



Programmed death-ligand 1 expression level as a predictor of EGFR tyrosine kinase inhibitor efficacy in lung adenocarcinoma

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Background: The main objective of this study was to investigate the impact of programmed death-ligand 1 (PD-L1) expression on the efficacy of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) in patients with advanced non-small cell lung cancer (NSCLC).

Methods: This study analyzed 108 patients with NSCLC who had received EGFR-TKI as first-line systemic treatment at Seoul National University Bundang Hospital and Seoul National University Hospital between December 2012 and October 2018. The National Cancer Center Research Institute (NCCRI) and The Cancer Genome Atlas (TCGA) datasets were analyzed to investigate the mechanisms underlying EGFR-TKI-resistance in tumors with high PD-L1 expression.

Results: Among the 108 patients, 55, 37, and 16 had negative (PD-L1 Tumor proportion score <1%), weak (1–49%), and strong (≥50%) PD-L1 expression, respectively. Patients with strong PD-L1 expression had significantly shorter median progression-free survival (PFS; 7.07 months) than patients with weak (14.73 months, $P < 0.001$) or negative (12.70 months, $P = 0.001$) PD-L1 expression. After adjustment for covariates by Cox regression, PD-L1 expression remained a significant indicator of adverse prognosis. In EGFR-TKI-refractory patients, the frequency of T790M mutation and the PFS following treatment with third-generation EGFR-TKI and PD-1 antibody were similar in the three groups. TCGA and NCCRI database analysis showed that high PD-L1 expression in EGFR-mutated NSCLCs correlated with IL-6/JAK/STAT3 signaling and high *MUC16* mutation frequency.

Conclusions: Strong PD-L1 expression in tumors might be a surrogate indicator of poor response to first-line EGFR-TKIs in NSCLC patients with sensitizing EGFR mutations, and may reflect a *de novo* resistance mechanism involving JAK-STAT signaling.

Keywords: Non-small cell lung cancer (NSCLC); epidermal growth factor receptor (EGFR); programmed death-ligand 1 (PD-L1); EGFR tyrosine kinase inhibitor (EGFR TKI); JAK-STAT pathway

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Introduction

In patients with advanced non-small cell lung cancer (NSCLC) whose tumors harbor activating epidermal growth factor receptor (*EGFR*) mutations (exon 19 deletions or L858R point mutations), treatment with EGFR tyrosine kinase inhibitors (TKIs) is recommended (1-4). Patients with *EGFR*-mutated advanced NSCLC initially respond to first-generation EGFR-TKIs, such as gefitinib or erlotinib, but the majority of them usually show disease progression within one year after treatment initiation (5). The reason for acquired drug resistance is the development of a second mutation in exon 20 of the *EGFR* gene, causing a threonine (T)-to-methionine (M) substitution at codon 790 (T790M). This mutation is observed in 50–60% of the patients with acquired resistance to first-generation EGFR-TKIs (6). Although a second-generation EGFR-TKI, afatinib, has been developed to solve this issue, various clinical studies showed its limited efficacy against lung cancers resistant to first-generation EGFR-TKIs (7,8). Subsequently, a third-generation EGFR-TKI, osimertinib, has been approved for the treatment of T790M-positive lung tumors with acquired resistance to gefitinib, erlotinib or afatinib (9). More recently, the clinical practice guidelines of the National Comprehensive Cancer Network recommended osimertinib as the preferred EGFR-TKI option for first-line treatment (2).

Recent studies of cancer immunology have brought about a novel therapeutic concept in oncology. Tumor cells may escape the immune system by expressing programmed death-1 ligand (PD-L1) on their surface. The binding of tumor cell PD-L1 to programmed death-1 (PD-1) on the surface of activated T cells downregulates the immune response reducing the production and cytotoxic activity of inflammatory cytokines (10). This mechanism of immune evasion is supported by the following observations: (I) in a substantial proportion of NSCLC patients, PD-L1 expression in the tumor increases after platinum-based neoadjuvant chemotherapy, and this upregulation is associated with poor clinical outcome (11); (II) NSCLC patients with survival time shorter than 3 years after lobectomy surgery show relatively high expression of PD-L1 (12). Immune checkpoint inhibitors targeting the PD-1/PD-L1 axis represent a new paradigm for the treatment of advanced NSCLC. Recent studies have shown that PD-1/PD-L1 blockade increases the number of effector T cells, as well as the cytotoxic activity of tumor-specific T cells (13). Immune checkpoint inhibitors targeting the PD-1/PD-L1

axis revert the immune inhibitory effect of tumors, allowing immune cells to kill cancer cells. Notably, in patients with advanced NSCLC treated with immune checkpoint inhibitors, PD-L1 expression in tumor cells is a positive predictor of survival (14).

Although most patients with advanced NSCLC carrying *EGFR* mutations show a durable response to EGFR-TKIs, some of them undergo rapid disease progression or primary drug resistance. Specific features are associated with a relatively high probability to benefit from EGFR-TKIs: Asian origin, female gender, adenocarcinoma histology, and non-smoking status (15). However, the relationship between tumor cell-specific PD-L1 expression and the clinical outcome of advanced NSCLC patients treated with EGFR-TKI is unclear. We here investigated whether PD-L1 expression in tumor cells could be used to predict the duration of treatment, as well as the emergence of the T790M mutation, in patients with advanced NSCLC receiving EGFR-TKI as a first-line systemic treatment. Publicly available transcriptome datasets of lung adenocarcinoma were analyzed to interpret the findings of this study. We present the following article in accordance with the REMARK reporting checklist (available at <http://dx.doi.org/10.21037/tlcr-20-893>).

Methods

Patients

This study analyzed 108 patients with advanced NSCLC who had received EGFR-TKI as a first-line systemic treatment at Seoul National University Bundang Hospital and Seoul National University Hospital between December 2012 and October 2018 (*Figure 1*). Demographic characteristics and clinical data, including age, gender, smoking status, Eastern Cooperative Oncology Group Performance Status (ECOG PS), baseline *EGFR* mutation status, type of EGFR-TKI treatment, progression-free survival (PFS), and patient response, based on the Response Evaluation Criteria in Solid Tumors (RECIST, Version 1.1), were extracted from retrospective electronic medical records. The rate of the T790M mutation following failure of EGFR-TKI treatment, as well as PFS and patient response to third-generation EGFR-TKIs and subsequent immunotherapy, were also reviewed. This study was performed in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Seoul National University

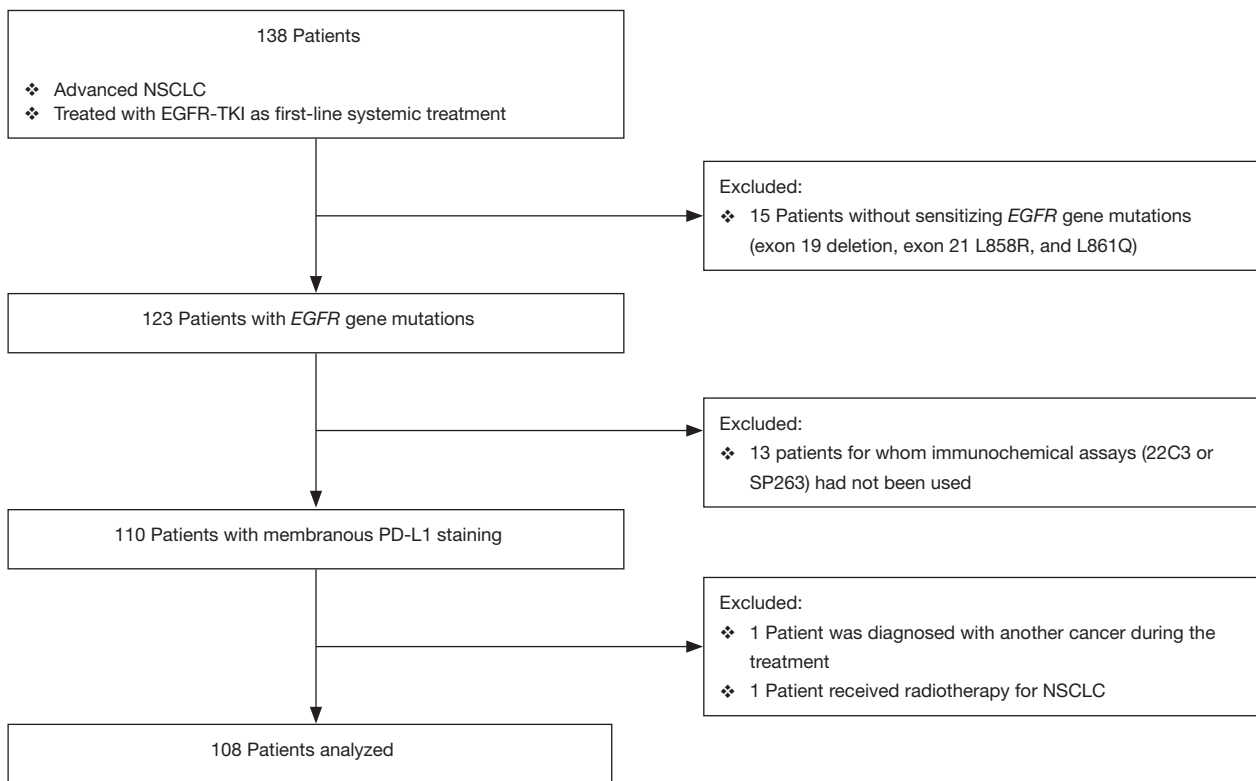


Figure 1 CONSORT diagram.

Bundang Hospital (IRB No. B-1808/487-101). Consent to participate was waived by the ethics board.

Analysis of EGFR mutations and PD-L1 expression

EGFR gene mutations were analyzed by a peptide nucleic acid (PNA) clamping-based sensitive method (PNA Clamp™ or PANAMutyper™ *EGFR* mutation detection kit) in formalin-fixed paraffin-embedded tissues (16-18). *EGFR*-activating mutations were defined as mutations associated with *EGFR*-TKI sensitivity, including exon 19 deletion, exon 21 L858R, and L861Q.

Diagnostic assays based on PD-L1 immunohistochemistry were performed according to the manufacturer's instructions. The following antibodies and detection systems were utilized: mouse monoclonal primary anti-PD-L1 antibody, 22C3 pharmDx (prediluted, clone 22C3, Dako, Carpinteria, CA, USA) with an Autostainer Link 48 and an EnVision DAB Detection System (Agilent Technologies, Santa Clara, CA, USA); rabbit monoclonal primary anti-PD-L1 antibody, Ventana SP263 (prediluted, Ventana Medical Systems, Tucson, AZ, USA) with a Benchmark XT staining system and

Ultra with OptiView Universal DAB Detection Kit (Ventana Medical Systems). The staining results were interpreted by expert lung pathologists. The percentage of tumor cells with membranous PD-L1 staining of any intensity (tumor proportion score, TPS) was categorized into three ranges and the corresponding groups (<1%, negative expression; 1-49%, weak expression; ≥50%, strong expression).

Analysis of open source databases of RNA expression

To evaluate the impact of PD-L1 expression on the efficacy of *EGFR*-TKI treatment in patients with lung adenocarcinoma carrying *EGFR* mutations, we used two publicly available transcriptome datasets of lung adenocarcinoma: the National Cancer Center Research Institute (NCCRI) dataset and The Cancer Genome Atlas (TCGA) dataset (19,20). The NCCRI dataset was downloaded from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>, accession number GSE31210), and the *EGFR* mutational status of patients in the NCCRI dataset was available from the literature. The TCGA transcriptome dataset and curated mutation data were

Table 1 Baseline characteristics of patients

Characteristic	Number of patients (%)
Age	
Median	65
Range	34–84
Sex, n (%)	
Female	71 (65.7)
Male	37 (34.3)
Smoking history, n (%)	
Never smoker	73 (67.6)
Current/former smoker	35 (32.4)
ECOG PS, n (%)	
<2	96 (88.9)
≥2	12 (11.1)
Brain metastases at diagnosis, n (%)	
No	45 (41.7)
Yes	63 (58.3)
EGFR mutation, n (%)	
Exon 19 deletion	60 (55.6)
Exon 21 L858R	48 (44.4)

ECOG PS, Eastern Cooperative Oncology Group Performance Status; EGFR, epidermal growth factor receptor.

downloaded from the cBioPortal (<https://www.cbioportal.org/>) (21). Expression values were log₂-normalized to genes using Cluster 3.0 software (22).

The PD-L1 status of samples was classified according to the level of *CD274* (PD-L1) mRNA expression; samples with upper 50% values throughout the whole samples were defined as PD-L1-high, and the remaining lower 50% values were defined as PD-L1-low (23). Then, synonymous and benign mutations were excluded, and samples with potentially pathogenic *EGFR* mutations were selected. Only samples for which transcriptomic data were available and the EGFR mutational status was known were included in the analysis.

To compare pathway enrichment in PD-L1-high and PD-L1-low samples, Gene Set Enrichment Analysis was performed (GSEA_4.0.2, <http://software.broadinstitute.org/gsea/index.jsp>) (24,25). Hallmark gene sets from the Molecular Signatures Database (MsigDB) were analyzed (26). In the GSEA analysis, pathways with nominal

P value <0.05 and FDR q value <0.25 were considered as significantly enriched.

Statistical analysis

The correlation between tumor PD-L1 expression and other patient characteristics was analyzed by either chi-square test or Fisher's exact test. PFS was defined as the time from the initiation of EGFR-TKI treatment to the detection of disease progression or any cause of death, and was evaluated with the Kaplan-Meier method. The log-rank test was applied to compare the cumulative survival time between patient groups. Multivariate analysis was performed using a logistic regression model incorporating age, sex, smoking history, ECOG PS, status of brain metastasis at diagnosis, and PD-L1 expression. The mutational profiles of PD-L1-high and PD-L1-low patients were compared by using the TCGA dataset. To establish whether a certain gene was more frequently mutated in a specific group, Fisher's exact test was applied. All statistical analyses were performed with R software version 3.6.0 (<https://www.r-project.org/>), and P values <0.05 were considered indicative of statistical significance.

Results

Outcome of patients treated with EGFR-TKI as a first-line systemic therapy

A total of 108 patients were included in the analysis; the demographic and clinical characteristics of participants are shown in *Table 1*. The median age was 65 years (range, 34–84 years), 71 (65.7%) patients were female, 73 (67.6%) never-smokers, 96 (88.9%) displayed ECOG PS <2, and 45 (41.7%) had no brain metastases when diagnosed. In addition, 60 (55.6%) patients presented exon 19 deletions and 48 (44.4%) exon 21 deletions. All patients had been treated with EGFR-TKI as appropriate.

Among the 108 lung adenocarcinoma specimens analyzed, 55 (50.9%), 37 (34.3%), and 16 (14.8%) exhibited negative (TPS <1%), weak (1–49%), and strong (≥50%) PD-L1 expression, respectively. PD-L1 expression was not correlated with age, sex, smoking history, ECOG PS, presence of brain metastasis at diagnosis, type of EGFR mutation, or type of EGFR-TKI used (*Table 2*).

In univariate analysis, the median PFS of EGFR-TKI-treated patients was 12.70 months (95% CI, 9.66–15.74 months) for patients with negative PD-L1

Table 2 Association between PD-L1 expression and clinicopathologic features

PD-L1 expression	Negative (n=55, 100%)	Weak (n=37, 100%)	Strong (n=16, 100%)	P value
Age				0.293
<65	29 (52.7)	14 (37.8)	9 (56.25)	
≥65	26 (47.3)	23 (62.2)	7 (43.75)	
Sex				0.331
Female	38 (69.1)	21 (56.8)	12 (75.0)	
Male	17 (30.9)	16 (43.2)	4 (25.0)	
Smoking history				0.164
Never smoker	39 (70.9)	21 (56.8)	13 (81.3)	
Current/former smoker	16 (29.1)	16 (43.2)	3 (18.8)	
ECOG PS				0.208
<2	51 (92.7)	30 (81.1)	15 (93.8)	
≥2	4 (7.3)	7 (18.9)	1 (6.3)	
Brain metastases at diagnosis				0.764
No	22 (40.0)	15 (40.5)	8 (50.0)	
Yes	33 (60.0)	22 (59.5)	8 (50.0)	
Oncogenic driver				0.560
Exon 19 del	33 (60.0)	18 (48.6)	9 (56.3)	
Exon 21 L858R	22 (40.0)	19 (51.4)	7 (43.8)	
First-line EGFR-TKIs				0.071
Gefitinib	30 (54.5)	11 (29.7)	6 (37.5)	
Erlotinib	23 (41.8)	24 (64.9)	10 (62.5)	
Afatinib	2 (3.6)	2 (5.4)	0 (0.0)	
Responses to first-line EGFR-TKI				0.133
Partial response	42 (76.4)	29 (78.4)	13 (81.3)	
Stable disease	7 (12.7)	7 (18.9)	0 (0.0)	
Progressive disease	6 (10.9)	1 (2.7)	3 (18.8)	
2nd Mutation				0.838
T790M	21 (38.2)	13 (35.1)	7 (43.8)	
Subsequent treatment				<0.001
Immunotherapy	7 (12.7)	2 (5.4)	9 (56.3)	
Response rate ^a				
EGFR-TKIs	42/55 (76.4)	29/37 (78.4)	13/16 (81.3)	1.000
Third generation EGFR-TKI	6/23 (26.1)	3/10 (30.0)	4/7 (57.1)	0.335
Immune checkpoint inhibitors	0/6 (0.0)	0/2 (0.0)	1/7 (14.3)	1.000

^a, number of patients with partial response/total number of patients. PD-L1, programmed death-ligand 1; ECOG PS, eastern cooperative oncology group performance status; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

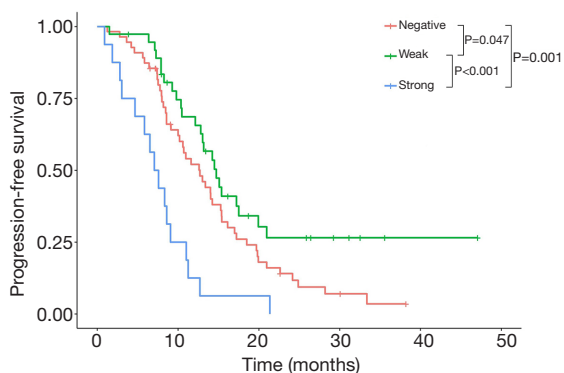


Figure 2 Kaplan-Meier curve of progression-free survival in patients receiving first-line EGFR-TKI therapy. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

expression, 14.73 months (95% CI, 12.13–17.33 months) for patients with weak PD-L1 expression, and 7.07 months (95% CI, 4.98–9.16 months) for patients with strong PD-L1 expression (log rank; negative *vs.* weak PD-L1 expression, $P=0.047$; negative *vs.* strong PD-L1 expression, $P=0.001$; weak *vs.* strong PD-L1 expression, $P<0.001$) (Figure 2). Patients with strong PD-L1 expression had a significantly shorter PFS compared to those with weak or negative PD-L1 expression.

When adjusted for age, smoking history, sex, ECOG PS, and status of brain metastasis at diagnosis in the Cox regression model, PD-L1 expression remained a significant prognostic indicator of short-term PFS (hazard ratio for negative *vs.* strong PD-L1 expression, 2.788; 95% CI, 1.536–5.059; $P=0.001$) (Table 3).

Early progression (within 6 months after first-line EGFR-TKI treatment) rates were 12.7%, 2.7%, and 37.5% in patients with negative, weak, and strong PD-L1 expression respectively (Fisher's exact test; negative *vs.* weak *vs.* strong PD-L1 expression, $P=0.004$). Notably, most cases of early progression were observed among patients with high PD-L1 expression (Figure 3).

There were no statistically significant PD-L1 expression-related differences in the best treatment response of EGFR-TKI, based on RECIST 1.1 criteria (negative *vs.* weak *vs.* strong PD-L1 expression, $P=0.133$) (Table 2). However, the disease control rate (partial response + stable disease) was numerically lower in strong PD-L1 expression group (81.3%) compared to those of negative (89.1%) and weak (97.3%) group.

Acquired resistance to first-line EGFR TKI treatment

Of 108 patients with advanced NSCLC who had received

with EGFR-TKI as first-line systemic treatment, biopsies were performed again in 66 patients after showing acquired resistance. The rate of T790M mutation was analyzed to identify possible differences related to PD-L1 expression. Among EGFR-TKI-refractory patients, the rate of T790M mutation was between the three PD-L1 expression groups (38.2% in negative, 35.1% in weak, and 43.8% in strong PD-L1 expression group; $P=0.838$). The rate of T790M mutation was associated with neither subtype of EGFR mutation or pretreated EGFR-TKIs (Tables S1,S2).

Among patients treated with third-generation EGFR-TKI ($n=47$), the median PFS was 6.63, 8.13, and 9.77 months for negative, weak and strong PD-L1 expression group respectively. However, these differences were not statistically significant (log rank test: negative *vs.* weak PD-L1 expression, $P=0.987$; negative *vs.* strong PD-L1 expression, $P=0.710$; weak *vs.* strong PD-L1 expression, $P=0.516$) (Figure S1). Moreover, no statistically significant differences related to PD-L1 expression were detected in the response to third-generation EGFR-TKI, based on RECIST 1.1 criteria (negative *vs.* weak *vs.* strong PD-L1 expression, $P=0.530$).

Outcome of patients treated with immune checkpoint inhibitors following EGFR-TKI failure

After EGFR-TKI treatment failure, 18 patients were treated with anti-PD-1 antibody (Nivolumab; $n=11$, Pembrolizumab; $n=7$). The median PFS after immune checkpoint inhibition was 1.33 months in negative, 0.43 months in weak, and 1.53 months in strong PD-L1 expression group (log rank; negative *vs.* weak PD-L1 expression, $P=0.326$; negative *vs.* strong PD-L1 expression, $P=0.559$; weak *vs.* strong PD-L1 expression, $P=0.138$) (Figure S2). At six months, the PFS rate was highest in patients with strong PD-L1 expression (negative PD-L1 expression, 16.7%; weak PD-L1 expression, 0%; strong PD-L1 expression, 41.7%). However, based on RECIST (Version 1.1), the level of PD-L1 expression did not significantly affect the response to immune checkpoint inhibitors ($P=0.736$).

Use of public databases to explore the mechanism of EGFR-TKI resistance

A total of 127 (50 PD-L1-high) and 64 (25 PD-L1-high) samples of lung adenocarcinoma carrying the EGFR

Table 3 Multivariate analysis of clinicopathological features for progression-free survival

Factors	HR	95% CI	P value
Age			
<65	Reference		
≥65	1.105	0.668–1.827	0.436
Sex			
Male	Reference		
Female	1.105	0.668–1.827	0.697
Smoking history			
Never smoker	Reference		
Current/former smoker	0.921	0.553–1.534	0.752
ECOG PS			
<2	Reference		
≥2	1.548	0.715–3.351	0.268
Brain metastases at diagnosis			
No metastasis	Reference		
Metastasis	0.994	0.640–1.544	0.977
PD-L1 expression			
Strong (≥50%)	Reference		
Weak (1–49%)	0.202	0.101–0.401	<0.001
Negative (<1%)	0.359	0.198–0.651	0.001

ECOG PS, Eastern Cooperative Oncology Group Performance Status; PD-L1, programmed death-ligand 1.

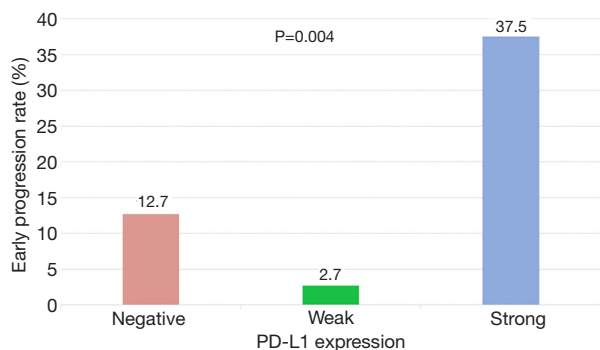


Figure 3 Early progression rate according to PD-L1 expression. PD-L1, programmed death-ligand 1.

mutation were analyzed in the NCCRI and TCGA dataset, respectively. GSEA analysis revealed a significant enrichment of 14 (Table S3) and 8 (Table S4) pathways in PD-L1-high tumors, according to the NCCRI and the

TCGA dataset, respectively. Among these pathways, 8 were significantly enriched in both datasets and most of them were related to immune functions (Figure 4A,B). Notably, the “HALLMARK_IL6_JAK_STAT3_SIGNALING” pathway, which is known to be associated with EGFR-TKI resistance, was consistently and highly enriched in both datasets (normalized enrichment scores of 1.73 and 1.69 in the NCCRI and TCGA dataset, respectively, Figure 4C,D) (27,28).

The comparison of mutational profiles in PD-L1-high and PD-L1-low tumors allowed to identify 26 genes with significantly higher mutation rate in one of the two groups (Table S5). Notably, PD-L1-high samples exhibited a higher frequency of the *MUC16* mutation compared to the other samples (44.0% vs. 30.8%, P=0.034, Figure 4E), which is involved in tumorigenesis and metastasis of lung cancer cells through the regulation of the JAK2-STAT3 axis (29).

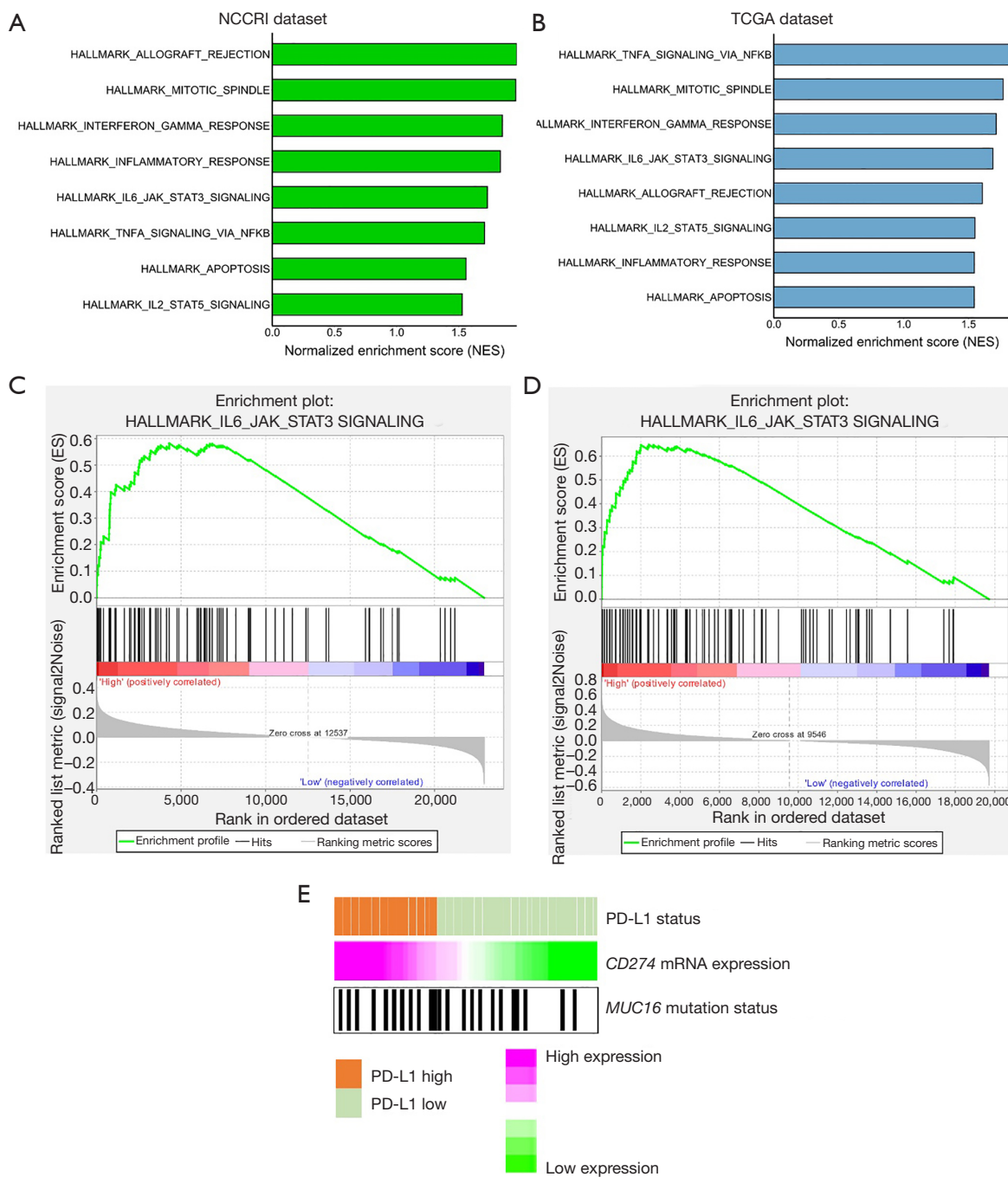


Figure 4 Open-source database analysis focusing on the mechanism of EGFR-TKI resistance in PD-L1-high lung adenocarcinoma. (A) Bar plot showing normalized enrichment score in the NCCRI dataset. The 8 pathways enriched in both the NCCRI and TCGA datasets are shown. (B) Bar plot showing the normalized enrichment score in the TCGA dataset. The 8 enriched pathways common to both datasets are shown. (C) GSEA enrichment plot of the “HALLMARK_IL6_JAK_STAT3_SIGNALING” pathway in the NCCRI dataset. (D) GSEA enrichment plot of the “HALLMARK_IL6_JAK_STAT3_SIGNALING” pathway in the TCGA dataset. (E) Graph showing the status of PD-L1 expression and *MUC16* mutation in each sample. Each column represents one sample; the columns are arranged in order of decreasing *CD274* (PD-L1) mRNA expression. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PD-L1, programmed death-ligand 1; NCCRI, the national cancer center research institute; TCGA, the cancer genome atlas; GSEA, gene set enrichment analysis; JAK, Janus kinase; STAT, signal transducer and activator of transcription; mRNA, messenger ribonucleic acid.

Discussion

In this study, we demonstrated that high PD-L1 expression was associated with poor clinical outcome in patients treated with first-line EGFR-TKI. Early cancer progressors with high PD-L1 expression didn't have significantly more frequent T790M resistance mutation compared to those with low PD-L1 expression.

To explore the mechanism underlying the different efficacy of EGFR-TKI in patients with PD-L1-high and PD-L1-low, *EGFR*-mutated lung adenocarcinoma, public datasets were employed. A comprehensive analysis of TCGA and NCCRI databases consistently showed that high PD-L1 expression in *EGFR*-mutated lung adenocarcinoma was correlated with the activation of the IL-6/JAK/STAT3 pathway, as well as with a high frequency of *MUC16* mutation.

The association between high PD-L1 expression and *de novo* resistance to EGFR-TKI of lung adenocarcinoma has been described by previous studies (30-32). The proportion of PD-L1 positive patients ranged between 45.5% and 66.7% among those with primary resistance to EGFR-TKI, and between 12.3% and 30.2% among those with disease control or acquired resistance (30,31). In addition, it was reported that, after treatment with EGFR-TKI, patients with PD-L1 expression $\geq 50\%$ exhibited median PFS of 1.6, 3.8, and 5.9 months, while median PFS of 7.3, 9.5, and 12.5 months were observed in patients with PD-L1 expression $< 1\%$, respectively (30-32), which was consistent with our findings. More recently, FLAURA trial (33) reported that, after first-line treatment with gefitinib or erlotinib, median PFS was 6.9 months in patients with PD-L1 expression $\geq 1\%$, while median PFS was 10.9 months in patients with PD-L1 expression $< 1\%$, which was consistent with our results. However, interestingly, after first-line treatment with osimertinib, median PFS was unaffected by PD-L1 expression status.

One of the above-mentioned studies demonstrated the efficacy of immune checkpoint inhibitors in the treatment of *EGFR*-mutated PD-L1 high tumors, as these tumors are highly positive to CD8+ T cells (30,34); moreover, one of these patients showed PFS longer than 5 months following treatment with an immune checkpoint inhibitor (30). Although the level of PD-L1 expression did not seem to significantly affect PFS in EGFR-TKI-resistant patients that had been treated with immune checkpoint inhibitor, some patients with high tumor expression of PD-L1 did show more favorable outcomes,

with PFS > 6 months, suggesting that subsets of PD-L1-high patients might benefit from treatment with immune checkpoint inhibitors. Therefore, although immunotherapy showed limited overall efficacy against *EGFR*-mutated lung adenocarcinoma (35,36), the specific effects of the immune check point inhibitors in patients with high PD-L1 expression may deserve further investigation.

In addition, immune checkpoint inhibitor in combination with platinum-based chemotherapy may be a possible therapeutic option in EGFR-TKI-resistant patients as the recent IMpower 150 trial reported that chemo-immunotherapy plus bevacizumab improved PFS when compared to chemotherapy alone in EGFR-mutant patients who had progressed on a EGFR-TKI therapy (37). The IMpower 130 trial failed to demonstrate a clinical benefit for the addition of immune checkpoint inhibitor to chemotherapy without bevacizumab in EGFR-mutant NSCLC (38). Clinical benefit between immune checkpoint inhibitor alone and in combination with chemotherapy in EGFR-TKI-resistant patients is not yet studied well.

We showed that the rate of T790M mutation in EGFR-TKI-resistant patients did not significantly differ between groups with different levels of tumor PD-L1 expression. This finding contrasts with a previous report (32), showing a higher frequency of the mutation in PD-L1-negative compared to PD-L1-high patients. This contradiction could be due to differences in the number of patients who underwent a re-biopsy to verify the acquisition of resistance, as well as to the application of distinct first-line EGFR-TKI regimens although previous studies focusing on the rate of T790M acquisition after treatment with various EGFR-TKIs have reported inconsistent results (39,40). However, we observed that treatment with third-generation EGFR-TKIs resulted in similar PFS in the three PD-L1 expression groups, and the data were comparable to those of a previous clinical trial of 2nd-line osimertinib (9). These findings implicate that although PD-L1 expression may indicate the occurrence of *de novo* resistance to *EGFR* pathway inhibitors, the T790M mutation may still be acquired, and in that case *EGFR* pathway inhibition may still be effective.

A possible mechanism of *de novo* resistance to EGFR-TKI in patients with PD-L1 high lung adenocarcinoma with *EGFR* mutation has been previously suggested, involving *MET* amplification and *KRAS* mutations (30). In this study we showed that PD-L1 high *EGFR* mutant samples were significantly enriched with JAK-STAT pathway and *MUC16* mutation using NCCRI and TCGA datasets. The JAK-STAT pathway mediates cytokine signaling and involved

in promoting cell proliferations (41,42). While JAK-STAT pathway upregulates PD-L1 expression (43,44), JAK-STAT pathway was also shown to be associated with resistance to EGFR mutant lung cancer in a preclinical study (28). In addition, *MUC16* mutation is associated with JAK-STAT3 pathway in tumorigenesis and metastasis (29). Altogether, these findings suggest that PD-L1 expression may indicate the activation of JAK-STAT pathway which can act as a *de novo* resistance mechanism to EGFR-TKI.

Combinations of EGFR-TKIs and anti-PD-1 antibodies to treat PD-L1-high, *EGFR*-mutated lung adenocarcinoma are being currently tested by clinical trials. However, treatment failure has frequently been reported due to the elevated incidence of pulmonary and hepatic toxicity (45). In light of our findings, combination therapies based on the inhibition of both EGFR and JAK-STAT pathways may prove effective for selected patients with PD-L1-high, *EGFR*-mutated lung adenocarcinoma, especially those developing primary resistance to first-line EGFR-TKI therapy. Preclinical studies have already demonstrated that JAK2 inhibition sensitizes resistant *EGFR* mutant lung adenocarcinoma to EGFR-TKI (46). A phase 1b clinical trial demonstrated that the combination of ruxolitinib, a JAK inhibitor, and afatinib is well tolerated but show modest therapeutic efficacy, with an overall response rate of 23.3% (47). Another clinical trial is ongoing to evaluate the efficacy of the combination of itacitinib, a JAK1 inhibitor, and osimertinib (ClinicalTrials.gov Identifier: NCT02917993). Should the efficacy of these combination therapies be confirmed, our study may pave the way for the use of PD-L1 expression as a new biomarker to assess the effect of these treatments.

Conclusions

This study has some limitations mostly derived from a retrospective analysis. However, our findings were consistent with previous studies and supported by genomic and transcriptomic analysis of open-source databases. The antibodies to test PD-L1 expression were not identical throughout patients, although the two assays used in this study (22C3 and SP263) had been reported to have fair concordance rates (48).

In conclusion, we demonstrated that PD-L1 strong expression predicts poor response to first-or second-generation EGFR-TKI in treatment naïve advanced NSCLC, and that PD-L1 expression may indicate underlying *de novo* resistance mechanism including JAK-

STAT pathway. To illustrate the association of PD-L1 expression and resistance to EGFR-TKI more in depth, comprehensive genomic and transcriptomic analyses on these tumors along with annotated clinical features and treatment outcome data are warranted.

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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 study was performed in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the

Institutional Review Board of Seoul National University Bundang Hospital (IRB No. B-1808/487-101). Consent to participate was waived by the ethics board.

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