EPHA5 mutation predicts the durable clinical benefit of immune checkpoint inhibitors in patients with lung adenocarcinoma

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Abstract

Immune checkpoint inhibitor (ICI) therapy has shown remarkable clinical benefit in lung adenocarcinoma (LUAD) patients. Genomic mutations may be applicable to predict the response to ICIs. Eph receptor A5 (EPHA5) is frequently mutated in breast cancer, lung cancer, and other tumors; however, its association with outcome in patients who receive immunotherapy remains unknown. In this study, we report that EPHA5 mutations were associated with increased tumor mutation burden (TMB), neoantigen load, levels of immune-related gene expression signatures, and enhanced tumor-infiltrating lymphocytes (TILs) in LUAD. LUAD patients with EPHA5 mutations in the immunotherapy cohort achieved a longer progression-free survival (PFS) time than patients with wild-type EPHA5. Immune response pathways were among the top enriched pathways in samples with EPHA5 mutations. In addition, patients with EPHA5 mutations tended to be more sensitive to certain targeted molecular inhibitors, including serdemetan, Iox2, and PF1-1. Collectively, our results suggest that identifying mutations in the EPHA5 gene may provide insight into the genome-wide mutational burden and may serve as a biomarker to predict the immune response of patients with LUAD.

Introduction

Currently, lung cancer is still the leading cause of cancer-related deaths worldwide [1]. The majority of lung cancer patients are non-small-cell lung cancer (NSCLC) and are in an advanced stage [2]. NSCLC can be histologically classified into lung adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC), with LUAD being the most common subtype, comprising more than 50% of all NSCLCs [3]. At present, a therapeutic plateau has been reached, with a response rate of only approximately 20% in LUAD patients who receive traditional treatment [4]. Therefore, new selective treatments are urgently needed to improve the outcome of patients with advanced LUAD.

Immune checkpoint inhibitors (ICIs), including anti-PD-(L)1 monotherapy and anti-cytotoxic T-lymphocyte-4 (anti-CTLA-4), have emerged as therapeutic landscape for patients with advanced cancers, including LUAD [5–8]. However, only 20–30% of LUAD patients respond to ICIs, with most patients not achieving objective responses [9]. The mechanisms underlying variations in the response to ICIs among LUAD patients are still unclear, and potential biomarkers that

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are predictive of the benefit of ICIs are needed to elucidate the mechanisms of resistance in ICI nonresponders.

To date, the only Food and Drug Administration-approved predictive biomarkers of the response to ICIs in NSCLC patients are programmed death-ligand 1 (PD-L1) expression, the microsatellite instability (MSI) status, and mismatch repair deficiency [10, 11], but the sensitivity and specificity of these approaches are still modest [12]. Tumor mutation burden (TMB) [13], the intensity of CD8+ T-cell infiltrates [14], copy number variants (CNVs), and the predicted neoantigen load [15] have also been proposed as distinct biomarkers of the response to ICI therapies in NSCLC patients. Moreover, many studies have found that mutations in genes associated with genomic integrity, such as EGFR and P53 [16], could cause replication stress and genomic instability, resulting in a high TMB and neoantigen burden in NSCLC patients. This raises the question of whether other mutations simultaneously affect the above factors to provide stronger predictive value for therapeutic outcomes in LUAD patients.

The Eph receptor tyrosine kinase (RTK) family is the largest group of surface receptors and is classified into the EphA or EphB subfamily [17]. Eph receptors have been proven to play an important role in tumor immunity [18]. For example, CD4+ T cells recognize the EphA3 epitope and then elicit selective immunoreactivity against melanoma cells [19]. In addition, CD4+ and CD8+ T cells recognize multiple epitopes of EPHA2 and enhance the immune activity of CD8+ T cells in cancers [20, 21]. In addition to inducing immunologic responses, Eph receptors also regulate immune cell trafficking. EphA1 and EphA4 are expressed in T cells and promote the chemotaxis and migration of CD4+ and CD8+ T cells by recruiting multiple kinase pathways [22–24]. Furthermore, a recent study showed that mutations in the EPHA5 gene impair natural killer (NK) cell-mediated cytotoxicity against NSCLC cells [25], suggesting that Eph receptors affect the tumor immune microenvironment. Thus, evaluating the roles of the Eph receptor in the response to ICIs, especially those that are driven by genetic changes, may provide additional insight into the tumor immune microenvironment.

In the present study, we first examined EPHA5, a member of the Eph/ephrin family, as a new biomarker for the response to ICIs in patients with LUAD. By comparing the gene mutation profiles of ICI responders and non-ICI responders based on the ICI-treated LUAD cohort reported by Rizvi [13], we identified a high mutation frequency of EPHA5 in ICI-responsive patients. We then analyzed the relationship of EPHA5 mutation and the prognosis of LUAD patients in both the ICI-treated LUAD cohort and the non-ICI-treated LUAD cohort. We also investigated whether EPHA5 mutation is associated with the immune microenvironment in LUAD. Our current evidence suggests that EPHA5 alterations yield promising predictive value for ICI treatment in LUAD patients.

**Subjects and methods**

**Public datasets used in this study**

An ICI-treated cohort comprising 240 NSCLC patients with a detailed clinical annotation, response data and mutation data (mutation data were obtained from targeted next-generation sequencing (NGS)) described in the cBioPortal for Cancer Genomics at http://www.cbioportal.org (240MSKCC ICI-treated cohort) was analyzed. All patients were enrolled from Memorial Sloan Kettering Cancer Center (MSKCC) and treated with anti-PD-(L)1 monotherapy alone or anti-PD-(L)1 monotherapy combined with anti-CTLA-4. The efficacy of tumor immunotherapy was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 and defined as durable clinical benefit (DCB) or no durable benefit (NDB) [13].

Somatic mutation data, gene expression data (Illumina HiSeq, RNA-Seq) and overall survival (OS) data from the TCGA-LUAD cohort were downloaded from the Genomic Data Commons (https://portal.gdc.cancer.gov/) by using the R package TCGAbiolinks [26], while disease-free survival (DFS) data were downloaded from cBioPortal [27]. All TCGA-LUAD patients were enrolled from Biospecimens Core Resource (BCR) at Nationwide Children’s Hospital and were not treated with any Immune checkpoint inhibitor. Of the 494 LUAD patients included in this cohort, 254 patients received chemotherapy and 240 patients underwent radiotherapy. We thus defined the TCGA-LUAD cohort as non-ICI-treated LUAD subgroup in the present study.

The GDSC-LUAD dataset contains somatic mutation data and drug sensitivity data on multiple LUAD cell lines and was downloaded from the Genomics of Drug Sensitivity in Cancer (GDSC) database (https://www.cancerrxgene.org) [28].

**Comparison of the prognosis of LUAD patients with mutant EPHA5 and those with wild-type EPHA5**

A subset of ICI-treated LUAD patients (n = 187, from the 240MSKCC ICI-treated cohort) with annotated clinical records was divided into two subgroups, EPHA5-MT (mutant type) and EPHA5-WT (wild-type), and PFS, defined as the date the patient began immunotherapy to the date of progression, was assessed by Kaplan–Meier (KM) analysis and compared between the two subgroups. We also calculated the DFS and OS of 494 non-ICI-treated patients from the TCGA-LUAD cohort depending on the status of EPHA5 mutations by KM analysis.

**EPHA5 mutation and tumor immunogenicity**

The somatic mutation data from the ICI-treated LUAD cohort were analyzed by targeted NGS (MSK-IMPACT) as
described by Hira Rizvi. In the TCGA-LUAD and GDSC-LUAD cohorts, TMB was determined by using the non-synonymous mutations as the raw mutation count and dividing by 38 Mb [29], while the TMB score was then applied to the ICI-treated LUAD cohort. We used the ComplexHeatmap [30] package in R to visualize the genetic mutations in the top 20 genes with the highest alteration frequencies and clinical characteristics of patients in the ICI-treated LUAD and TCGA-LUAD cohorts. The maftools [31] package in R was used to visualize EPHA5 mutations in the ICI-treated LUAD and TCGA-LUAD cohorts.

**Tumor-infiltrating lymphocyte (TIL) analysis and T-cell-inflamed gene expression (RNA) profiling**

The CIBERSORT algorithm [32] (http://cibersort.stanford.edu/) was used to compare the abundance of 22 TILs between the EPHA5-MT and EPHA5-WT subgroups in the TCGA-LUAD cohort. In addition, differences in the mRNA expression of T-cell-inflamed genes associated with antigen presentation, adaptive immune resistance, chemokine expression, and cytolytic activity between the EPHA5-WT and EPHA5-MT subgroups in the TCGA-LUAD cohort were also compared [33]. The differences in mRNA expression were calculated as follows: \( p \)-value < 0.05 and \( \log_2(\text{fold-change}) > 0.58 \) or \( \log_2(\text{fold-change}) < -0.58 \).

**Drug sensitivity analysis of the EPHA5-WT and EPHA5-MT subgroups**

According to the mutation status of EPHA5, the cell lines corresponding to code “LUAD” in the GDSC database were divided into two groups, and then the two groups of cells were compared for their sensitivity to compounds including chemotherapy and molecular-targeted drugs.

**CNV analysis**

The Affymetrix SNP 6.0 microarray data of the TCGA-LUAD cohorts were downloaded from the Broad GDAC Firehose (http://gdac.broadinstitute.org/). We then used GenePattern [34] (https://cloud.genepattern.org/gp/pages/index.jsf) to analyze the downloaded CNV segments with GISTIC 2.0. We set the confidence level to 0.99 and did not exclude the X chromosome before analysis. All other GISTIC 2.0 parameters were default values.

**Pathway enrichment analysis**

The gene expression profiles of the TCGA-LUAD cohort were downloaded and partitioned into two groups according to the mutation status of EPHA5. The differentially expressed genes between the two groups were obtained by using the R package edgeR [35] and then fed into the R-function in the clusterProfiler [36] package for gene set enrichment analysis (GSEA). A gene set with a nominal \( p \)-value less than 0.05 was considered significantly enriched in Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways.

**EPHA5 mutation and DNA damage repair (DDR) gene mutation**

The gene sets associated with the DDR pathways were obtained from the Molecular Signatures Database (MSigDB) of the Broad Institute [37]. The numbers of nonsynonymous mutations in DDR pathway-related genes were compared between the EPHA5-WT subgroup and the EPHA5-MT subgroup in the ICI-treated LUAD cohort, TCGA-LUAD cohort, and GDSC-LUAD cohort.

**Statistical analysis**

The comparisons of TMB, neoantigen load, immune cell abundance, immune-related gene expression, age, pack years, MSI score and number of mutations in DDR pathway genes between the EPHA5-WT and EPHA5-MT subgroups were performed using the Mann–Whitney U test. Differences in the distributions of baseline characteristics, including gene mutation status, sex, smoking history, treatment stage, and response, between subgroups were investigated using the Chi-square test or Fisher’s exact test. Clinical outcomes, including OS, PFS, and DFS, were calculated using KM analysis and compared using the log-rank test. All \( p \)-values are two-sided, with \( p \leq 0.05 \) taken as significant. All statistical analyses were performed using R software ver. 3.6.1 (R Foundation, Vienna, Austria). The ggpubr package (https://CRAN.R-project.org/package=ggpubr) was used to generate boxplots for visualization. The maftools package [31] was used to visualize CNV, and the false discovery rate (FDR) was set to 0.05. Significant \( p \)-values are represented as *\( p \leq 0.05 \), **\( p \leq 0.01 \), ***\( p \leq 0.001 \), and ****\( p < 0.0001 \), and ns indicates no significance.

**Results**

**Association of the EPHA5 mutation status with clinical characteristics of patients with LUAD**

To identify the mutated genes that might be related to the response to ICIs in advanced LUAD patients, we first investigated genes with different mutation frequencies between patients who achieved a DCB and those who achieved NDB among all patients who received ICIs in the ICI-treated LUAD cohort. The gene mutation pattern showed that EPHA5 was one of the top 20 frequently
mutated genes in patients who achieved DCB compared to patients who achieved NDB. EPHA5 encodes an RTK involved in cancer development, and EPHA5 was found to be mutated in multiple cancers. We thus speculated that EPHA5 may be related to the response to ICIs. To verify our hypothesis, we initially analyzed the mutation characteristics of EPHA5 based on the ICI-treated LUAD, TCGA-LUAD, and TCGA-LUSC datasets. The results revealed that the mutation sites of EPHA5 spanned the entire gene, including the ligand binding site and receptor activity sites, which might affect the protein structure and function of EPHA5 (Fig. 1a). We then divided the three cohorts into the EPHA5-WT and EPHA5-MT subgroups and analyzed the correlation between the EPHA5 mutation status and clinical characteristics of patients. As shown in Fig. 1b, c, and Fig. S1, analysis of the three datasets showed that a history of tobacco exposure exhibited the strongest association with the EPHA5 mutation status among all clinical variables considered in LUAD, but not in LUSC. Interestingly, EPHA5 mutation was also associated with TMB and neoantigen load of NSCLC patients. EPHA5 mutation also showed a positive correlation with the mutation frequency of several common mutations found in LUAD, such as missense mutations in TP53, KEAP1, and LRP1B, which affect the efficacy of immunotherapies, suggesting that EPHA5 mutations may be related to the response to ICIs in patients with LUAD.

Fig. 1 Landscapes of EPHA5 gene mutations in LUAD. a Mutation sites of EPHA5 (as protein schematics) are shown. b, c Mutation patterns of EPHA5 in relation to genes associated with genomic instability and clinical characteristics of patients in the ICI-treated LUAD cohort (b) and the non-ICI-treated TCGA-LUAD cohort (c).
EPHA5 mutation is associated with the clinical benefit of ICIs in LUAD patients

Since TMB and neoantigen load serve as predictive markers of the response to ICIs, we first aimed to determine whether EPHA5 mutation in LUAD patients affects the whole TMB and alters tumor antigenicity. By analyzing the ICI-treated LUAD and non-ICI-treated TCGA-LUAD datasets, we determined that the EPHA5-MT subgroups had a higher TMB and neoantigen load (Fig. 2a), suggesting a potential relationship between mutated EPHA5 and the response to ICIs in LUAD patients. We then assessed the association of EPHA5 mutation with the prognosis of LUAD patients. The results showed that patients in the EPHA5-MT subgroup experienced longer PFS than those in the EPHA5-WT subgroup when analyzing the ICI-treated LUAD cohorts ($p = 0.039$) (Fig. 2b); however, there was no significant difference in either DFS or OS between the EPHA5-WT subgroup and the EPHA5-MT subgroup in the non-ICI-treated TCGA-LUAD cohort ($p > 0.05$) (Fig. 2c). This finding suggests that LUAD patients with EPHA5 mutations benefit from ICI treatment but not non-ICI treatment.

EPHA5 mutation is associated with high CNV counts

The acquisition of tumor aneuploidy correlates with markers of immune evasion and is associated with reduced immune-mediated cytotoxic function, and high CNV counts have been confirmed to be associated with poor clinical outcomes regarding NSCLC immunotherapy [38, 39]. Therefore, we explored the associations of the EPHA5 mutation status with CNVs at the chromosome arm level in the TCGA-LUAD cohorts. As shown in Fig. 3, we found that CNV counts were relatively lower in the EPHA5-MT subgroup than in the EPHA5-WT subgroup at 1q21.2, 1q22, 8q24.21, 9p21.3, and 14q13.3. However, CNV counts could not be validated in the ICI-treated LUAD dataset due to limited genomic coverage of the targeted panel.

EPHA5 mutation is associated with activated antitumor immunity

TILs are important markers for predicting the response to ICI treatment. We thus continued to survey the relationships between EPHA5 mutation and common immune infiltrates...
in the LUAD-TCGA cohort. Our results showed that tumor-infiltrating T lymphocytes, including CD8$^+$ T cells, CD4$^+$ activated memory T cells and macrophage M1 cells, were generally more abundant in the EPHA5-MT tumors than in the EPHA5-WT tumors, as evaluated by the expression levels of immune cell marker genes and CIBERSORT analysis (Fig. 4a, b). The immune signature analysis also revealed significantly increased expression levels of chemokines and cytolytic activity-associated gene signatures (CXCL10, CXCL9, CD8A, CD8B, and GZMB) in EPHA5 mutant tumors compared to wild-type EPHA5 tumors. We also found that the levels of LAG3, an immune checkpoint marker, were higher in the EPHA5 mutation subgroups (Fig. 4c). These results indicate that EPHA5 mutation is strongly associated with a relatively hot immune micro-environment, which firmly supports its ability to predict the response to ICIs in LUAD patients.

**EPHA5 mutation is associated with high mutation frequencies of DDR pathway genes**

Considering that DDR gene alterations are associated with the response to ICIs in tumors, we further examined the association of the EPHA5 mutation status and DDR gene mutations in both the ICI-treated LUAD cohort and the non-ICI-treated TCGA-LUAD cohort. We observed a trend towards an enrichment of DDR gene alterations in the EPHA5 mutation subgroups. Although this trend was not observed in the base excision repair (BER) pathway in the ICI-treated LUAD cohort, eight other pathways (i.e., homologous recombination (HR), mismatch repair (MMR), Fanconi anemia (FA), nucleotide excision repair (NER), nonhomologous end joining (NHEJ), DDR, DNA double-strand breaks (DSBs), and single-strand breaks (SSBs)) demonstrated enrichment for mutations in the EPHA5-MT subgroups versus the EPHA5-WT subgroups (Fig. 5).

**EPHA5 mutation and drug selection of LUAD cells**

In addition to examining the role of EPHA5 mutation in disease progression, we also investigated the role of EPHA5 mutation in chemotherapy and molecular-targeted drug therapy in LUAD patients. By analyzing the GDSC database, we found that the mutation status of EPHA5 had no effect on cell sensitivity to common chemotherapy drugs, such as cisplatin and irinotecan (Fig. 6a). Interestingly, the EPHA5-WT subgroup and the EPHA5-MT subgroup had different sensitivities to 37 molecular-targeted drugs, of
which serdemetan, lox2 and PF1-1 were relatively sensitive in the EPHA5-MT subgroup, while the other 34 drugs were more resistant in the EPHA5-MT subgroup (Fig. 6b and Fig. S2). This result may provide a reference for the treatment choice in LUAD patients with EPHA5 mutations.

Pathway enrichment analysis of EPHA5 mutation

Finally, we investigated whether EPHA5 mutation is involved in immune-related pathways using the LUAD-TCGA cohorts. GSEA showed that the insulin receptor,
negative regulation of Notch signaling, MHC II antigen presentation, regulation of p53, and NK-cell-mediated cytotoxicity pathways, which enhance antitumor immune activity in cancers, were significantly enriched in with the EPHA5-MT subgroup, while the positive regulation of the ERK1 and ERK2 cascade, PI3K cascade, FGFR2 function, and NF-kappaB signaling pathways were significantly enriched in the EPHA5-WT subgroup. In addition, consistent with previous studies showing that microRNAs (miRNAs) may play a role in regulating tumor immunity through the direct regulation of genes involved in immune activation [40], our results revealed a significant enrichment of gene silencing by miRNAs in the EPHA5-MT subgroup (Fig. 7). These results suggest that EPHA5 mutation might affect tumor immunity in LUAD patients by influencing multiple pathways.

**Discussion**

In this study, we observed that EPHA5 mutation was enriched in patients who respond to ICIs and is strongly predictive of DCB among LUAD patients treated with ICIs. Based on the analysis of the ICI-treated LUAD dataset and the non-ICI-treated TCGA-LUAD dataset, as well as the GDSC-LUAD dataset, EPHA5 mutation is significantly associated with enhanced tumor immunogenicity, activated antitumor immunity, a high mutation burden of DDR genes, and low CNV counts at multiple chromosomal arms. We also determined that LUAD patients with EPHA5 mutations are more sensitive to certain molecular-targeted drugs, such as serdemetan, lox2 and PF1-1. Furthermore, samples with EPHA5 mutations were characterized by the upregulation of signaling pathways involved in a hot immune
microenvironment, such as MHC II antigen presentation and NK-cell-mediated cytotoxicity. These results suggest that EPHA5 mutation may be a predictor of the immune response and promote the immune response in patients with LUAD.

The Eph/ephrin family is the largest known family of RTKs in mammals [41]. The Eph/ephrin family of receptors consists of nine EphA members and five EphB members classified according to sequence homology [42]. There is evidence that Eph receptors may mediate immune cell activation [18, 43], but data regarding the potential correlation between genomic alterations of Eph/ephrin family genes and the response to ICIs in LUAD patients are not available. Our present study represents the first report on the association between the response to ICIs and specific mutated genes belonging to the Eph/ephrin family in LUAD patients. Among the multiple tumor-related genes identified, we found that EPHA5 mutation was strongly associated with prolonged PFS in patients who received ICI treatment. These findings add great value to the robust link between the Eph/ephrin family and immunotherapy.

Previous studies have observed that EPHA5 is frequently mutated in several cancers, including NSCLC [25, 44, 45]. Mutations in the EPHA5 gene could impair NK-cell-mediated cytotoxicity against cancer cells [25] and predict the overall prognosis of cancer patients [46]. In addition to the effects on gene mutations, changes in EPHA5 transcription levels also affect tumor progression (e.g., enhanced drug resistance, invasion and metastatic ability of cancer cells) [47–49]. In our present study, we focused on the role of genomic alterations of EPHA5 in the response to ICIs. We examined the clinical relevance of EPHA5 alterations in the MSKCC and TCGA databases. We observed that mutated EPHA5 was beneficial for LUAD patients who received ICIs. However, for patients who did not receive ICIs, mutated EPHA5 was not significantly associated with prognosis, suggesting that EPHA5 mutation may be related to the tumor immune environment. The results of the subsequent analysis were consistent with our expectations. EPHA5 mutations are associated with a high tumor TMB, neoantigen load, T lymphocyte infiltration, and expression of immune-related signatures. EPHA5 mutations are also related to CNV in certain chromosome arms and mutations in genes related to the DDR signaling pathways. Through GSEA, we further revealed the potential mechanisms by which mutant EPHA5 regulates the immune response in LUAD. This evidence suggests that mutations in a single gene may be used to predict the response to ICIs in LUAD patients.

This retrospective analysis has several limitations. First, the relatively small number of patients treated with ICIs limits the statistical power of our analysis. Second, the ICI-treated cohort includes patients who received anti-PD-1 as well as anti-PD-L1 [anti-PD-(L)1]-based combination...
therapy. A difference in treatment may lead to a difference in response. Third, since a single targeted NGS panel was used in the ICI-treated LUAD dataset and gene expression data are lacking, this dataset could not be used to assess the correlation of EPHA5 mutations with immune cell infiltration, CNV counts and related pathways. However, we assessed the correlation of EPHA5 mutations with immune characteristics in LUAD by analyzing the non-ICI-treated TCGA-LUAD dataset. Although our study provides some interesting findings, it should still be tested in a larger and more uniformly treated cohort.

In summary, our study provides solid evidence that EPHA5 mutation is associated with a high objective response rate, good DCB, and prolonged PFS in patients receiving ICIs. Therefore, EPHA5 mutation can serve as a novel predictive biomarker for the response to ICIs in LUAD patients. Further exploration of molecular mechanisms and prospective clinical trials are warranted.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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