNA repair, especially in MMR, which supported the notion that MMR deficiency acted as a favorable agent for PD-1 blockade (7).

It is well known that smoking-related lung cancers were characterized by greater mutation burden, higher rate of transversion, and more frequent *KRAS* mutation than that occurred in never smokers (21, 32, 46, 47). More recently, studies have demonstrated the association of PD-L1 expression with significant smoking history (48). In our study, we discovered that *TP53* mutation, especially *TP53*/*KRAS* comutation, showed increased PD-L1 expression and augmented tumor immunogenicity. To confirm whether these correlations are more related to tobacco exposure, a multivariate linear regression analysis of mutation count and PD-L1 expression stratified by smoking status was performed. We demonstrated that *TP53* mutation was responsible for increased mutation burden and PD-L1 expression independent of smoking status (Supplementary Tables S4 and S5). Recent studies based on subgroup analysis demonstrated those with a history of current or ever smoking showed much better benefits of PD-1 blockade than non-smokers. So we can imagine current or ever-smoker patients with *TP53* and/or *KRAS* mutation may be the optimal population for PD-1 blockade immunotherapy.

Co-occurring mutations in *TP53* and *KRAS* have recently been defined as a specific cluster associated with activation of antitumor immunity and immune tolerance/escape (49). Interestingly, our study identified *TP53* and *KRAS* comutant tumors manifested exclusive increased expression of PD-L1 and a highest proportion of $PD\text{-}L1^+/CD8A^+$ than *TP53* or *KRAS* single mutation. Meanwhile, *TP53*/*KRAS* dual mutation showed predominant increased mutation burden and enriched in the TH subset. Consistent with these preclinical predictions, the clinical analysis on the base of MSKCC and our center database had further confirmed that those with co-occurring mutations in *TP53* and *KRAS* showed remarkable clinical benefit from pembrolizumab. These results implicated a possibility that *TP53* and *KRAS* mutation played a role with synergistic and complementary in regulating immune biomarkers, which gave rise to a responsive TME with adaptive immune resistance and high immunogenicity. However, these findings were established in a relatively small cohort and even fewer patients with *TP53* and *KRAS* comutation. Based on the preliminary evidence, a prospective study with a larger sample size of *TP53*/*KRAS* mutation and PD-L1 expression for response to PD-1 blockade is warranted in the future.

Taken together, the results of this study provided an insight into immune regulation driving by some common mutations of lung adenocarcinoma. We discovered a prominent significance of *TP53* and *KRAS* mutation in boosting PD-L1 expression, facilitating T-cell infiltration, and augmenting tumor immunogenicity. This work provided evidence that *TP53* and *KRAS* mutation in lung adenocarcinoma might be served as a pair of potential predictive factors in guiding PD-1 blockade immunotherapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** Z.-Y. Dong, Y.-L. Wu

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Z.-Y. Dong, W.-Z. Zhong, X.-C. Zhang, S.-Y. Liu, H.-Y. Tu, H.-J. Chen, Y.-L. Sun, Q. Zhou, X.-N. Yang, J.-X. Lin, H.-R. Zhai, L.-X. Yan, R.-Q. Liao

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Z.-Y. Dong, W.-Z. Zhong, X.-C. Zhang, J. Su, Z. Xie, S.-Y. Liu, H.-Y. Tu, H.-J. Chen, Y.-L. Sun, Q. Zhou, H.-H. Yan, L.-X. Yan

**Writing, review, and/or revision of the manuscript:** Z.-Y. Dong, W.-Z. Zhong, J. Su, S.-Y. Liu, H.-Y. Tu, H.-J. Chen, Q. Zhou, X.-N. Yang, J.-X. Lin, H.-H. Yan, L.-X. Yan, Y.-L. Wu

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Z.-Y. Dong, J. Su, Z. Xie, J.-J. Yang, H.-R. Zhai

**Study supervision:** Z.-Y. Dong, W.-Z. Zhong, X.-C. Zhang, Y.-L. Wu

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Clinical Cancer Research

# Potential Predictive Value of *TP53* and *KRAS* Mutation Status for Response to PD-1 Blockade Immunotherapy in Lung Adenocarcinoma

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