Original Article

Effect of mesenchymal-epithelial transition amplification on immune microenvironment and efficacy of immune checkpoint inhibitors in patients with non-small cell lung cancer

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Background: Immune checkpoint inhibitors (ICIs) have brought clinical benefits to patients with various histological types of lung cancer. Previous studies have shown an association between mesenchymal-epithelial transition (MET) and the immunotherapy response in non-small cell lung cancer (NSCLC) but there is a lack of clinical data on the correlation of MET amplification with the ICI response in NSCLC.

Methods: Copy number alteration (CNA), somatic mutation, and clinical data from two immunotherapy cohorts (Rizvi *et al.* cohort and our local cohort) were collected and pooled to further investigate the key role of MET amplification in patients with NSCLC receiving ICIs. The correlations between MET amplification and tumor immunogenicity and antitumor immunity were further investigated in The Cancer Genome Atlas (TCGA)-NSCLC [lung adenocarcinoma (LUAD)/lung squamous cell carcinoma (LUSC)] data-set.

Results: In the immunotherapy cohorts, MET amplification was associated with longer progression-free survival (PFS) times in patients receiving ICI treatment (P=0.039; HR =0.37; 95% CI: 0.18–0.73). In the TCGA-NSCLC data-set, MET amplification was associated with high MET mRNA and protein levels, tumor mutation burden (TMB), neoantigen load (NAL), immune-activated cell patterns, immune-related gene expression levels, and the number of gene alterations in the DNA damage response and repair (DDR) pathway. Gene set enrichment analysis (GSEA) results indicated significant up-regulation of the immune response-related pathways in the MET-amplification group.

Conclusions: Our results suggest that MET amplification may be a novel predictive marker for immunotherapy efficacy in NSCLC.

Keywords: Mesenchymal-epithelial transition amplification (MET amplification); immunotherapy; immune microenvironment; immune checkpoint inhibitors (ICIs); non-small cell lung cancer (NSCLC)

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Introduction

In recent years, immune checkpoint inhibitors (ICIs), represented by anti-programmed cell death receptor ligand 1 (anti-PD-L1), its ligand PD-L1, and anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4), have opened a new chapter for the treatment of advanced non-small cell lung cancer (NSCLC). A series of clinical trials confirmed the therapeutic effect of ICIs in NSCLC (1), and in advanced NSCLC, the response rate of anti-PD-1/PD-L1 monotherapy was 17–21%, although some patients responded very persistently. Therefore, the identification of suitable biomarkers to screen the dominant population for immunotherapy efficacy is particularly important (2).

Increasingly, studies have shown that inflammatory tumor immune microenvironment is associated with the effectiveness of immunotherapy (3-7). High expression of PDL1, infiltration of inflammatory cells such as CD8+ T lymphocytes and Expression of inflammatory cytokines are all regarded as the important element of inflammatory tumor immune microenvironment (8-10). Therefore, the gene which can remodel the tumor immune microenvironment to be inflammatory type, it could be the efficacy biomarker to predict the efficacy of immunotherapy.

To date, studies have suggested that specific gene mutations may be potential predictors of ICI treatment response (11-14). *TP53*, mesenchymal-epithelial transition (*MET*) and *KRAS* driver mutations in lung adenocarcinoma (LUAD) have been found can regulate immune cell infiltration and PD-L1 expression, both of which may represent potential predictors guiding ICI treatment (11). Similarly, a recent study found that recurrent mutations in TET1 (TET1-MT) were predictive of a relatively good durable clinical benefit from ICIs and an improvement in overall survival (OS) across multiple cancer types (12).

High-level *MET* amplification, *MET* exon 14 skipping alterations (*MET*Δ14), or *MET* overexpression are the different type of *MET* gene alternation. *MET* amplification has been reported as a valuable marker for poor prognosis in advanced unresectable tumor such as colorectal cancer, breast carcinoma and gastric carcinomas but not in NSCLC patients (15,16). Studies have suggested that patients with *MET* mutations and *MET* amplification may be more responsive to immunotherapy than to targeted therapy (15,17,18), as *MET* mutation, amplification, or overexpression can upregulate coinhibitory molecule expression and downregulate costimulatory molecule expression (17). However, most of these studies were based on the cell molecular biology and immunohistochemistry, lacking the data of clinical immunotherapy especially for Chinese patients. Therefore, the correlation between *MET* amplification and the efficacy of NSCLC immunotherapy remains unclear.

In this study, we collected copy number alteration (CNA), somatic mutation, and clinical data of patients with NSCLC who were treated with ICIs to further assess the correlation between *MET* amplification and the efficacy of ICIs in patients with the disease. We found that in patients with *MET*-amplified NSCLC, ICI treatment was associated with longer progression-free survival (PFS) times, enhanced immunogenicity, and activated antitumor immunity. Our study suggests that *MET* amplification may be a novel predictive marker for immunotherapy efficacy in NSCLC. We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/atm-21-4543).

Methods

Clinical cohorts and cancer cell lines

To assess the correlation between *MET* amplification and the efficacy of NSCLC immunotherapy, data from ICI-treated patients in two NSCLC clinical cohorts were collected and processed as shown in Figure S1. The first ICI-treated cohort from Rizvi *et al.* consisted mainly of 240 NSCLC samples (n=240) with CNA, somatic mutation, and clinical data (19). Samples from the Rizvi *et al.* cohort (n=240) were sequenced using the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) panel. As the ICI-treated cohort from Samstein *et al.* (20) consisted of only four patients with *MET* amplification, we collected CNA, tumor mutation burden (TMB), and clinical data from another ICI-treated cohort from Zhujiang Hospital of Southern Medical University, Guangzhou Chest Hospital and First People's Hospital of Chenzhou. A total of 10 patients received ICIs (anti-PD-1 monotherapy, ≥3 treatment lines) to investigate the effect of *MET* amplification on the prognosis of NSCLC immunotherapy. Detailed clinical characteristics of patients with *MET* amplification are listed in https://cdn.amegroups.cn/static/public/atm-21-4543-1.docx. This research was performed in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the ethics committee of Zhujiang Hospital of Southern Medical University, Guangzhou Chest

Hospital and First People's Hospital of Chenzhou. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

Clinical and sample data (mRNA expression and somatic mutation data) from The Cancer Genome Atlas (TCGA)-LUAD and TCGA-lung squamous cell carcinoma (LUSC) datasets were downloaded from the Genomic Data Commons (https://portal.gdc.cancer.gov/) using the R package TCGAbiolinks (21). The TCGA-LUSC and TCGA-LUAD datasets were combined into the TCGA-NSCLC dataset for subsequent analysis. Gene expression and protein expression levels in the TCGA-NSCLC dataset were log2 normalized [fragments per kilobase of exon model per million mapped fragments (FPKM) +1] and were expressed as the normalized reverse-phase protein array (RPPA) values.

In addition, we used cBioPortal (22) (https://www.cbioportal.org) to download CNA data from the TCGA-LUAD and TCGA-LUSC datasets. The units of CNA were GISTIC 2.0 copy number.

We downloaded CNA and drug sensitivity data for 69 NSCLC cell lines from the Genomics of Drug Sensitivity in Cancer (GDSC) database (23), and the units of CNA and drug sensitivity data were GISTIC 2.0 copy number and in half-maximal inhibitory concentration (IC_{50}) value.

Clinical samples and classification of lung cancer cell lines

Consistent with a previous study (22), for the TCGA-NSCLC cancer immunotherapy datasets (from Rizvi *et al.* and the local cohort), a GISTIC score of ≥2 was defined as *MET* amplification, and a score of <2 was defined as non-*MET* amplification. Similarly, in the CNA data for the GDSC-NSCLC cell lines, a GISTIC score of ≥2 was defined as *MET* amplification, and a score of <2 was defined as non-*MET* amplification.

mRNA expression profiling, RPPA analysis, and drug sensitivity analysis

Methods for tumor RNA extraction, mRNA library preparation, sequencing, quality control, and subsequent data processing to quantify gene expression in TCGA-NSCLC samples have been previously reported (24). *MET* protein expression in the TCGA-NSCLC dataset was based on the RPPA of the TCGA database (24). Drug sensitivity data for human NSCLC cell lines are available from GDSC (https://www.cancerrxgene.org/).

Immunological characteristics and correlation analysis of tumor immunogenicity

We used the CIBERSORT web portal (http://cibersort.stanford.edu/) (25) to analyze mRNA expression data from the TCGA-NSCLC dataset to estimate the abundance of 22 immune cell types in the TCGA-NSCLC dataset. The list of immune-related genes and the neoantigen data for the TCGA-NSCLC dataset were obtained from Thorsson *et al.* (26), and the expression levels of these genes were quantified as log2 (FPKM +1) values.

TMB refers to the total number of substitutions and insertion/deletion mutations per megabase in the exon-coding region of a tumor gene in the tumor cell genome (27). TMB was defined as nonsynonymous mutations in the TCGA-NSCLC and local cohorts. We used the somatic mutation data in the TCGA-NSCLC dataset as the raw mutation count and 38 Mb as the estimate of the exome size (28).

Functional and pathway enrichment analyses

For gene annotation enrichment analysis using the clusterProfiler R package, differences in Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Reactome pathways for which $P<0.05$ were considered significant. The gene set enrichment analysis (GSEA) gene set was obtained from the Broad Institute Molecular Signatures Database (MSigDB) (29).

Gene set related to DNA damage response and repair (DDR) pathway analysis

We used the DDR pathway gene set from the Broad Institute MSigDB database (29), which includes the following eight DDR-related pathways: "R-HSA-6783310_REACTOME_Fanconi_Anemia_Pathway", "hsa03450_KEGG_Non_Homologous_End_Joining", "R-HSA-73884_REACTOME_Base_Excision_Repair", "hsa03430_KEGG_Mismatch_Repair", "R-HSA-5696398_REACTOME_Nucleotide_Excision_Repair", "R-HSA-5696398_REACTOME_Double_Strand_Break_Repair", "GO:0003697_Single_Stranded_DNA_Binding", and "hsa03440_KEGG_Homologous_Recombination". Detailed gene sets related to DDR pathway analysis are listed in https://cdn.amegroups.cn/static/public/atm-21-4543-2.xlsx.

Statistical analysis

The Mann-Whitney test was used to compare TMB, neoantigen load (NAL), immune cell content, and immune gene mRNA and protein content between the *MET*-amplification and non-*MET*-amplification groups. Fisher's exact test was used to compare correlations in smoking history, clinical stage, and sex between the groups, and the correlation between the *MET* amplification status and sex was compared using the chi-square test. PFS and OS in the *MET*-amplification and non-*MET*-amplification groups were analyzed by the Kaplan-Meier method with a log-rank test and by Cox proportional hazards regression analysis. P<0.05 was considered statistically significant, and all statistical tests were two-sided. R software (version 3.6) was used for statistical analysis, and the R package "ComplexHeatmap" (30) was employed to visualize the mutation and immune cell landscape.

Results

Mutation profile of the study population

We used cBioPortal to obtain a published study of NSCLC immunotherapy from Rizvi *et al.* comprising 240 patients receiving ICI treatment [anti-PD-(L)1 monotherapy or combination treatment with anti-CTLA-4 therapy]. This included CNA, somatic mutation, and clinical data for patients with NSCLC, and was used to further investigate the critical role of *MET* amplification in the prognosis of NSCLC immunotherapy. In order to explored Chinese patients data, we integrated the copy number variation, somatic mutation, and clinical data of NSCLC patients (n=10) with *MET* amplification from Zhujiang Hospital of Southern Medical University, Guangzhou Chest Hospital and First People's Hospital of Chenzhou. In addition, we used the TCGA-NSCLC dataset to further investigate the mutation characteristics, immune cell infiltration characteristics, immune-related gene expression profiles (GEPs), tumor immunogenicity, and antitumor immunity in patients with *MET* amplification (Figure S1).

The gene mutation landscape in the ICI-treated cohort (Rizvi *et al.*) is shown in *Figure 1A*, along with information for other genes mutated in the *MET*-amplification group (1.67%, 4/240), such as *TP53* (75%, 3/4), *KEAP1* (50%, 2/4), *SMARCA4* (50%, 2/4), *PTPRT* (50%, 2/4), *EGFR* (25%, 1/4), and *PTPRD* (25%, 1/4). Most were missense mutations (6/11, 54.5%) and frameshift mutations (3/11, 27.3%). The gene mutation landscape in the non-ICI-treated cohort (TCGA-NSCLC) is shown in *Figure 1B*. In the *MET*-amplification group (2.58%, 24/929), the *TP53* (70.83%, 17/24), *TTN* (50%, 12/24), *CSMD3* (45.83%, 11/24), *LRP1B* (41.67%, 10/24), *ZFHX4* (41.67%, 10/24), *XIRP2* (37.5%, 9/24), *COL11A1* (33.33%, 8/24), *MUC16* (33.33%, 8/24), *PCDH15* (33.33%, 8/24), *NAV3* (29.17%, 7/24), and *RYR2* (29.17%, 7/24) genes had the highest mutation frequency.

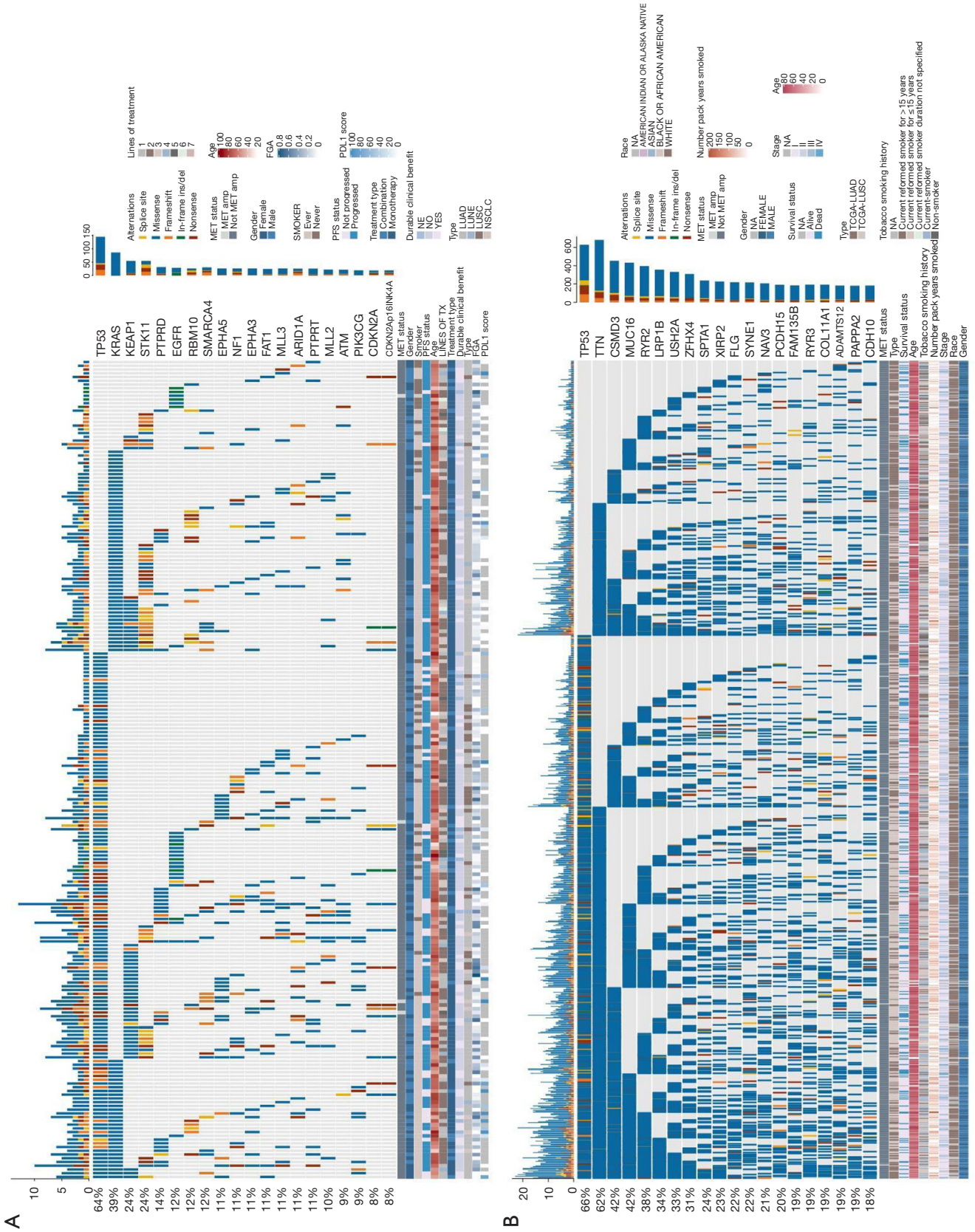
Survival analyses based on the MET status

To further explore the correlation between *MET* amplification and PFS on immunotherapy in patients with NSCLC, we collected and pooled two ICI-treated cohorts—the Rizvi *et al.* cohort (n=240) and our local cohort (n=10). In this integrated ICI-treated cohort, the *MET*-amplification group had a trend toward a longer PFS than the non-*MET*-amplification group (*Figure 1C*; P=0.149, HR =0.56, 95% CI: 0.30–1.04). As the local cohort consisted of patients with *MET* amplification who were treated with ≥3 treatment lines, we adjusted for the treatment lines and further performed the comparison in the integrated ICI-treated cohort restricted to ≥3 treatment lines (n1=62, n2=10). This showed *MET* amplification was associated with prolonged PFS after immunotherapy (*Figure 1D*; P=0.039, HR =0.37, 95% CI: 0.18–0.73).

To confirm the efficacy of *MET* amplification for predicting the PFS and OS of non-ICI-treated patients with NSCLC, we further evaluated the survival difference between patients with *MET* amplification and those without *MET* amplification in the TCGA-NSCLC LUAD/LUSC cohort and found no differences in OS or PFS between patients with *MET* amplification and those without *MET* amplification (TCGA-NSCLC LUAD/LUSC) (*Figure 1E-1J*).

Association of MET gene amplification with upregulated MET mRNA and protein expression

In NSCLC, *c-MET* gene amplification further leads to abnormal activation of the *c-MET* pathway via overexpression of the *c-MET* protein. Abnormal activation of the *c-MET* pathway ultimately promotes tumor cell proliferation and metastasis (31). We found higher *MET* mRNA and protein expression in the *MET*-amplification group than in the non-*MET*-amplification group in the TCGA-NSCLC LUAD/LUSC cohort (*Figure 2A-2I*). However, the regulatory effect of *MET* amplification on the



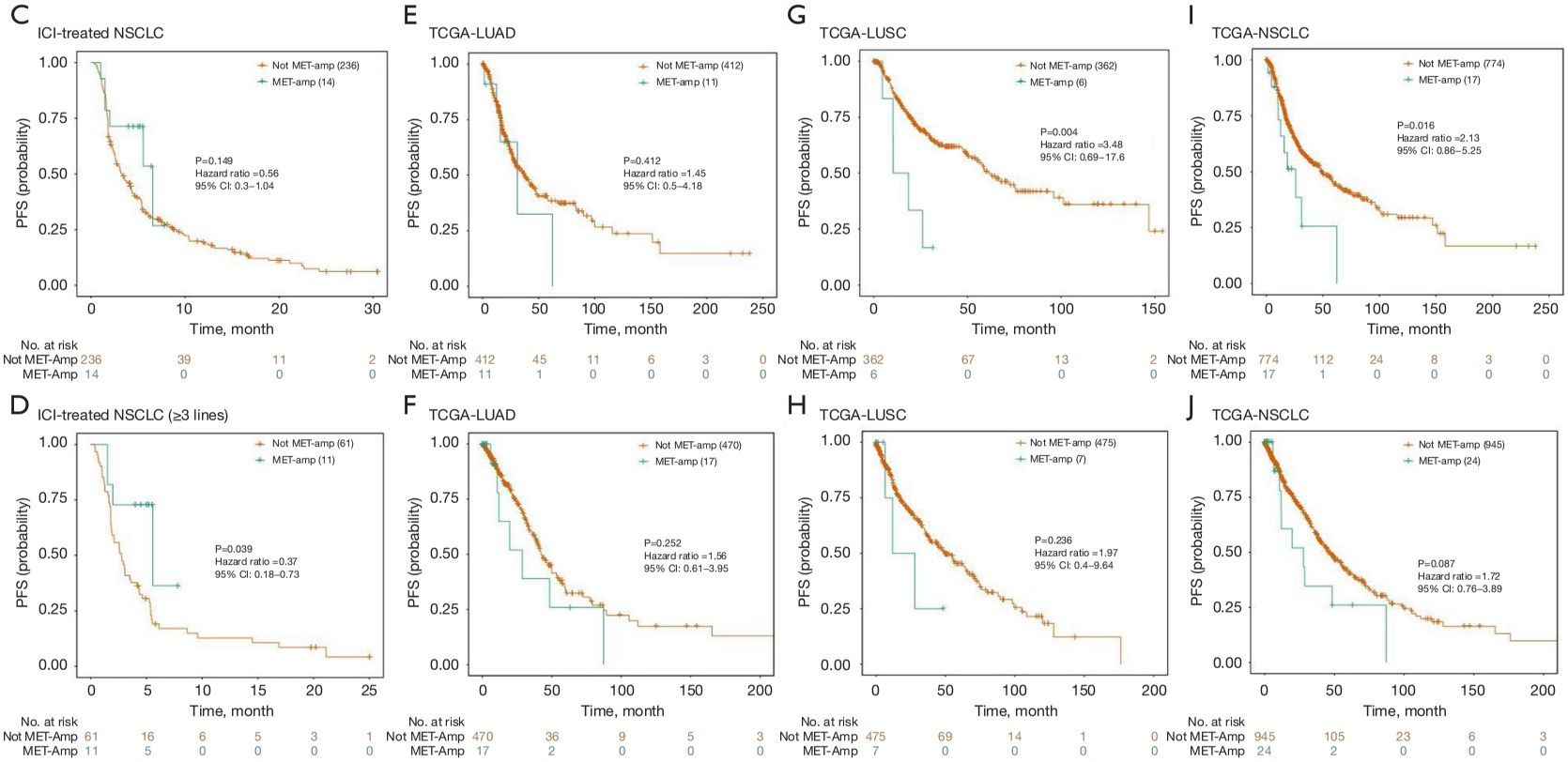


Figure 1 Mutation landscape and survival information of NSCLC patients stratified by the *MET* amplification status. (A) Top 20 frequently mutated genes in NSCLC in the Rizvi cohort (top panel). Genes are ranked by the mutation frequency. The alteration type, *MET* status, sex, cancer stage, smoking status, PFS status, treatment type, durable clinical benefit, histological subtype, lines of treatment, age, FGA, and PD-L1 score are annotated. (B) Top 20 frequently mutated genes in NSCLC in the TCGA-NSCLC cohort (bottom panel). Genes are ranked by the mutation frequency. The alteration type, survival status, *MET* status, histological subtype, clinical stage, age, race, tobacco smoking history, and number of pack-years smoked are annotated. (C) Kaplan-Meier estimates of PFS in the ICI-treated cohort comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=250, log-rank test). (D) Kaplan-Meier estimates of PFS in the ICI-treated cohort (≥3 treatment lines) comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=72). Patients who harbored *MET* amplification showed a better prognosis for ICI-based immunotherapy (P=0.039, log-rank test). (E) Kaplan-Meier estimates of PFS in the TCGA-LUAD cohort comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=423, log-rank test). (F) Kaplan-Meier estimates of OS comparing patients in the TCGA-LUAD cohort with *MET* amplification with their respective counterparts without *MET* amplification (n=487, log-rank test). (G) Kaplan-Meier estimates of PFS in the TCGA-LUSC cohort comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=368, log-rank test). (H) Kaplan-Meier estimates of OS in the TCGA-LUSC cohort comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=483, log-rank test). (I) Kaplan-Meier estimates of PFS in the TCGA-NSCLC (TCGA-LUAD + TCGA-LUSC) cohort comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=891, log-rank test). (J) Kaplan-Meier estimates of OS in the TCGA-NSCLC (TCGA-LUAD + TCGA-LUSC) cohort comparing patients with *MET* amplification with their respective counterparts without *MET* amplification (n=969, log-rank test). NSCLC, non-small cell lung cancer; MET, mesenchymal-epithelial transition; PFS, progression-free survival; FGA, fraction of copy number-altered genome; PD-L1, programmed cell death receptor ligand 1; TCGA, The Cancer Genome Atlas; ICI, immune checkpoint inhibitor; LUAD, lung adenocarcinoma; OS, overall survival; LUSC, lung squamous cell carcinoma.

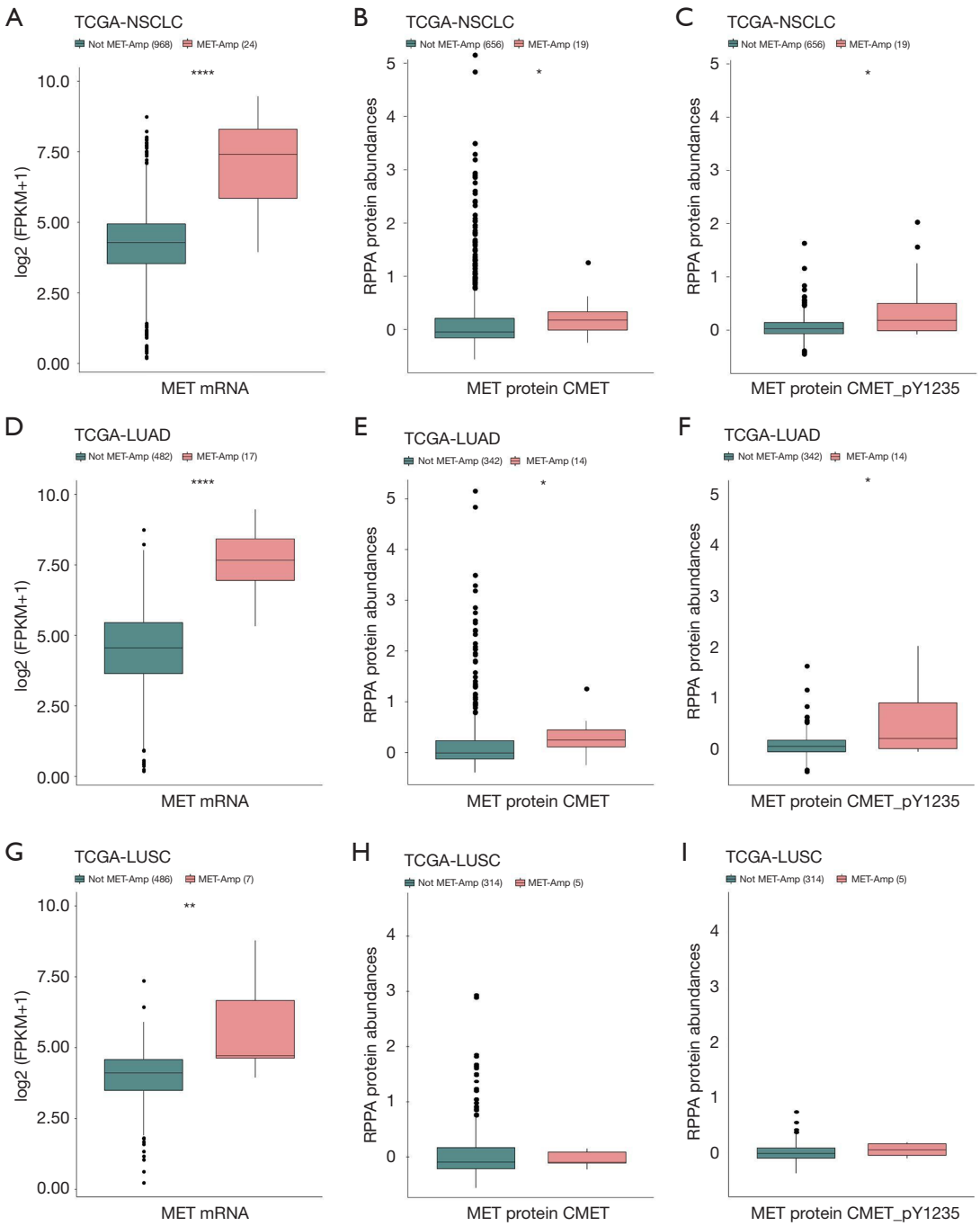


Figure 2 *MET* mRNA and protein expression is upregulated in *MET*-amplified NSCLC. Expression of *MET* mRNA (A) and *MET* proteins CMET (B) and CMET_pY1235 (C) in the *MET*-Amp and non-*MET*-Amp groups in the TCGA-NSCLC cohort. Expression of *MET* mRNA (D) and *MET* proteins CMET (E) and CMET_pY1235 (F) in the *MET*-Amp and non-*MET*-Amp groups in the TCGA-LUAD cohort. Expression of *MET* mRNA (G) and *MET* proteins CMET (H) and CMET_pY1235 (I) in the *MET*-Amp and non-*MET*-Amp groups in the TCGA-LUSC cohort. (*, P<0.05; **, P<0.01; and ****, P<0.0001). The *MET* protein list was obtained from the Human Protein Atlas. The lines and boxes indicate the medians and upper and lower quartiles, respectively.) MET, mesenchymal-epithelial transition; NSCLC, non-small cell lung cancer; MET-Amp, MET-amplification; TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

expression of *MET* mRNA and protein during the evolution of NSCLC is unclear. Our analysis based on the TCGA-NSCLC LUAD/LUSC cohort showed that the *MET*-amplification NSCLC group (2.41%, 24/992) had higher expression levels of *MET* mRNA and *MET* proteins CMET and CMET_PY1235 than the non-*MET*-amplification group (*Figure 2A-2C*; P<0.0001, P<0.05, and P<0.05, respectively). Similarly, the *MET*-amplification LUAD group had higher expression levels of *MET* mRNA and *MET* proteins CMET and CMET_PY1235 than the non-*MET*-amplification LUAD group (*Figure 2D-2F*; P<0.0001, P<0.05, and P<0.05, respectively). In addition, the *MET*-amplification LUSC group had higher expression levels of *MET* mRNA (*Figure 2G*, P<0.01) and a trend toward higher expression levels of the *MET* proteins CMET and CMET_PY1235 (*Figure 2H,2I*) than the non-*MET*-amplification LUAD group.

Most current treatment strategies for abnormal activation of the *c-MET* pathway use multikinase *MET* inhibitors such as crizotinib and cabozantinib. The GDSC database contains data for approximately 1,000 human cancer cell lines. Regarding the susceptibility to different drugs, we further investigated the correlation between *MET* amplification and the sensitivity to common drugs in 69 NSCLC cell lines from the GDSC database (Figure S2). There was no significant difference in common chemotherapeutic drugs (cisplatin, paclitaxel, docetaxel, vinorelbine, and gemcitabine) and *c-MET* receptor kinase inhibitors (cabozantinib; crizotinib; foretinib; and PHA-665752), between the *MET*-amplification and non-*MET*-amplification NSCLC groups. In addition, NSCLC cell lines with *MET* amplification were resistant to common targeted drugs, such as *c-MET* receptor kinase inhibitors (e.g., crizotinib). These drug sensitivity data showed that *MET*-amplified NSCLC may be less responsive to chemotherapy or targeted therapy.

Landscape of immune cells and transcriptome traits based on MET status

To further investigate differences in the immune cell infiltration status and potential biological mechanisms between NSCLC with and without *MET* amplification, we used the CIBERSORT algorithm to estimate the immune cell infiltration status in 992 samples from the TCGA-NSCLC cohort (*Figure 3*). The heatmap indicated that the immune cells and some clinical features were significantly different between the *MET*-amplification and non-*MET*-amplification groups. For example, CD8+ T cells (P<0.05), M1 macrophages (P<0.05), gamma delta T cells (P<0.05), and activated CD4+ memory T cells (P<0.0001) were more abundant in the *MET*-amplification group. This pattern indicated that immune-activated cells were significantly enriched in the *MET*-amplification group. In addition, patients in the *MET*-amplification group had a longer duration of smoking (median: *MET* amplification *vs.* non-*MET* amplification: 45 *vs.* 30 years, P<0.05) and were more likely to have a history of smoking (P<0.01).

As *MET* amplification plays a key role in NSCLC progression and prognosis, we analyzed the underlying biological mechanisms of *MET* amplification in the TCGA-NSCLC dataset. The TCGA-NSCLC dataset was divided into two groups according to the *MET* amplification status, and GSEA was performed using the gene set obtained from the MSigDB. When the *MET*-amplification group was compared with the non-*MET*-amplification group, immune response-related pathways, such as downstream signaling events of B cell receptors (BCRs), positive regulation of interferon-gamma (IFN-γ) production, immune response to tumor cells, and downstream TCR signaling, were significantly upregulated in the *MET*-amplification group (*Figure 4A*). Additionally, oncogenic signaling and metabolic-related pathways, such as response to fibroblast growth factor, *ERK1* and *ERK2* cascade, and fatty acids, were significantly downregulated in the *MET*-amplification group (*Figure 4B*).

Association of MET gene amplification with enhanced immunogenicity and activated antitumor immunity

To better identify the immune profile, we analyzed differences in immune-related gene expression patterns between the *MET*-amplification and non-*MET*-amplification groups in the TCGA-NSCLC database (*Figure 5A-5D*). Consistent with the elevated immune cell infiltration, the expression of many immunostimulation-related genes, such as chemokine genes (*CXCL9*, *CXCL10*, and *CCL5*) and cytolysis-related genes (*PRF1* and *GZMA*), was significantly upregulated in the *MET*-amplification group (*Figure 5A,5B*). The *MET*-amplification NSCLC group exhibited significant upregulation of immune checkpoint-related genes (such as *LAG3* and *PDCD1LG2*) compared to the non-*MET*-amplification NSCLC group. In addition, the *MET*-amplification NSCLC group exhibited significantly upregulated expression of antigen presentation-related genes, such as *HLA-A*, *HLA-B*,

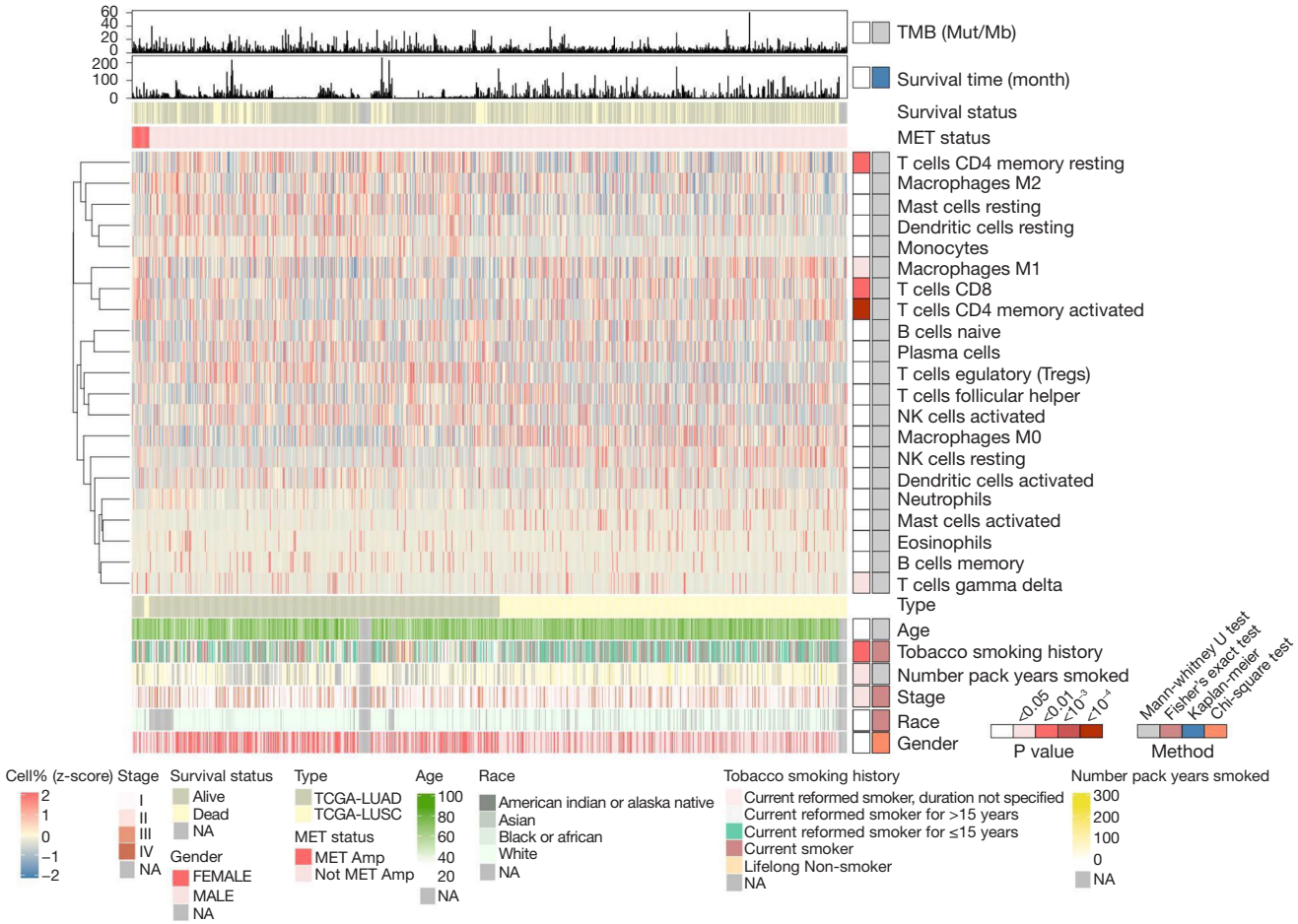


Figure 3 Landscape of the immune cells and clinical features in NSCLC (TCGA cohort). TMB, 22 immune cell types, and age were analyzed using the Mann-Whitney U test. Tobacco smoking history and clinical stage were analyzed using Fisher's exact test and sex was analyzed using the chi-square test. The TME-infiltrating cells, TMB, survival status, *MET* status, histological subtype, clinical stage, age, race, tobacco smoking history, number of pack-years smoked, and statistical methods are shown as patient annotations. The corresponding levels of significance are displayed as a heatmap in the right panel. NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas; TMB, tumor mutation burden.

HLA-C, and *MICB* (*Figure 5A*). Further subgroup analysis showed that the *MET*-amplification TCGA-LUAD group also had upregulated immune-related gene expression patterns such as chemokine-related, cytotoxicity-related and immune checkpoint-related genes (*Figure 5A*,*5B*). These results suggest that *MET* amplification is closely associated with enhanced tumor immunogenicity and an activated immune microenvironment, providing a theoretical basis for the benefit of immunotherapy in patients with *MET*-amplified NSCLC.

We further compared tumor immunogenicity and antitumor immunity between patients with and without *MET* amplification in the TCGA-NSCLC cohort. *Figure 5E* shows that 10 patients with *MET* amplification (local cohort, ICI-treated) had a higher TMB than those without (TCGA-NSCLC, non-ICI-treated; P<0.0001), whereas all patients with *MET* amplification had a trend toward a higher TMB than those without (TCGA-NSCLC, non-ICI-treated; P<0.01). Subgroup analysis (*Figure 5F*) showed that the *MET*-amplification LUAD group (local cohort) had a higher TMB than the non-*MET*-amplification (P<0.001) and *MET*-amplification (P<0.05) TCGA-LUAD groups, whereas the *MET*-amplification TCGA-LUAD group also had a higher TMB than the non-*MET*-amplification

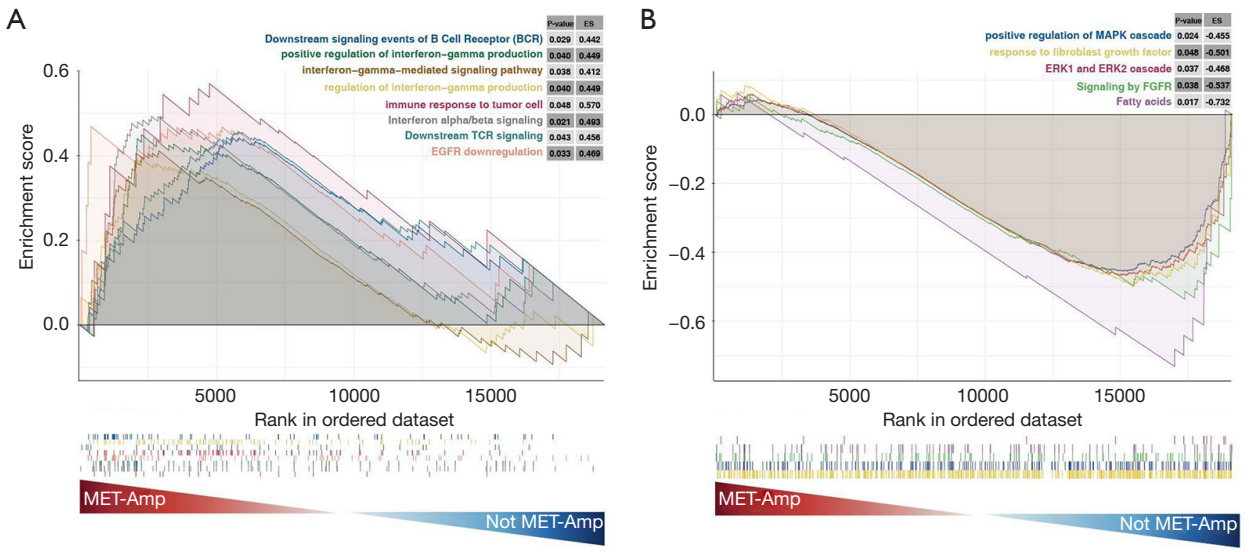


Figure 4 Transcriptome biological function traits of *MET*-Amp and non-*MET*-Amp tumors in the TCGA-NSCLC cohort. (A,B) GSEA of hallmark gene sets downloaded from the MSigDB. All transcripts are ranked by the log2 (fold change) between the *MET*-Amp and non-*MET*-Amp tumors in the TCGA-NSCLC cohort. Each run was performed with 1,000 permutations. Enrichment results with significant associations between *MET*-Amp and non-MET-Amp tumors are shown. MET, mesenchymal-epithelial transition; MET-Amp, MET-amplification; TCGA, The Cancer Genome Atlas; NSCLC, non-small cell lung cancer; GSEA, gene set enrichment analysis; MSigDB, Broad Institute Molecular Signatures Database.

TCGA-LUAD group (P<0.05). Similarly, the *MET*-amplification LUSC group (local cohort) had a higher TMB than the non-*MET*-amplification TCGA-LUSC group (P<0.05, *Figure 5G*). In addition, the *MET*-amplification TCGA-NSCLC group had a trend toward a higher NAL than the non-*MET*-amplification TCGA-NSCLC group (*Figure 5H*). Further subgroup analysis showed that the levels of NAL between the *MET*-amplification and non-*MET*-amplification in the TCGA-LUAD group (*Figure 5I*) and TCGA-LUSC group (*Figure 5J*).

Increasingly, studies have shown that alterations in DNA damage response- or DNA damage repair-related pathways are associated with immunotherapeutic efficacy (32-34). For example, the number of DDR pathway gene mutations is positively correlated with markers of tumor immunogenicity, such as TMB and NAL. In addition, DDR may be a biomarker for predicting the efficacy of immunotherapy (32). Therefore, we used the DDR gene set from the MSigDB (https://cdn.amegroups.cn/static/public/atm-21-4543-2.xlsx) to compare differences in the number of mutations in the DDR-related pathway between the *MET*-amplification and non-*MET*-amplification groups in the TCGA-NSCLC/LUAD/LUSC cohort and in GDSC-NSCLC cell lines (Figure S3). The number of mutations in multiple DDR pathways was significantly higher in the *MET*-amplification TCGA-NSCLC LUAD group than in the non-*MET*-amplification TCGA-NSCLC LUAD group.

Discussion

MET amplification plays critical roles in the NSCLC mutagenic process, contributing to subclonal diversification, intratumor heterogeneity. Here, our study focused on the association between *MET* amplification and the efficacy of ICIs for NSCLC treatment, and the results showed it was associated with superior PFS times in the ICI-treated cohort (n=72; P=0.039, HR =0.37, 95% CI: 0.18–0.73) but not in the non-ICI-treated cohort (e.g., the TCGA-NSCLC LUAD/LUSC cohort). Thus, *MET* amplification can distinctly function in NSCLC to predict the prognosis of immunotherapy. Through analyses of immune cells, immune-related gene expression, immunogenicity, and antitumor immunity, we identified significant correlations between *MET* amplification and immune-related gene expression, increased immune cell infiltration, enhanced immunogenicity, activated antitumor immunity, and

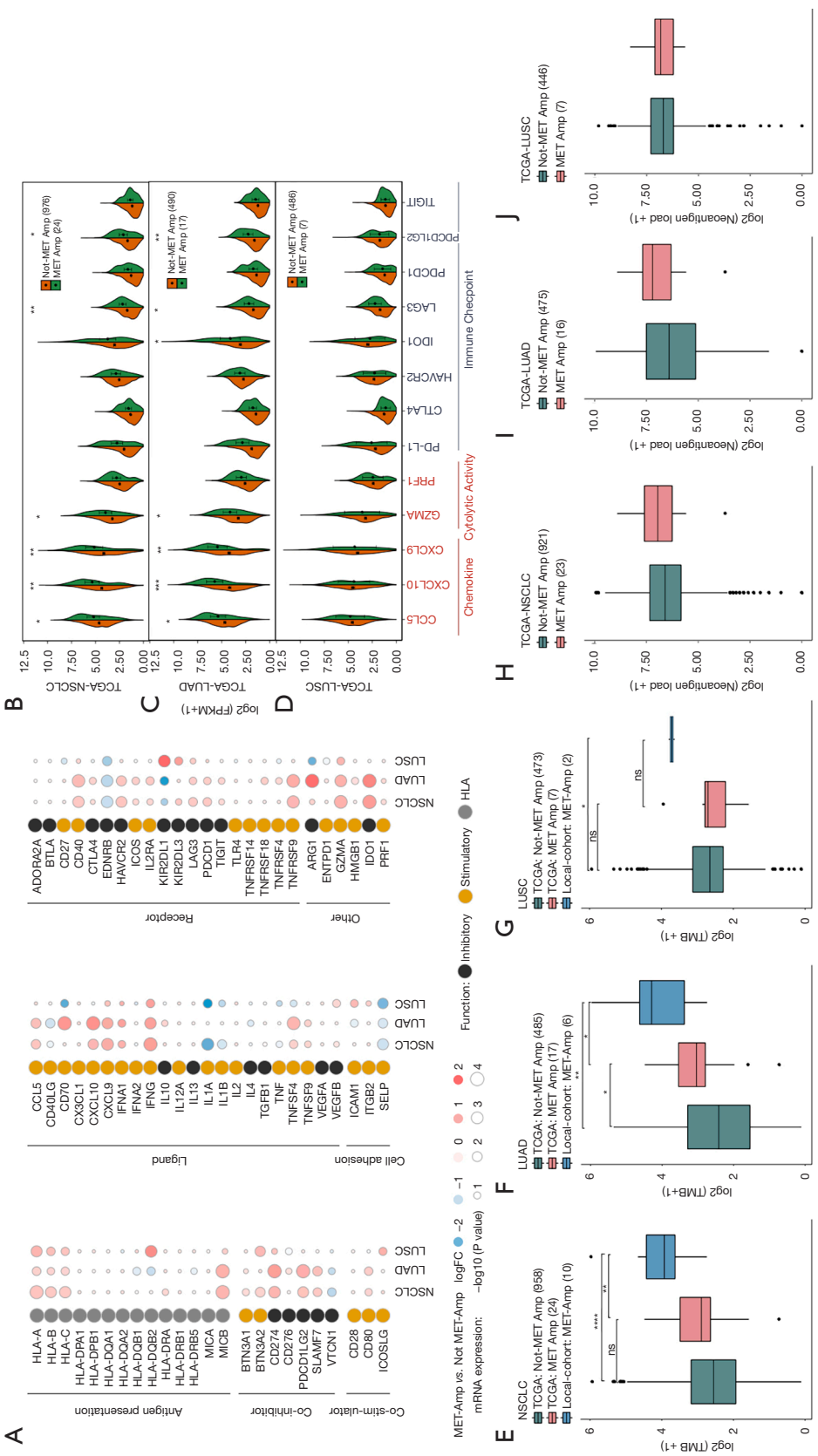


Figure 5 *MET*-Amp is associated with enhanced tumor immunogenicity and activated antitumor immunity. (A) Bubble plot depicting mean differences in the expression of immune-related genes between the *MET*-Amp and non-*MET*-Amp tumors in the TCGA-NSCLC cohort. The X-axis of the bubble plot indicates TCGA-NSCLC, TCGA-LUAD, and TCGA-LUSC, and the Y-axis indicates immune-related gene names. Each circle represents the fold change of immune-related genes between the *MET*-Amp and non-*MET*-Amp tumors. Red indicates upregulation, while blue indicates downregulation Violin plots showing the expression levels of immune-related genes, such as chemokines, cytolytic activity-associated genes, and immune checkpoints between the *MET*-Amp and non-*MET*-Amp tumors in the TCGA-NSCLC (B), TCGA-LUAD (C), and TCGA-LUSC (D) cohorts. Comparison of TMB between the *MET*-Amp and non-*MET*-Amp tumors in the TCGA-NSCLC (E), TCGA-LUAD (F), and TCGA-LUSC (G) cohorts. Comparison of NAL between the *MET*-Amp and non-*MET*-Amp tumors in the TCGA-NSCLC (H), TCGA-LUAD (I), and TCGA-LUSC (J) cohorts. (Mann-Whitney U test; *, P<0.05; **, P<0.01; and ****, P<0.0001; the line and box represent the median and upper and lower quartiles, respectively). MET, mesenchymal-epithelial transition; MET-Amp, MET-amplification; TCGA, The Cancer Genome Atlas; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NAL, neoantigen load; TMB, tumor mutation burden.

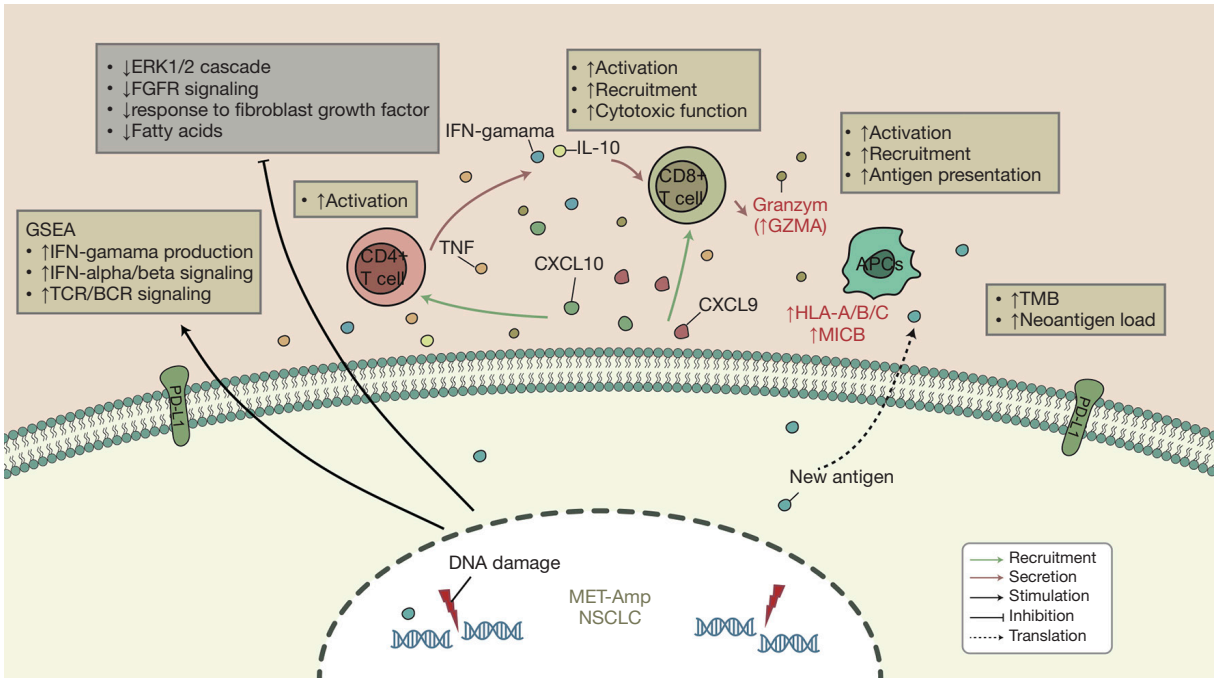


Figure 6 Possible mechanism underlying the improved efficacy and prognosis in *MET*-Amp NSCLC patients receiving ICIs. MET, mesenchymal-epithelial transition; MET-Amp, MET-amplification; NSCLC, non-small cell lung cancer; ICI, immune checkpoint inhibitor.

decreased DDR efficacy. In addition, in the GDSC database, the *MET*-amplification group exhibited a trend toward resistance to many common chemotherapeutic drugs, such as *c-MET* receptor kinases (e.g., crizotinib). Studies have shown that patients with *MET* overexpression may be more responsive to ICI immunotherapy than to targeted therapy (15), and *MET* amplification, mutation, or overexpression may be manifested by upregulation of PD-L1 and other immunosuppressive molecules at the mRNA and protein levels (17). Furthermore, we summarized the possible mechanisms underlying the improved efficacy and prognosis in *MET*-amplified NSCLC patients receiving ICIs (*Figure 6*).

Tumor immunogenicity is the developmental basis of tumor immunity, and many somatic mutations can produce antigens to activate CD8+ cytotoxic T cells, thereby exerting a T cell-mediated antitumor effect. In addition, IFN-γ is generally produced by effector T cells or antigen-presenting cells (APC) to support antigen presentation and the recruitment of other immune cells, thereby initiating antiproliferative and apoptotic effects on the tumor. To date, T cell-inflamed GEPs, specific gene mutations, high MSI (MSI-H), TMB, NAL, and the TME (such as the CD8+ T cell abundance) have gradually become potential markers for the immunotherapy response (3,10,35). When TMB increases, it promotes the production of new antigens in tumors which are presented to APC, which can lead to the transformation of T cells into mature and activated T cells and increase the sensitivity of patients to treatment with PD-1 and CTLA-4 inhibitors (36). Recently, mutations in DDR pathway-related genes have attracted attention in immunotherapy (37). For example, functional mutations in the DDR pathway reduced genome stability and increased tumor immunogenicity via the accumulation of DNA damage to increase the efficacy of immunotherapy (37,38). This effect suggests ICI therapy may be a potential strategy for patients with *MET*-amplified NSCLC. However, no clinical correlation has been identified for *MET* immunotherapy in NSCLC.

As is known to all, *MET* amplification is a *de novo* driver gene in NSCLC about 5%. So far, there are several c-*MET* inhibitors approved by FDA/CFDA for *MET* exon 14 skipping in advanced NSCLC. However, for *de novo MET* amplification, MET-TKI monotherapy demonstrated a short PFS of 4 months in NSCLC clinical study. In a subgroup analysis of PROFILE 1001, patients with high level *MET* amplification demonstrated an objective response rate (ORR) of 38.1% and a long PFS of

6.7 months. These result suggested *MET* inhibitors maybe only efficacy to patients with high level *MET* amplification in NSCLC. And our study demonstrated immunotherapy maybe another selective strategy for patients with low level *MET* amplification. On the other side, *MET* amplification is an acquired resistance gene closed to 20% after using EGFR-TKI. In a phase I/II clinical study "INSIGHT", tepotinib combined with gefitinib demonstrated a dramatic PFS of 16.6 months which provided a new therapy for patients with resistance from EGFR-TKI, especially from third EGFR-TKI in future. In this study we found *MET* gene amplification was associated with inflammatory tumor microenvironment, but whether it can remodel the immune desert type of tumor microenvironment in EGFR-mutant NSCLC has not been known. Whether these patients can be benefit from immunotherapy still need further research.

Different from target therapy and chemotherapy, immunotherapy in advanced NSCLC is focus on the long-term survival. Existing studies suggest that RECIST 1.1 evaluation underestimated the benefit of immunotherapy, further research is required to optimize iRECIST and establish some criteria for selecting patients who will benefit from continued immunotherapy beyond PD per RECIST 1.1. Tumor micrometastasis maybe one of key point about recurrence after lung cancer surgery. It was reported that tumor immune microenvironment is predictive of prognosis after surgery in NSCLC. This suggested immunotherapy may impact on tumor micrometastasis and improve the prognosis of early lung cancer. Lambrechts *et al.* (39) reported that the expression profiles of tumor stromal cell marker genes and tumor stromal cell subsets differed between LUAD and LUSC, and that low expression of CD8+ T cell cluster marker genes in LUAD was associated with an improved survival prognosis. In contrast, CD8+ T cell cluster marker genes expressed in LUSC were associated with a worse survival prognosis in LUSC. All these studies suggested some special patients maybe benefit from immunotherapy after surgery. To date, clinical research about evaluation of immunotherapy after lung cancer surgery are going on. In this study, we found *MET* amplification is closely associated with enhanced tumor immunogenicity and an activated immune microenvironment. Therefore, it worth waiting for whether patients with *MET* amplification after surgery can benefit from checkpoint inhibitors.

This study has some limitations. First, our analysis did not compare *MET* amplification with non-*MET* amplification in patients with NSCLC on first-line immunotherapy. Second, unlike the NSCLC ICI-treated cohort from Rizvi *et al.*, our local datasets represent only the Chinese population, and differences in genetic backgrounds between ethnicities may affect the results of the analysis. Third, we found patients in the *MET*-amplification NSCLC group (local cohort) had a higher TMB than those in the *MET*-amplification TCGA-NSCLC group, which showed, TMB may be a predictive marker of the clinical immunotherapy response in the *MET*-amplification NSCLC group (local cohort) to select patients for immunotherapy. Fourth, *MET* amplification has been reported to show intratumoral heterogeneity in a number of cases (40), and intratumoral heterogeneity should be considered when interpreting our results on *MET* and phospho-*MET* protein expression. Fifth, the threshold used to define *MET* amplification varies among studies. Sixth, because of the difference in structure, there still a certain difference in the efficacy of different ICIs. But in this study, the number of patients treated with ICIs was unfortunately very small. So we cannot to explore it further. Finally, our analysis considered only the two most important subtypes of NSCLC, and the remaining subtypes were not considered. Therefore, more research involving a large number of samples and diverse ethnic groups is needed for analysis and validation.

Conclusions

Our study provides evidence that *MET* amplification is associated with long PFS times and with known immunotherapy response markers, including TMB, NAL, immune-related genes, and the high infiltration of specific immune cells. Therefore, *MET* amplification could be a predictive biomarker for ICIs. A series of prospective clinical studies and molecular mechanistic explorations is required.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This research was performed in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the ethics committee of Zhujiang Hospital of Southern Medical University, Guangzhou Chest Hospital and First People's Hospital of Chenzhou. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

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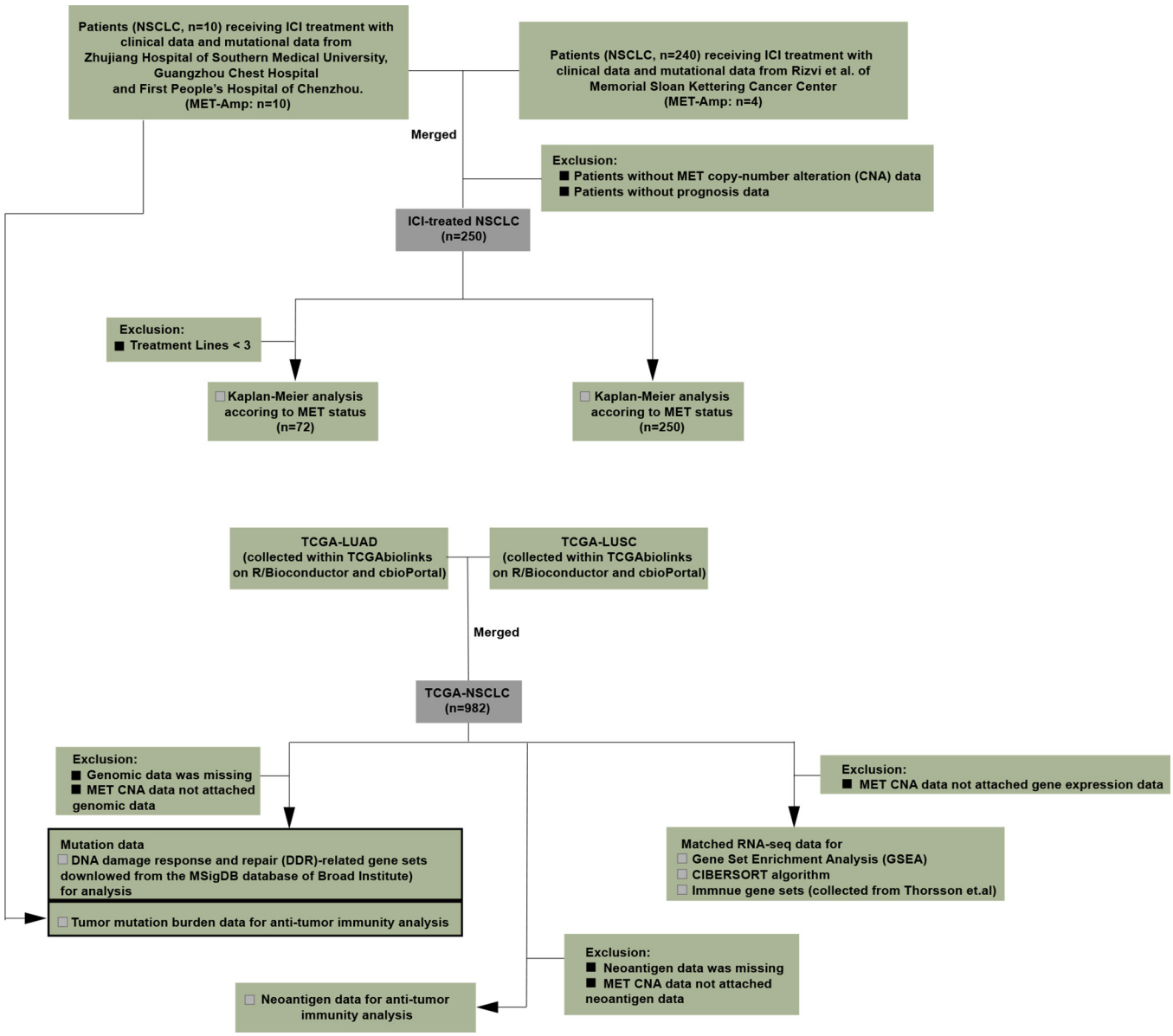

Figure 1 Mutation landscape and survival information of NSCLC patients stratified by the *MET* amplification status. (A) Top 20 frequently m**Figure S1** Flowchart of data processing of the TCGA dataset and the ICI-treated NSCLC cohort. TCGA, The Cancer Genome Atlas; ICI, immune checkpoint inhibitor; NSCLC, non-small cell lung cancer.

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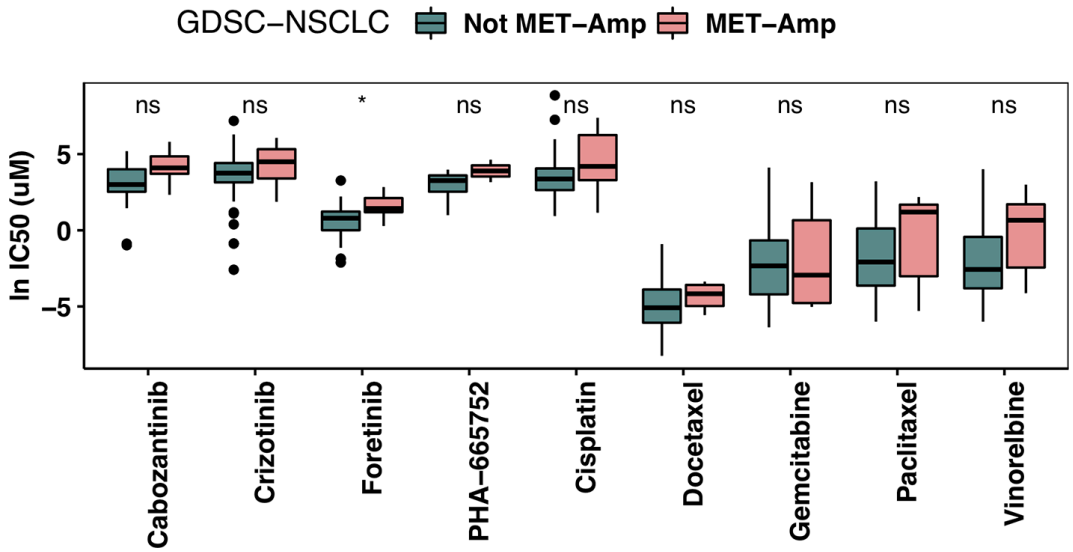

Figure S2 Comparison of the drug sensitivity of cell lines from the GDSC-NSCLC database between *MET*-Amp and non-*MET*-Amp cell lines. (Mann-Whitney U test; *, P<0.05). GDSC, Genomics of Drug Sensitivity in Cancer; NSCLC, non-small cell lung cancer; MET, mesenchymal-epithelial transition; MET-Amp, MET-amplification.

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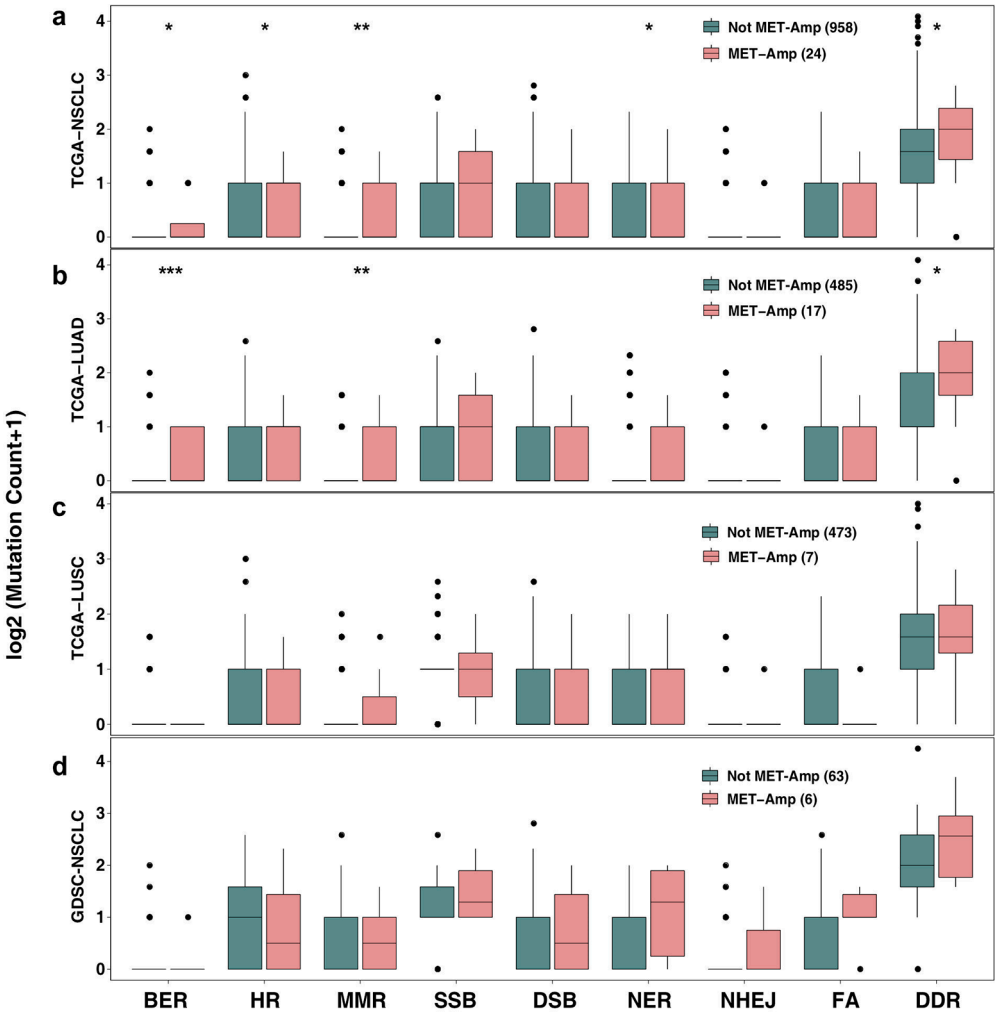

Figure S3 Comparison of DNA damage-related gene set alterations in cell lines from the TCGA-NSCLC (A), TCGA-LUAD (B), TCGA-LUSC (C), and GDSC-NSCLC (D) databases between the *MET*-Amp and non-*MET*-Amp groups. (Mann-Whitney U test; *, P<0.05; **, P<0.01; ***, P<0.001). TCGA, The Cancer Genome Atlas; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; GDSC, Genomics of Drug Sensitivity in Cancer; MET, mesenchymal-epithelial transition; MET-Amp, MET-amplification.

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