TGFBR2 mutation predicts resistance to immune checkpoint inhibitors in patients with non-small cell lung cancer

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Abstract

Background: Resistance or even hyper-progression to immune checkpoint inhibitors (ICIs) manifesting as accelerated disease progression or death has impeded the clinical use of ICIs. The transforming growth factor beta (TGFβ) receptor pathway has been identified in contributing to immune dysfunction, which might be associated with resistance to ICIs. We aimed to explore the role of TGFβ in the resistance to ICIs in non-small cell lung cancer (NSCLC) in this study.

Methods: Public cohorts with patients treated with ICIs or chemotherapy including POPLAR/OAK (n = 853), MSKCC (n = 1662) and Van Allen (n = 57) and TCGA (n = 3210) cohorts were obtained and analyzed.

Results: The expression of immune-checkpoint related genes, including programmed death-ligand 1 (PD-1), lymphocyte-activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death ligand 1 (PDCD1), and programmed cell death ligand 2 (PDCD1LG2) were significantly upregulated in transforming growth factor beta TGFβ receptor 2 (TGFβR2)-mutated patients than those with wild-type TGFβR2 (p < 0.05). In the POPLAR/OAK cohort, TGFβR2-mutated patients showed shorter progression-free survival (PFS) (p = 0.004; hazard ratio (HR), 2.83; 95% confidence interval (CI), 1.34–6.00) and overall survival (OS) (p = 0.0006; HR, 3.46; 95% CI, 1.63–7.35) than those with wild-type TGFβR2 when treated with ICIs but not chemotherapy.

In the merged MSKCC and Van Allen cohorts, a similar result was observed that the OS was inferior in patients with mutated TGFβR2 compared with those with wild-type TGFβR2 (p = 0.007; HR, 2.53; 95% CI, 1.25–5.12). The association between TGFβR2 mutation and survival remained significant in multivariable cox regression in both POPLAR/OAK cohort (p = 0.02; HR, 2.53; 95% CI, 1.17–5.45) and merged cohort (p = 0.008; HR, 2.63; 95% CI, 1.29–5.35). We further evaluated the association between TGFβR2 mutations and OS in multiple types of tumors. The association between TGFβR2 mutations and OS remained significant in NSCLC (p = 0.02; HR, 2.47; 95% CI, 1.16–5.26), but not in other type of tumors.

Conclusions: We identified that TGFβR2 mutation predicted the resistance to ICIs in NSCLCs. The clinical delivery of ICIs should be cautious in those patients.

Keywords: immune checkpoint inhibitors, immune resistant, non-small cell lung cancer, TGFβR2

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Introduction

Immune checkpoint inhibitors (ICIs) have revolutionized the treatment of non-small-cell lung cancer (NSCLC).1,2 Despite this, the therapeutic outcome of monotherapy remains unsatisfactory for the majority of patients. Single-agent
immunotherapy demonstrates an objective response rate (ORR) of 20–30% in the subsequent management, representing a major unmet medical need in NSCLC.\(^3,4\) Previous efforts have been made to identify the clinical, histopathological, and genetic biomarkers of responses to ICIs.

In this regard, several predictors, including programmed cell death ligand 1 (PD-L1) expression,\(^5\) tissue tumor mutational burden (TMB)\(^6,7\) or blood TMB (bTMB),\(^8\) Notch signaling,\(^9\) the epoxide hydrolase (EPHA) gene family,\(^10\) and tumor protein 53 (TP53) and kirsten rat sarcoma virus (KRAS) co-mutation,\(^11\) are currently under investigation to identify patients that can benefit from ICIs. Meanwhile, several combination strategies including ICIs combined with chemotherapy have been developed to increase the response rate for ICIs.\(^12,13\) However, it is of equal significance to identify why some tumors fail to respond or even hyper-progress after ICIs; this may offer the first step in developing novel strategies to overcome drug resistance.

In a minority of patients, a novel pattern of hyperprogressive disease (HPD) has been observed during immunotherapy.\(^14,15\) HPD was defined as progressive disease on the first computed tomography scan during immunotherapy according to RECIST version 1.1 criterion and the tumor growth rate (TGR) exceeding 50% compared with baseline, corresponding to an absolute increase in the TGR exceeding 50% per month.\(^14\) HPD may happen in multiple types of tumors, as well as in patients receiving ICIs monotherapy or in combination with chemotherapy in the first-line or later treatment.\(^14\) The role for mouse double minute 2/4 homolog (MDM2/4) amplification, janus kinase 1/2 (JAK1/2) loss-of-function mutations and epithelial growth factor receptor (EGFR) mutations in HPD has recently emerged, which may partly result from the dysfunction of the biological processes critical to antitumor immune responses; despite this, the exact mechanisms remain inexplicable.\(^15,16\) The incidence of HPD ranged from 8.0% to 30.4% in NSCLCs after treatment with ICIs monotherapy.\(^14,17,18\) Previous studies have demonstrated the role of kelch-like ECH-associated protein 1 (KEAP1)\(^19\) serine/threonine kinase 11 (STK11)\(^20\) and EGFR\(^17\) alterations in the immunotherapeutic resistance in NSCLC. However, the underlying mechanism has not been fully explored.

Tumor growth factor β (TGF-β) is widely known to have pleiotropic effects in cancer cells, including roles in cytostasis, epithelial-mesenchymal transition, stemness, invasion and metastasis; it can be tumor suppressive or oncogenic depending on the context.\(^21,22\) Meanwhile, secretion of TGF-β is a strategy commonly used by tumors to inhibit cellular immune responses by preventing the maturation of professional antigen-presenting cells (APCs) and inhibiting T-cell proliferation, cytokine release, and cytolytic activity.\(^23\) Previous studies have demonstrated the increased TGF-β signaling in non-responders to ICIs in urothelial cancer,\(^24\) breast cancer,\(^25\) and so on.

The TGF-β family members signal via heteromeric complexes of type I and type II (TGFBR1, TGFBR2) receptors, which activate members of the Smad family of signal transducers. TGFBR2 is a transmembrane protein that has a protein kinase domain, recruits and phosphorylates TGFBR1, forming a heterodimeric complex, and binds TGF-β.\(^26,27\) It is usually required for tumor growth and metastasis to have lost TGF-β signaling in tumor cells owing to mutations in TGFBRF1, TGFBR2, or Smad family member 4 (SMAD4).\(^21\) Meanwhile, the TGF-β signaling was unaffected or even increased in immune cells, which further inhibited the cellular immune response. Mutations in TGFBR2 along with APC, KRAS, and TP53 in mice have developed colorectal tumors with increasing metastatic potential and high phospho-Smad family member 3 (SMAD3) staining in the stromal margins with an immune-excluded phenotype.\(^28\) Genetic deletion of TGFBR2 in CD4⁺ T cells suppressed the tumor growth,\(^29\) further supported the controversial roles of TGFBR2 in tumor cells and immune cells. However, since TGFBR2 mutations are less prevalent in NSCLC (around 1–2%\(^30,31\)) little is known about the role of TGFBR2 mutations in immunotherapy in patients with NSCLC.

In light of the mechanisms above, we hypothesized that TGFBR2 mutations in tumors might be associated with the resistance to ICIs in NSCLC; TGFBR2 mutations might identify patients that cannot benefit or who will even hyper-progress from ICIs in NSCLC.

**Methods**

**Patients and data source**

We obtained whole-exome sequencing (WES) and mRNA expression data of patients with lung adenocarcinoma (LUAD), squamous cell lung
carcinoma (LUSC), head and neck carcinoma (HNSC), bladder carcinoma (BLCA), esophageal carcinoma (ESCA), skin cutaneous melanoma (SKCM), and colorectal cancer (COAD) from The Cancer Genome Atlas (https://portal.gdc.cancer.gov/) to investigate these mechanisms. (Supplemental Table S1). Another three independent public cohorts were also analyzed to study the association between TGFBR2 mutations and survival in the present study, including the POPLAR/OAK,7 MSKCC,32 and Van Allen33 cohorts. The data for the three independent cohorts was retrieved from the published articles (detailed features are displayed in the Supplemental Table S2). (1) The POPLAR/OAK cohort consisted of 853 patients with advanced NSCLC who were treated with either atezolizumab or docetaxel as a second-line treatment from a phase II trial POPLAR and a phase III trial, OAK. All patients in the POPLAR/OAK cohort implemented a genomic profiling analysis of circulating tumor DNA with Foundation One panel (315-gene panel, 1.1 Mb). (2) The MSKCC cohort was composed of 1,662 patients with a variety of cancer types who had received at least one dose of ICIs therapy, including 350 patients with NSCLC. Tumor tissues were profiled with a MSK-IMPACT panel (341-gene panel, 0.98 Mb, 56 patients; 410-gene panel, 1.06 Mb, 239 patients; 468-gene panel, 1.22 Mb, 55 patients). (3) The Van Allen cohort was defined as the NSCLC subpopulation of the pan-cancer research on microsatellite-stable (MSS) patients who were treated with ICIs. Tumor tissue samples were sequenced by WES. TGFBR2 mutations

The nonsynonymous mutations, including TRUNC (Frameshift del, Frameshift ins, nonsense, nonstop, splice region, splice site), INFRAME (Inframe del and Inframe ins), and the MISSENSE mutations of TGFBR2 were defined as TGFBR2 mutations in this study. 

Gene set enrichment analysis

For gene set enrichment analysis, the java gene set enrichment analysis (GSEA) Desktop Application (GSEA 4.0.1) was downloaded from http://software.broadinstitute.org/gsea/index.jsp. GSEA was used to compare the signaling pathway enrichment between TGFBR2 mutation and TGFBR2 wild-type groups. 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 4.0.1 of GSEA, using the GSEA preranked module. The normalized enrichment score (NES) is the primary statistic for examining gene set enrichment results. The p-value adjusted by FDR estimates the statistical significance of the enrichment score. A gene set with a FDR ≤ 0.05 was considered to be significantly enriched in genes. 

mRNA expression profiling and signaling pathway analysis

Comparison of the TGFBR1 and TGFBR2 mRNA expression between tumor and adjacent normal tissues was performed by http://gepia2.cancer-pku.cn/. The association between TGFBR2 mutation and selected genes was analyzed in the TCGA database, where both DNA sequencing and RNA sequencing data are available. The data was processed and analyzed using the R package (limma). Normalized gene expression data of LUSC, LUAD, HNSC, BLCA, ESCA, SKCM, and COAD in TCGA data sets were loaded into in silico Pathway Activation Network Decomposition Analysis (iPANDA). The software enables the calculation of the Pathway Activation Score (PAS), a value that serves as a quantitative measure of differential pathway activation between two statuses. We determined a quantitative measure of the signaling pathway activation scores in TGFBR2 mutated samples by using the TGFBR2 wide-type samples as a reference. The signaling pathway activation scores represent the intensity and direction of the pathway activation. Generally, positive scores that are considered upregulated, while negative scores correspond to downregulated pathways.34 The detail analysis approach for iPANDA was available in a previous study.35

Statistical analyses

Continuous variables were described as the median (range) and compared by Mann–Whitney U test. Categorical variables were described as a number (frequency) and compared by chi-square test or fisher exact test. Survival was estimated by Kaplan–Meier curves. Hazard ratios (HR) were determined through the use of univariable and multivariable Cox regression analysis. Variables with p < 0.1 in the univariable regression were also included into multivariable cox regression. All analyses were performed by R 3.6.0 and graphs were drawn by Graphpad prism 8.0. All
reported *p*-values were two-sided and a *p*-value < 0.05 was considered as statistically significant.

**Results**

*The identification of the association between TGFBR2 mutation and immune dysfunction*

We first compared the mRNA expression of TGFBR1 and TGFBR2 between tumors and adjacent normal tissues from TCGA. The expression of TGFBR2 was significantly lower in the tumor tissues of LUAD (Tumor: *n* = 483; Normal: *n* = 59) and LUSC (Tumor: *n* = 486; Normal: *n* = 50) compared with adjacent normal tissues instead of TGFBR1 (*p* < 0.05, Figure 1(a)), which was also observed in breast and colorectal cancer (Supplemental Figure S1A). These results suggested that the down-regulation of TGFBR2 in tumor might be associated with the tumorigenesis of NSCLC.

In general, the frequency of TGFBR2 mutation was relatively low in LUSC (2.1%) and LUAD (0.7%) compared with other tumors like esophageus cancer (4.9%) and head and neck squamous cancer (4.7%) (Supplemental Figure S1B). In total, 13 patients with NSCLC harboring 14 TGFBR2 mutations were identified from TCGA. The mutational sites of TGFBR2 from the NSCLC in TCGA are depicted in Figure 1(b), including 6 truncating mutations and 8 missense mutations mostly happened in ecTbetaR2 and kinase domains. One mutation, p.D35N, occurred beyond ecTbetaR2; kinase domains with unknown oncogenic effects were excluded from the following analysis. There are no significant associations between TGFBR2 mutations and other recurrent driver mutations in NSCLC (Supplemental Figure S1C).

We then used iPANDA to assess the level of TGF-β signaling between mutated TGFBR2 cases and wide-type TGFBR2 samples based on the TCGA data sets of LUAD and LUSC. Indeed, TGFβ signaling of tumors was significantly down-regulated in patients with mutated TGFBR2 compared with wild-type TGFBR2 [Mann–Whitney U test, *p* = 0.0003, Figure 1(d)]. Consistent with this, the interleukin-6 (IL6)-JAK-STAT signaling was also enriched in mutated TGFBR2 based on the GSEA results (Supplemental Figure S1D). The JAK-STAT signaling mediated by type I and type II interferons was widely reported to be associated with cytotoxic T lymphocyte inactivation by upregulating programmed death-ligand 1 (PD-L1) (CD274) expression.36,37 The phenomenon was subsequently observed through an increased mRNA expression of STAT1 and CD274 in patients with mutated TGFBR2 compared with wild-type TGFBR2 [Mann–Whitney U test, *p* < 0.05, Figure 1(d)]. We also observed the upregulation of other signaling in the TGFBR2 mutation group, including ErbB signaling, insulin signaling, Ras signaling, and VEGF signaling etc., compared with TGFBR2 wide-type group, suggesting the downregulation of TGFβ was associated with the tumor metastasis and angiogenesis, which is consistent with previous studies (Supplemental Figure S1E). In addition, we further investigated the association between TGFBR2 mutation and other immune checkpoints. The mRNA expression of LAG3, TIGIT, PDCD1, and PDCD1LG2 were increased in patients with mutated TGFBR2 compared with those with wild-type TGFBR2 [Mann–Whitney U test, *p* < 0.05, Figure 1(e)], suggesting the immune inhibition in patients with mutated TGFBR2.

We further explored the relationship between TGFBR2 mutation and immune dysfunction score, which is a candidate resistance regulator to predict the immunotherapeutic responsiveness.38 The immune dysfunction score tended to be increased in patients with mutated TGFBR2; however, no significant difference was observed (Mann–Whitney U test, *p* = 0.23). This is probably due to the small sample size of mutated TGFBR2 [Figure 1(f)]. However, the upward tendency of immune dysfunction score was consistent with that in the patients with EGFR mutation, which may be served as a negative indicator responding to ICIs. All of these results suggest that TGFBR2 mutations might be associated with immunotherapy resistance.

The negative association between TGFBR2 mutation and survival in the training cohort

We further studied the association between TGFBR2 mutations and survival in NSCLC...
Figure 1. The potential mechanisms associating with TGFBR2 mutation. (a) The mRNA expression of TGFBR1 and TGFBR2 between tumor and normal tissues in LUAD and LUSC. (b) The mutational sites of TGFBR2 in the NSCLC from TCGA. (c) The difference of TGFβ signaling between patients with mutated and wild-type TGFBR2. (d) The difference of JAK-STAT signaling and CD274 mRNA expression between TGFBR2 mutation group and TGFBR2 wide-type group. (e) The difference of immune checkpoints between TGFBR2 mutation group and TGFBR2 wide-type group. (f) The difference of dysfunction score between TGFBR2 mutation group and TGFBR2 wide-type group.

*p < 0.05 by t-test.

JAK-STAT, janus kinase-signal transducer and activator of transcription proteins; LUAD, lung adenocarcinoma; LUSC, squamous cell lung cancer; NSCLC, non-small cell lung cancer; TGFBR1/2, transforming growth factor, beta receptor I/II.
patients treated with ICIs. We first used POPLAR/OKA cohorts \( (n = 853) \) as the training set. In total, there were 14 (1.6%) patients with TGFBR2 mutations, among whom seven patients were treated with ICIs and the rest were treated with chemotherapy. The incidence rate of TGFBR2 mutations in the POPLAR/OAK cohorts was consistent with the NSCLC dataset in TCGA. In patients treated with ICIs \( (n = 429) \), patients with mutated TGFBR2 had a shorter progression free survival (PFS) [hazard ratio (HR), 2.83; 95% confidence interval (CI), 1.34–6.00, \( p = 0.004 \); Figure 2(a)] and OS [HR, 3.46; 95% CI, 1.63–7.35, \( p = 0.0006 \); Figure 2(b)] than patients with wild-type TGFBR2. In contrast, in patients treated with chemotherapy \( (n = 424) \), there was no difference in PFS [HR, 0.86; 95% CI, 0.41–1.82, \( p = 0.69 \); Figure 2(c)] and OS [HR, 1.21; 95% CI, 0.57–2.57, \( p = 0.61 \); Figure 2(d)] between patients with mutated and wild-type TGFBR2. These results suggested that TGFBR2 was a negative predictor instead of a prognostic factor for ICIs.

In order to exclude the possibility that the negative predictive ability of TGFBR2 was due to the co-occurrence of other negative predictors, including EGFR mutations, ALK alterations, and JAK1/2 loss-of-function mutations, etc, we further depicted the OncoPrint for patients with mutated TGFBR2 [Figure 2(e)]. The most mutated genes in those patients were LRP1B (64%) and TP53 (64%). There were no previous reported negative genes in those patients.

In addition, to exclude the potential confounder of the established robust predictors, we wanted to further evaluate the association between TGFBR2 mutation and PD-L1 expression and plasma-based tumor mutational burden (bTMB) \( (n = 429) \). However, no significant association was observed between TGFBR2 mutations and bTMB [Mann–Whitney U test, \( p = 0.13 \); Figure 2(f)] and PD-L1 expression (negative: TC0 and IC0 versus positive: TC1/2 and/or IC1/2 and TC3 or IC3) [Fisher exact test, \( p = 0.48 \); Figure 2(g)].

We further performed multivariable cox regression to minimize the influence of potential confounding factors. In the univariable analyses, besides TGFBR2 mutations, several other alterations were indexed, such as Eastern Cooperative Oncology Group (ECOG) score \( (1 \text{ versus } 0) \), line of treatment \( (1 \text{ versus } 2) \), metastatic sites \( (>3 \text{ versus } \leq 3) \), and bTMB \( (\geq 16 \text{ versus } <16) \) were also associated with the immunotherapeutic PFS. The HRs were \( (95\% \ CI) \) 1.30 (1.05–1.61), 0.80 (0.64–1.01), 1.45 (1.17–1.80), and 0.81 (0.64–1.03), respectively (Table 1). In addition, ECOG score \( (1 \text{ versus } 0) \), histology (no-squamous versus squamous), metastatic sites \( (>3 \text{ versus } \leq 3) \), smoking status (ever versus never), and TP53 and bTMB \( (\geq 16 \text{ versus } <16) \) were also associated with the immunotherapeutic OS. The HRs were \( (95\% \ CI) \) 1.75 (1.35–2.27), 0.71 (0.55–0.91), 1.43 (1.03–1.98), 1.47 (1.16–1.86), and 1.58 (1.24–2.00), respectively (Table 1). In the multivariable analyses, the association between TGFBR2 mutations and PFS or OS remained significant in patients treated with immunotherapy (PFS: HR, 2.21; 95% CI, 1.03–4.73, \( p = 0.01 \); OS: HR, 2.53; 95% CI, 1.17–5.45, \( p = 0.02 \) after adjusting bTMB, sex, ECOG score, line of treatments, TP53, metastatic sites, and histology (Table 1). All of our results suggested that TGFBR2 mutations were independent negative predictors for immunotherapeutic efficacy.

The association between TGFBR2 mutations and survival in the validation cohort

We further validated the above findings in other, independent, validation cohorts. In the MSKCC cohort, 350 patients with NSCLC were analyzed, while in the Van Allen cohort, 57 patients were analyzed. There were eight patients (2.3%) with mutated TGFBR2, while there was one patient (1.8%) with mutated TGFBR2 in the Van Allen cohort [Figure 3(a)]. TGFBR2 mutations were associated with poorer OS in both the MSKCC cohort [HR, 2.11; 95% CI, 1.0–4.50, \( p = 0.04 \); Figure 3(b)] and the Van Allen cohort [HR, not applicable, \( p < 0.001 \); Figure 3(c)]; however, the sample size for the Van Allen cohort was relatively small. Under this consideration, we further combined the MSKCC cohort and the Van Allen cohort \( (n = 407) \). In the merged cohort, the OS was still shorter in patients with mutated TGFBR2 compared with those with wild-type TGFBR2 [HR, 2.53; 95% CI, 1.25–5.12, \( p = 0.007 \); Figure 3(d)]. In the multivariable cox regression, the association between TGFBR2 mutations and OS was still significant (HR, 2.63; 95% CI, 1.29–5.35, \( p = 0.008 \) by adjusting TMB and treatment (Supplemental Table S3). These results further confirmed that TGFBR2 mutations might be negative predictors for ICIs in NSCLC.
Figure 2. The association between TGFBR2 mutation and survival of immune checkpoint inhibitors in the training cohort. 
(a) Kaplan–Meier survival curves of PFS comparing the TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy. 
(b) Kaplan–Meier survival curves of OS comparing the TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy. 
(c) Kaplan–Meier survival curves of PFS comparing the TGFBR2 mutation group and TGFBR2 wide-type group treated with chemotherapy. 
(d) Kaplan–Meier survival curves of OS comparing the TGFBR2 mutation group and TGFBR2 wide-type group treated with chemotherapy. 
(e) OncoPrint depicts mutated genes with prevalence >20% in TGFBR2 mutation group. Reported frequencies include a composite of missense, nonsense, and splice mutations for each gene. Horizontal ordinate represents the mutation frequencies across different genes. Summary rows of each case at top include annotation for total number of mutations, age, sex, histology, smoking status and treatment. Patients without gene mutations are depicted in light gray on the OncoPrint. 
(f) Comparison of blood tumor mutational burden between TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy. 
(g) Comparison of PD-L1 expression between TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy.

PD-L1, programmed death-ligand 1; PFS, progression free survival; TGFBR2, transforming growth factor β receptor 2.
Table 1. Univariable analysis and multivariable cox regression analyses of PFS and OS in patients with ICIs treatment in training cohort.

| Parameter                          | PFS Univariable analysis | PFS Multivariable analysis | OS Univariable analysis | OS Multivariable analysis |
|-----------------------------------|--------------------------|-----------------------------|-------------------------|---------------------------|
|                                   | HR (95% CI) p-value       | HR (95% CI) p-value         | HR (95% CI) p-value     | HR (95% CI) p-value       |
| Age (>=60 versus <60)             | 1.05 (0.85–1.29) 0.66     | 1.34 (0.60–3.00) 0.47       | 1.01 (0.79–1.28) 0.97   |                           |
| Sex (male versus female)          | 0.84 (0.68–1.03) 0.10     | 0.92 (0.83–1.03) 0.13       | 0.89 (0.79–1.01) 0.08   | 0.99 (0.75–1.30) 0.93     |
| ECOG (1 versus 0)                 | 1.30 (1.05–1.61) 0.02     | 1.29 (1.03–1.61) 0.02       | 1.75 (1.35–2.27) <0.001 | 1.69 (1.30–2.20) <0.001   |
| Histology (no-squamous versus squamous) | 0.92 (0.74–1.15) 0.46     | 0.71 (0.55–0.91) 0.007      | 0.69 (0.53–0.90) 0.005  |                           |
| Smoke (ever versus never)         | 0.86 (0.66–1.11) 0.24     | 1.43 (1.03–1.98) 0.03       | 1.31 (0.91–1.90) 0.14   |                           |
| Line of treatments (1 versus 2)   | 0.80 (0.64–1.01) 0.06     | 0.77 (0.61–0.97) 0.03       | 0.94 (0.72–1.22) 0.63   |                           |
| Metastatic sites (>3 versus <=3)  | 1.45 (1.17–1.80) 0.001    | 1.16 (1.08–1.25) 0.001      | 1.58 (1.24–2.00) 0.001  | 1.59 (1.24–2.05) <0.001   |
| bTMB (>=16 versus <16)            | 0.81 (0.64–1.03) 0.092    | 1.06 (0.81–1.38) 0.68       | 0.62 (0.26–1.47) 0.28   | 0.86 (0.64–1.15) 0.32     |
| PDL1 expression (<1% reference)   | 0.83                      |                             | 0.66                    |                           |
| >=1%                              | 0.93 (0.74–1.18) 0.56     | 0.89 (0.68–1.22) 0.41       |                           |                           |
| unknown                           | 0.94 (0.72–1.23) 0.65     | 0.95 (0.73–1.35) 0.82       |                           |                           |
| TP53                              | 1.15 (0.94–1.41) 0.17     | 1.47 (1.16–1.86) 0.01       | 1.31 (1.01–1.70) 0.04   |                           |
| LRP1B                             | 1.06 (0.85–1.32) 0.62     | 1.07 (0.83–1.38) 0.62       |                           |                           |
| TGFBR2 (mut versus WT)            | 2.81 (1.33–5.95) 0.007    | 2.21 (1.03–4.73) 0.04       | 3.46 (1.63–7.35) 0.001  | 2.53 (1.17–5.45) 0.02     |

95% CI, 95% confidence interval; bTMB, blood tumor mutation burden; ECOG, Eastern Cooperative Oncology Group; HR, hazard Ratio; LRP1B, low-density lipoprotein receptor-related protein 1B; OS, overall survival; PDL1, programmed death ligand 1; PFS, progression-free survival; TGFBR2, transforming growth factor beta receptor; TP53, tumor protein 53; WT, wild type.

The association between TGFBR2 mutation and ICIs efficacy in multiple types of tumors

To investigate the negative predictive efficacy of TGFBR2 mutations for ICI treatment in pan-cancer, we further evaluated the association between TGFBR2 mutations and OS in multiple types of tumors (n = 1662). However, no statistically significant association was observed between OS and TGFBR2 status in other tumors, except in NSCLC [Figure 4(a)]. After adjusting for TMB (top 20% versus the rest), the association between TGFBR2 mutation and OS remained significant in NSCLC (HR, 2.47; 95% CI, 1.16–5.26; p = 0.02), but not in esophagogastric cancer, melanoma, colorectal cancer, bladder cancer, and head and neck cancer [Figure 4(b)].

We further investigated the associations between immune-checkpoint genes between the TGFBR2 mutation group and the TGFBR2 wide-type group in ESCA (n = 182), COAD (n = 594), BLCA (n = 413), SKCM (n = 448), and HNSC (n = 523) using the data from TCGA. The results showed that there were no significant differences in immune-checkpoint genes stratified by TGFBR2 status in these tumors, except the
LAG3, TIGIT, PDCD1, and PDCD1LG2, where expression was increased in the TGFBR2 mutation group in SKCM (Figure S2, Supplemental Table S4). Moreover, iPANDA analysis showed there was no significant differences of JAK-STAT signaling between the TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy in the SKCM cohort. (c) Kaplan–Meier survival curves of OS comparing the TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy in the Van Allen cohort. (d) Kaplan–Meier survival curves of OS comparing the TGFBR2 mutation group and TGFBR2 wide-type group treated with immunotherapy in the merged cohort.

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; OS, overall survival; TGFBR2, tumor growth factor β receptor 2.

Discussion
In this study, we demonstrated that TGFBR2 mutation was associated with poor survival of immunotherapy in NSCLC in the training and validation cohorts. In addition, the association between TGFBR2 mutation and OS remained significant in NSCLC, but not in other type of tumors in MSKCC cohort. All together, these results suggested that TGFBR2 mutation might predict immunotherapeutic resistance in NSCLC.

Previous studies regarding immunotherapeutic predictive biomarkers have paid more attention to the identification of the population who would benefit from ICIs, such as TMB, PD-L1 expression etc. It should be noted that screening these patients who will benefit from immunotherapy is important; however, with the development of
combination strategies, it is also important to identify patients who may exacerbate or hyper-progress after immunotherapy. This may maximize the opportunity for patients to benefit from alternative therapies and provide more insights into the development of combination strategies. In the present study, we identified that TGFBR2 mutation was an independent, negative predictor for immunotherapy in NSCLC. The use of ICIs in those patients need to be cautious. The detection of TGFBR2 mutations can be easily performed in tumor tissue or ctDNA, as demonstrated in the MSKCC/Van Allen cohorts and POPLAR/OAK cohorts, respectively. The clinical development of TGF-β pathway inhibitors in cancer has been impeded by concerns regarding loss of tumor suppression and the lack of predictive biomarkers to guide patient selection. Our study
provides a rational of combination of TGF-β inhibitors and ICIs in patients with mutated TGFBR2. However, whether the combination of TGF-beta inhibitors and ICIs can effectively overcome the resistance to immunotherapy in patients harboring TGFBR2 mutation remains to be evaluated in prospective clinical trials.

Previous studies have demonstrated the distinct roles of TGF-β signaling in tumor and immune cells. In immune cells, the activation of TGF-β signaling is associated with T cell exclusion, decreased cytokine release, and decreased cytolytic activity. In contrast, in tumor cells, TGF-β signaling is required to suppress tumors and mutations in TGFBR2 in tumors would induce the loss-of-function of TGF-β signaling to facilitate tumorigenesis and metastasis. As a result, we hypothesized that there exists a negative feedback mechanism in tumors with mutated TGFBR2 with increased secretion of TGFB1. As a result, in tumors with mutated TGFBR2, even though the TGF-β signaling was decreased in tumor cells, the TGF-β signaling might be increased in the immune microenvironment to induce the immune exclusion due to the increased TGFB1. This is consistent with previous findings in colorectal cancer that mutations in TGFBR2 along with APC, KRAS, and TP53, in mice developed colorectal tumors with increasing metastatic potential and exerted an immune excluded phenotype with increased TGF-β signaling in the stromal margins. However, this hypothesis needs to be further studied.

JAK-STAT signaling was upregulated in patients with mutated TGFBR2, as well as STAT1 and PD-L1 expression. The JAK-STAT-PD-L1 axis has been reported to be associated with impaired cytotoxic T cell activation and the decreased efficacy of ICIs in pancreatic cancer. It should be noted that the mechanism of resistance to ICIs stays not wholly explicit, which consists of primary resistance, acquired resistance, and PD-L1 dependent or independent adaptive resistance. The JAK-STAT-PD-L1 axis mediated by type I and type II interferons was usually involved in primary and adaptive resistance. It should be noted that the INFγ-JAK-STAT signaling axis is a double-edged sword; it may regulate the balance between immune-mediated tumor elimination and escape and when the adaptive resistance dominates over immune activity alone. Meanwhile, the expression of PD-L1 and other immune checkpoints was also increased in mutated TGFBR2, adding more evidences to the hypothesis that inhibition of the PD-1/PD-L1 axis in TGFBR2 mutated patients result in the upregulation of alternative immunosuppressive checkpoints, thereby dampening the immune reaction against cancer cells. Meanwhile, we observed that there are no significant differences of immune-checkpoint genes and JAK-STAT signaling stratified by TGFBR2 status in other types of tumors, considering TGFBR2 mutation predict an inferior OS for patients who receiving ICIs treatment in NSCLC but not in other types of tumors, the predictive efficacy may be NSCLC specific. However, the frequencies of TGFBR2 mutation were relatively low, with 2–10% in multiple types of tumors, and the immunotherapeutic effectiveness of TGFBR2 in other tumors need to be further validated.

It is noteworthy that in both training and validation cohorts, most of the patients with TGFBR2 mutations were dead or progressed rapidly within 2–3 months. Several gene alterations, such as MDM2/MDM4 amplification and EGFR driver mutations, were reported to be potentially associated to hyper-progression from ICIs in patients with advanced-stage solid cancers previously. KRAS and STK11 co-mutation, and KEAP1 mutation have been reported to be resistant to immunotherapy. However, these mechanisms cannot fully explain the immunotherapeutic resistance in NSCLC. Our results suggested the need for caution in applying ICIs in NSCLC patients with the presence of TGFBR2 mutations.

As for limitations, the retrospective nature of the study limited the interpretation of the results. However, the limitation of retrospective setting could be minimized by the training and validation cohorts. Secondly, the frequency of TGFBR2 mutations was relatively low, with 1–2% in NSCLC, which may lower the significance of testing TGFBR2 mutation in clinical practice and the low frequency of TGFBR2 mutation cannot fully explain the mechanism of HPD in NSCLC. However, considering the poor prognosis for immune resistance and hyper-progression after ICIs, the investigation about the negative biomarkers of ICIs remains significant even with a low prevalence. Thirdly, the mechanism explaining why TGFBR2 mutation would induce immune resistance was not fully explored in the study, which needs to be further studied.
In conclusion, our results demonstrate that TGFBR2 mutation is a negative predictor of ICIs in patients with NSCLC and the clinical use of ICIs should be cautious in those patients. The combination of ICIs and TGF-β signaling inhibitors might overcome the resistance for those patients harboring TGFBR2 mutations and unravel a possibility of personalized combinational strategy in the future clinical practice.

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Availability of data and materials
The training and validation cohorts (including OAK/POPLAR cohort, Van Allen cohort, MSKCC cohort) used in this study were publicly available as described in the Method section.

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The authors declare that there is no conflict of interest.

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Supplemental material
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