Original Article

The differential prognostic implications of PD-L1 expression in the outcomes of Filipinos with *EGFR*-mutant NSCLC treated with tyrosine kinase inhibitors

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Background: The tumor immune microenvironment influences tumor evolution in non-small cell lung cancer (NSCLC). Yet, the prognostic value of programmed death-ligand 1 (PD-L1) in epidermal growth factor receptor (EGFR)-mutant NSCLC remains controversial. Additionally, prognostic studies in Filipinos with EGFR-mutant NSCLC remain unexplored to this day.

Methods: We prospectively studied the outcomes of EGFR-mutant NSCLC in Filipino cohort, and retrospectively verified the survival trend using The Cancer Genome Atlas (TCGA) cohort. Kaplan-Meier method and generalized linear regression were used to assess survival. Expression and DNA methylation of cluster of differentiation 274 (*CD274*, gene that codes for PD-L1) were examined from TCGA tumor profiles. Pearson's correlation was used to correlate PD-L1 expression with outcomes associated with occurrence of EGFR mutations, tyrosine kinase inhibitor (TKI) types, and programmed cell death protein 1 (PD-1) expression. Proteome network analysis was used to examine the correlation between drug resistance and PD-L1.

Results: PD-L1 positivity was associated with significantly longer progression-free survival (PFS; P=0.0096) but had a significantly contrasting influence in the overall survival (OS; P=0.0011). PD-L1 positivity (in both protein and RNA) was associated with longer median OS (mOS) in exon21 L858R, whereas, negativity was associated with longer mOS in exon19 deletion (exon19del). Stratification (high, low, negative) of PD-L1 expression lacked significant prognostic value (all P>0.05). PD-L1/*CD274* expression (P<0.05) and DNA methylation (P<0.001) vary significantly among NSCLC subtypes and in different disease stages. Erlotinib treatment produced the longest median progression-free survival (mPFS; 874 days) relative to other EGFR-TKIs (137–311 days). PD-L1 lacked a significant correlation with EGFR-TKIs. Consistent with the immune-regulation activities of PD-1, higher expression leads to relatively shorter mOS. PD-1 correlated positively with PD-L1 expression and occurrence of exon21 L858R.

Conclusions: PD-L1 differentially influenced the outcomes of Filipinos with EGFR-mutant NSCLC. NSCLC subtypes, disease stage, and PD-1 expression may impact the collective outcomes associated with PD-L1 and EGFR-sensitizing mutations.

Keywords: Programmed death ligand-1 (PD-L1); non-small cell lung cancer (NSCLC); epidermal growth factor receptor mutations (*EGFR* mutations); Filipinos NSCLC; EGFR tyrosine kinase inhibitor (EGFR-TKI)

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Introduction

The high morbidity and mortality rates of lung cancer remain as global health problems. Lung cancer is the second most commonly diagnosed cancer and is the leading cause of cancer-related deaths worldwide (1,2). About 85% of lung neoplasms are non-small cell lung cancer (NSCLC) (3,4), with around 70% of patients already progressed to metastatic late stage (III and IV) upon diagnosis (5,6). Metastatic NSCLC normally has poor prognosis with about 5-year survival rate of 5% to 15% without targeted therapy (7-9).

Detection of driver mutations in the epidermal growth factor receptor (*EGFR*) gene has become a standard practice in lung cancer pathology (4,10,11). Approximately 32.3% of NSCLC are EGFR mutant (12), with aberrations occurring more frequently in the tyrosine kinase domain, such as exon19 deletion (exon19del) and exon21 L858R (13,14). Consequently, the discovery of tyrosine kinase inhibitor (TKI) drugs revolutionized the treatment landscape of EGFR mutant NSCLC. Patients treated with EGFR-TKIs have shown prolonged progression-free survival (PFS), better treatment tolerance, lower drug toxicity, and improved quality of life compared to patients who received conventional chemotherapies (10,11,15). Despite the positive clinical outcomes of EGFR-TKIs, clinical data showed that most NSCLC acquire resistance after 9–12 months of treatment with first- and second-generation TKIs, and 30–50% of these cases were due to the development of T790M mutation (16-19).

While the general objective of EGFR-TKIs is to counteract EGFR-driven cancer progression and induce cell-death, tumor eradication is partly but effectively contributed by efficient immune surveillance. The expression of immune-checkpoint protein, programmed death ligand-1 (PD-L1) or *CD274*, by tumors is a well-studied mechanism by which cancer evades immune destruction. Recent reports suggest that PD-L1 may play a role in the evolution of TKI-resistant NSCLC subclones. In fact, some studies demonstrated that PD-L1 expression correlate positively with the presence of EGFR mutations in NSCLC (20-22). PD-L1 has also been found to strongly associate with higher incidence of primary resistance to EGFR-TKIs (23). In addition, strong PD-L1 expression by naive advanced EGFR-mutants was associated with poor prognoses in Osimertinib-based first line therapy (24).

While a number of clinical studies report the prognostic significance of PD-L1 expression (20,22-26), some did not find correlation between PD-L1 and EGFR-TKI responses (19,27). Conflicting results from different population studies were reviewed elsewhere (28). With the high prevalence of EGFR-TKI resistance among NSCLC and the growing interest for immune checkpoint inhibitors, the prognostic implications of PD-L1 in EGFR-mutant NSCLC warrants further elucidation. The clinical correlations between PD-

Highlight box

Key findings

- Programmed death-ligand 1 (PD-L1) immunopositivity was associated with longer progression-free survival (PFS) in Filipinos with epidermal growth factor receptor (EGFR)-mutant non-small cell lung cancer (NSCLC).
- PD-L1 immunopositivity may prognose longer overall survival (OS) in patients with exon21 L858R, but not those with exon19 deletion.

What is known and what is new?

- PD-L1, an immune checkpoint protein, downregulates the anti-tumor activity of the immune system. Yet, different population studies report the inconsistent prognostic value of PD-L1 in NSCLC.
- PD-L1 expression differentially influences the outcomes of Filipinos with EGFR-mutant NSCLC. Disease subtypes, stages, and programmed cell death protein 1 (PD-1) expression levels may impact the collective outcomes associated with PD-L1 and EGFR mutations.

What is the implication, and what should change now?

- More standardization towards comprehensive molecular profiling and tumor biomarker analysis would benefit treatment outcomes of NSCLC patients. Further studies on identifying more biomarkers to predict the emergence of drug resistant NSCLC would help curb the growing number of lung cancer cases globally.

L1 and EGFR mutations among Filipinos with NSCLC are currently unknown. Therefore, we conducted a prospective study and performed an observational study on the prognostic significance of PD-L1 expression in disease progression, survival, and treatment response of NSCLC in Filipino cohort. We subsequently verified our findings using the survival data of NSCLC patients from The Cancer Genome Atlas (TCGA) cohort. We present this article in accordance with the REMARK reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-118/rc).

Methods

Study design and patients

This multicenter study was conducted in three institutions (Lung Center of the Philippines, National Kidney and Transplant Institute, and East Avenue Medical Center) in the Philippines. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Review Board (protocol number LCP-CS-001-2019) of the Lung Center of the Philippines. All participating hospitals/institutions were informed and agreed the study. All patients provided written informed consent for genetic testing, as well as use of their clinical data.

Forty-two Filipino patients with pathology-confirmed NSCLC at stage III to IV enrolled from April 2019 to May 2022 were prospectively studied. Participants were treated with tyrosine kinase inhibitors (gefitinib, erlotinib, afatinib, and osimertinib) based on physician's choice. Disease progression, overall survival (OS), and treatment response were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 (29). The following clinical data were collected: age, sex, smoking status (non-smoker, first-hand, second-hand smoker), clinical stage, type of EGFR mutation, presence of brain metastasis at baseline, type of TKI treatment, and PD-L1 tumor proportion scores (TPS) by immunohistochemistry (IHC). Observational endpoints were measured in terms of PFS and OS.

TCGA cohort

Clinical and survival data of NSCLC patients from the TCGA-lung adenocarcinoma (LUAD) and TCGA-lung squamous cell carcinoma (LUSC) projects of TCGA Research Network (https://www.cancer.gov/tcga) were retrospectively obtained through the GDC Data Portal (https://gdc.cancer.gov). A total of 45 patients with PD-L1 and EGFR mutation (inframe deletion in exon19 and L858R substitution in exon21) profiles were included in TCGA cohort. RNA-seq abundance reads [in fragments per kilobase million (FPKM)] of PD-L1 and programmed cell death protein 1 (PD-1) of patients in TCGA cohort were retrieved through the Pathology section of The Human Protein Atlas (https://www.proteinatlas.org). The median FPKM (1.7 and 2.6 for exon21 L858R and exon19del, respectively) cutoff was used to categorize expression levels into high (> median), low (≤ median), and none (0 FPKM). Comparison of *CD274* expression levels among tumors and normal tissues were achieved by comparing RNA-seq data (in TPM) from TCGA (LUAD and LUSC projects) with the Genotype-Tissue Expression (GTEx) cohort data through Gene Expression Profiling Interactive Analysis (GEPIA) web tool (http://gepia.cancer-pku.cn). *CD274* gene methylation profiles of normal and tumor tissues from TCGA (LUAD and LUSC projects) were characterized using the CpG-aggregated mean Beta methylation values, and the correlation between *CD274* expression and methylation was analyzed using Pearson's correlation through the SMART web tool (http://www.bioinfo-zs.com/smartapp/). Proteins involved in EGFR-TKI resistance and PD-L1 expression in NSCLC were identified using Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp) and the analysis of functional protein network was performed using STRING (https://string-db.org). Statistical analysis for each analysis were describe in figure legends.

Tissue collection and processing

The protocol for specimen collection was approved by the Single Joint Research Ethics Board (SJREB-2020-97). Blood samples (5–10 mL) were collected from recruited Filipino NSCLC patients (before treatment and during therapy upon follow-up every 3 months), and were immediately processed for plasma testing or cryopreservation at –80 ℃. Tumor tissue and adjacent normal tissue samples (>2 cm from cancer tissue edge) were taken during surgery. The tumor tissue samples were confirmed by a pathologist to be NSCLC pre-operatively by upfront lobectomy or intra-operatively by frozen section during surgery. The tissue samples were cut into pieces (at least 2–3 pcs, size >0.5×0.5 cm^2 per piece). The tissue specimens were immediately (less than 20 min after surgical removal) stored in liquid nitrogen.

Table 1 Clinicopathologic characteristics of NSCLC patients enrolled in the study

Characteristics	Frequency (N=30)
Age, years, median [range]	62 [38–87]
Sex, n (%)	
Male	6 (20.0)
Female	24 (80.0)
Smoking status, n (%)	
Non-smoker	14 (46.7)
First-hand	4 (13.3)
Second-hand	12 (40.0)
Stage (upon diagnosis), n (%)	
IIIB	2 (6.7)
IV	28 (93.7)
Brain metastasis at baseline, n (%)	
Yes	10 (33.3)
No	17 (56.7)
Unknown	3 (10.0)
EGFR mutations, n (%)	
Exon19del	17 (56.7)
Exon21 L858R	13 (43.3)
TKI treatment, n (%)	
Afatinib	3 (10.0)
Gefitinib	15 (50.0)
Erlotinib	8 (26.7)
Osimertinib	4 (13.3)
PD-L1 expression, n (%)	
Negative	12 (40.0)
Positive	18 (60.0)
Low TPS	10 (33.3)
High TPS	8 (26.7)

NSCLC, non-small cell lung cancer; *EGFR*, epidermal growth factor receptor; exon19del, exon19 deletion; TKI, tyrosine kinase inhibitor; PD-L1, programmed death ligand-1; TPS, tumor proportion score.

EGFR mutation and PD-L1 expression test

EGFR mutation of tumor tissues were assessed using the AmoyDx EGFR 29 Mutations Detection Kit, performed at the Pathology and Laboratory Medicine Department of the Lung Center of the Philippines. In brief, genomic DNA was extracted from sections of tissue biopsy. Approximately 10 nanograms of DNA was added into the polymerase chain reaction (PCR) mix with primers designed to amplify and detect mutations in exons 19–21 of the *EGFR* gene. Cut-off values, quality control assessments, and test results were evaluated using manufacturer's guidelines. Expression of PD-L1 by NSCLC was evaluated by IHC using the pharmDx 22C3 kit, described previously (25). At least two qualified pathologists confirmed the IHC results. PD-L1 expression was classified as negative, low, and high if the TPS of PD-L1 were <1%, 1–49%, and >50%, respectively.

Statistical analysis

Descriptive statistics (frequency analysis) were used to summarize the sociodemographic and clinical characteristics of the cohort. The median progression-free survival (mPFS) and median OS (mOS) were analyzed using the Kaplan-Meier method. The log-rank test was used to compare survival curves of patient groups. A generalized linear regression model was used to predict 1-year survival (PFS and OS) rates as previously described (30,31). The elapsed days (x-axis) were plotted against the proportion of survival (y-axis), and the estimation of 1-year PFS rates was derived using the equation of the predicted line. Correlations of clinical responses and survival between variables were analyzed using Pearson's correlation. All analyses were performed using GraphPad Prism 9 (Graph Pad, USA), NCSS 2022 (NCSS, LLC., USA), and JASP (University of Amsterdam, The Netherlands). A P value <0.05 was considered statistically significant.

Results

Characteristics of the cohorts

In total, 42 Filipino participants were enrolled and after molecular profiling, data from 30 patients were analyzed. Their clinicopathologic characteristics are shown in *Table 1*. The median age of the participants was 62 years (range, 38–87 years), with majority female (80%). Most patients were non-smokers (46.7%) and were diagnosed mostly at stage IV (93.7%). Among the patients, 56.7% had exon19del and 43.3% had exon21 L858R EGFR mutations. Half (50%) of the cohort received gefitinib, 26.7% had erlotinib, 10% had afatinib, and 13.3% had osimertinib. Majority of the participants (60%) had an expression of PD-L1 and within this group 33.3% had low PD-L1 expression,

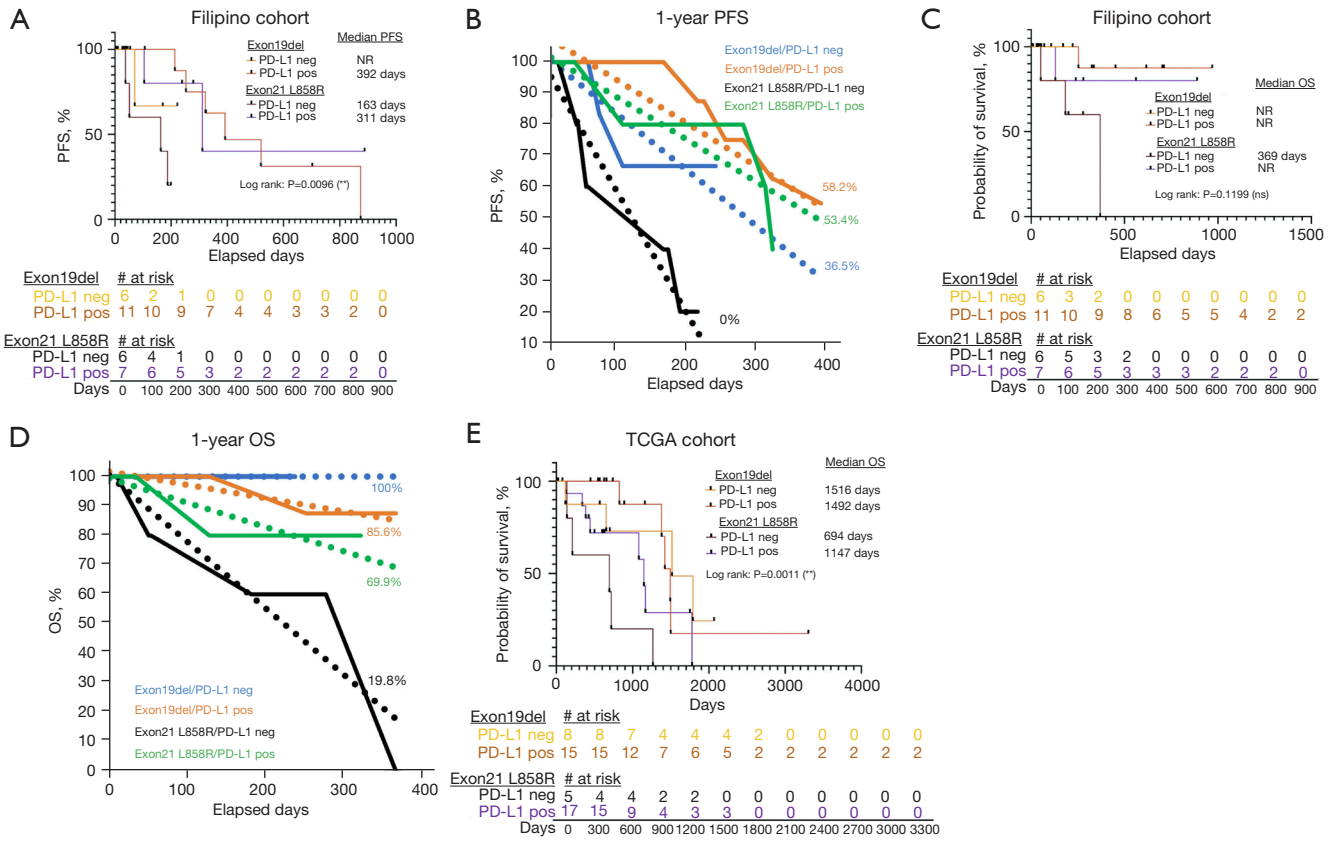

Figure 1 The influence of PD-L1 positivity and negativity in the survival of EGFR-mutant NSCLC with or without PD-L1 expression. The Kaplan-Meier method was used to analyze the median PFS and median OS. P values of the curves were determined using the log-rank test. A GLR model was used to evaluate 1-year survival rates. (A) KM curves showing the probability of PFS, and (B) the predicted 1-year PFS rates among Filipinos with EGFR-mutant NSCLC. (C) KM curves showing the probability of survival, and (D) the predicted 1-year OS rates among Filipinos with EGFR-mutant NSCLC. (E) KM curve showing the probability of survival among TCGA cohort. **, P<0.01; ns, not significant. In GLR, the survival proportions were plotted in continuous line and the predicted survival rates were plotted in broke line. PD-L1, programmed death-ligand 1; neg, negative; pos, positive; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; PFS, progression-free survival; OS, overall survival; KM, Kaplan-Meier; TCGA, The Cancer Genome Atlas; GLR, generalized linear regression.

while 26.7% had high expression.

A total of 45 patients were included in the survival analysis for TCGA cohort. Majority had exon19del (51.11%) and the rest had exon21 L858R (48.89%). In expression analysis, 969 NSCLC patients (483 LUAD and 486 LUSC) referencing to 685 normal tissues were examined. In DNA methylation analysis, 816 NSCLC patients (452 LUAD and 364 LUSC) referencing to 71 normal tissues were included.

PD-L1 positivity may predict better PFS

To identify the prognostic value of PD-L1 in disease outcomes of EGFR-mutant NSCLC, we first examined and compared the PFS of patients with or without PD-L1 expression. Patients tested positive for PD-L1 had significantly longer PFS compared to their PD-L1 negative counterparts (P=0.0096). The mPFS of PD-L1 positive exon19del and exon21 L858R were 392 and 311d, respectively. The mPFS of PD-L1 negative exon19del was not reached (NR), while the mPFS of exon21 L858R was 163 days (*Figure 1A*; Table S1). The incidence of acquired drug resistance to TKIs by NSCLC for a year is alarmingly high (19,23). Consistent with mPFS trend, the 1-year PFS rates of PD-L1 positive groups (exon19del =58.2% and exon21 L858R =53.4%) were higher compared to negative groups (exon19del =36.5% and exon21 L858R =0%) (*Figure 1B*). These results suggest that PD-L1 positivity in Filipinos

with NSCLC harboring EGFR-sensitizing mutations may prognose better PFS.

PD-L1 positivity differentially influence the OS of EGFR mutants

Next, we assessed the OS of patients with or without PD-L1 expression. The OS of patients with different PD-L1 profiles and EGFR mutations in Filipino cohort were not significant (P=0.1199). The mOS of PD-L1 negative exon21 L858R was 369 days, while the rest was NR mOS (*Figure 1C*; Table S1). Interestingly, PD-L1 positivity differentially influenced the 1-year OS rate of EGFR mutants. In exon19del, PD-L1 positive group had lower 1-year OS rate (85.6%) than those tested negative (100%). In exon21 L858R, PD-L1 positive patients had higher 1-year OS rate (69.9%) than PD-L1 negative patients (19.8%) as shown in *Figure 1D*. Due to small sampling size of the study, we further verified these trends from TCGA cohort. Interestingly, PD-L1 negative exon21 L858R from TCGA cohort had significantly the shortest mOS (694 days) compared to the rest (1,147 to 1,516 days, P=0.0011). The mOS of most groups in the TCGA cohort was greater than 1,000 days which may signify the limitation in the observation period in Filipino cohort.

Consistent with the trends in the 1-year survival rates of Filipino cohort, the mOS of exon19del positive for PD-L1 was shorter (1,492 days) compared to those negative for PD-L1 (1,516 days) in TCGA cohort. The mOS of exon21 L858R positive for PD-L1 was longer (1,147 days) compared to the negative group (694 days) as shown in *Figure 1E*. These results may suggest the differential influence of PD-L1 in the OS of NSCLC with different EGFR-sensitizing mutations.

PD-L1 gene expression profiling may lack prognostic value

We further stratified the expression of PD-L1 (both protein and RNA levels) into high, low, and negative. There was no significant difference in the PFS (P=0.4727 & 0.0995) and OS (P=0.2231 & 0.3317) of NSCLC patients with different PD-L1 protein expression in both exon19del and exon21L858R (*Figure 2A-2D*; Table S1). This trend was similar in TCGA cohort where OS of patients with different PD-L1 RNA expression did not reach significance (P=0.8923 & 0.3446) (*Figure 3A*,*3B*). These results may signify the lack of prognostic value of stratifying PD-L1 expression levels in EGFR-mutant NSCLC.

NSCLC is a heterogenous group of lung cancer which consists of morphologically and pathologically distinct subtypes—adenocarcinomas, squamous cell carcinomas, and large cell carcinomas. We hypothesized that PD-L1 expression levels vary among NSCLC subtypes. Thus, we examined and compared the expression levels of PD-L1 in LUAD and LUSC. Stage plot analysis showed that PD-L1 expression is relatively higher in LUSC (4.3 TPM) than in LUAD (3.7 TPM) and that the amount of PD-L1 transcript fluctuates among different stages (*Figure 3C*). Additionally, PD-L1 transcripts were significantly higher (P<0.05) in normal tissues than in tumors (*Figure 3D*) which signify that the different surface area of normal lung tissue that envelope the tumors may complicate the overall value of PD-L1 expression levels in prognosing disease outcomes in NSCLC. Consistent with the expression data, the promoter methylation profiles of *CD274* also varies among LUAD, LUSC, and normal tissues (*Figure 3E*). In LUSC, any hypermethylation of individual promoter CpG islands results in reduced expression. However, hypermethylation of each CpG island in LUAD does not always results in downregulation of PD-L1, but aggregated hypermethylation of the promoter reduces expression (*Figure 3F*). These results confirm that PD-L1 expression varies among NSLC which may provide additional insights about the shortcomings of previous studies that resulted in conflicting prognostic results.

EGFR-mutant NSCLC differentially responded to TKIs

To identify the efficacy of EGFR-TKIs in the management of EGFR-mutant NSCLC in Filipinos, we analyzed the survival of patients who received afatinib, gefitinib, erlotinib, and osimertinib. No significant differences were observed in the PFS (P=0.4671) and OS (P=0.1937) of patients in the four EGFR-TKI treated groups (*Figure 4A*,*4B*; Table S1). However, erlotinib treatment yielded relatively the longest mPFS (874 days), followed by those who received gefitinib (311 days), osimertinib (174.5 days), and afatinib (137 days). Consistent with the PFS trend, the mOS of patients treated with afatinib was 149.5 days which was relatively shorter compared to the rest, yet mOS was not reached (NR) for those remaining groups.

PD-L1 expression lacks correlation with TKI response

Next, we sought to understand the correlation among PD-L1 status, EGFR mutation, and TKI responses. Pearson's

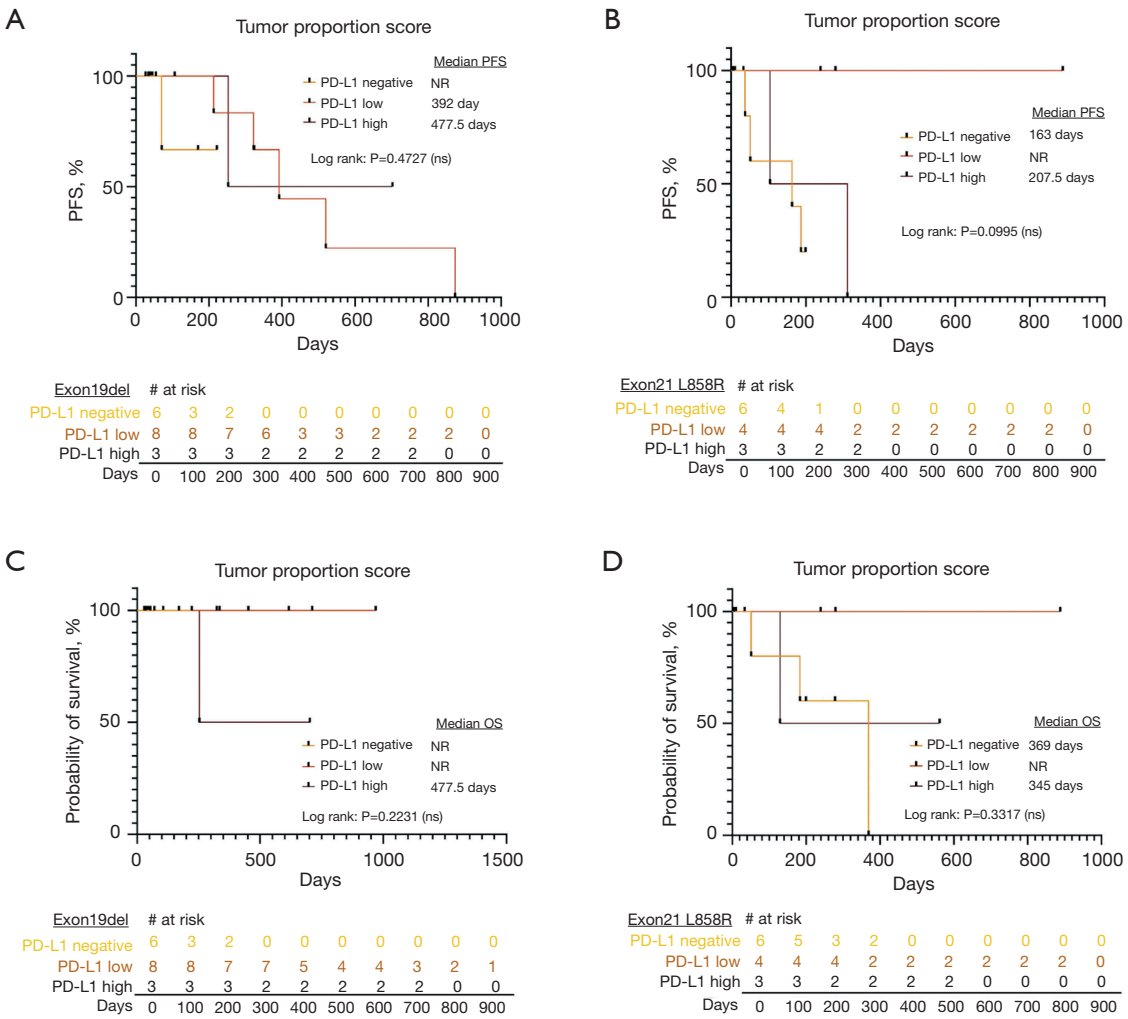

Figure 2 The influence of PD-L1 protein expression levels (negative, low, high) in the survival of Filipinos with EGFR-mutant NSCLC. Protein expression levels were stratified by TPS using IHC. The Kaplan-Meier method was used to analyze the median PFS of patients with (A) exon19del and (B) exon21 L858R and the median OS of patients with (C) exon19del and (D) exon21 L858R. The P values of the curves were determined using the log-rank test. ns, not significant. The median OS and median PFS of certain patient groups that had no 50% survival reduction throughout the observation period were considered NR. PD-L1, programmed death-ligand 1; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TPS, tumor proportion scoring; IHC, immunohistochemistry; PFS, progression-free survival; OS, overall survival; NR, not reached.

correlation (r) showed that gefitinib response positively correlated with the presence of exon19del (r=0.42), while erlotinib response correlated with the presence of exon21 L858R mutation (r=0.27). We did not observe sufficient correlation between PD-L1 status and EGFR-TKI treatment (Pearson's coefficients are almost similar at near zero, P>0.05) as shown in *Figure 4C*.

Functional proteome network of intracellular signaling pathways associated with EGFR-TKI resistance (KEGG: Hsa01521) and PD-L1 expression (KEGG: hsa05235) in NSCLC revealed that PD-L1 is associated with EGFR-TKI resistance pathway only through STAT3-dependent signaling. Majority of pathways involved in EGFR-TKI resistance such MAPK, mTOR, PI3K-Akt, mTOR and Jak-STAT signaling did not enrich with pathway for PD-L1 expression (*Figure 4D*). This may explain the lack of direct

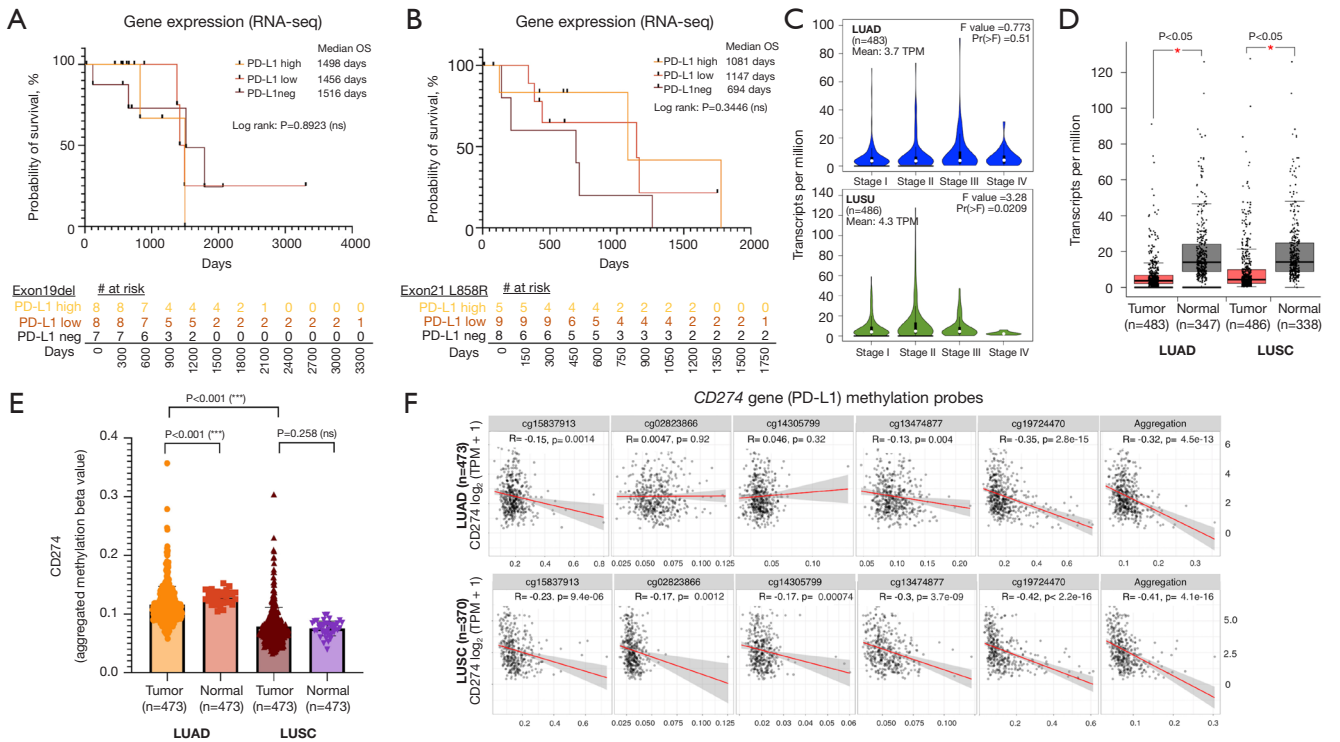

Figure 3 The influence of PD-L1 RNA expression levels (negative, low, high) in the survival of TCGA cohort with EGFR-mutant NSCLC. RNA expression levels were stratified using the mean cut off value of PD-L1 RNA-seq abundance reads in FPKM. The Kaplan-Meier method was used to analyze the median OS of patients with (A) exon19del and (B) exon21 L858R. The P values of the curves were determined using the log-rank test. ns, not significant. (C) Violin plot of PD-L1 expression expressed as TPM in two major NSCLC histology types, LUAD and LUSC, in various disease stages (I–IV). The expression levels were analyzed from NSCLC patients in the TCGA cohort using GEPIA web tool. (D) Scatter plot comparing the RNA expression levels (in TPM) between tumors and normal tissue. Differences in the expression were evaluated using one-way ANOVA through the GEPIA web tool. *, P<0.05. (E) *CD274* (also known as PD-L1) gene methylation profiles of NSCLC patients from TCGA cohort analyzed using the aggregated methylation mean Beta value of all five CpG probes within the promoter region of *CD274*. Comparison of methylation profiles were assessed using paired sample *t*-test. ***, P<0.001; ns, not significant. (F) Pearson's correlation between *CD274* DNA methylation and gene expression from TCGA cohort. The Pearson's coefficient (R) and P values (p) were measured in each cytosine-guanine (cg) sites that corresponded to a specific CpG probe. Methylation analysis were performed using SMART web interface. PD-L1, programmed death-ligand 1; neg, negative; TCGA, The Cancer Genome Atlas; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; FPKM, fragments per kilobase of exon per million reads mapped; OS, overall survival; TPM, transcripts per million; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; GEPIA, Gene Expression Profiling Interactive Analysis; ANOVA, analysis of variance; *CD274*, cluster of differentiation 274; SMART, Shiny Methylation Analysis Resource Tool.

correlation between PD-L1 expression with EGFR-TKIs.

PD-1 expression may influence the survival outcomes of EGFR mutant PD-L1 positive NSCLC

The interplay between cancer cells and the immune microenvironment represents a dynamic and a crucial event that defines the ability of tumors to evade immune destruction. The PD-1 is a known receptor of PD-L1, and the binding of these two proteins downregulates the immune system. We were then interested to identify the contribution of PD-1 in the survival of PD-L1 positive NSCLC with EGFR exon19del and exon21 L858R mutation. PD-1 expression levels had no significant influence in the OS of TCGA cohort (P=0.4531). However, the mOS of patients with low PD-1 was relatively longer compared to those with high expression in both exon19del (1,492 *vs.* 1,438.5 days) and exon21 L858R (1,147 *vs.* 1,081).

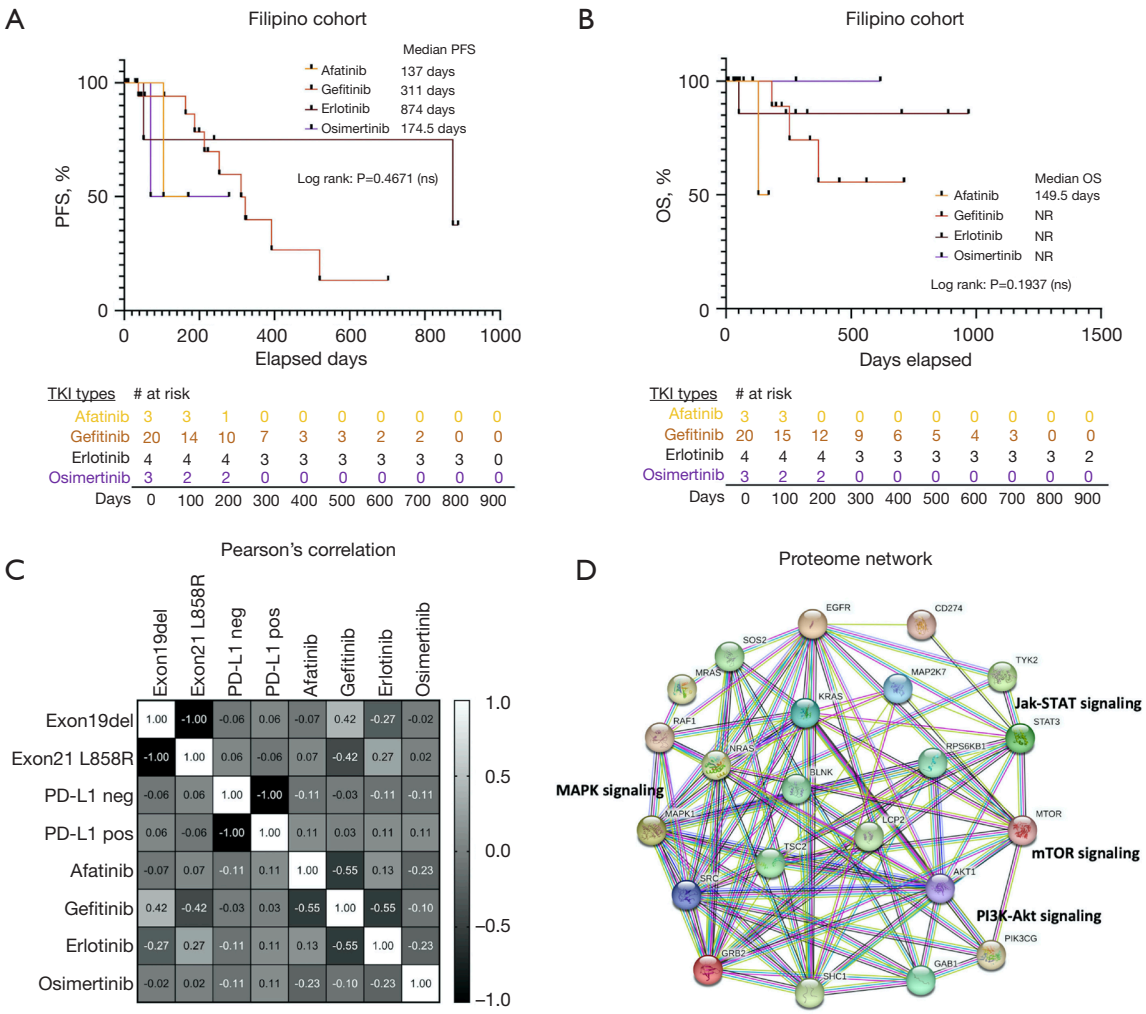

Figure 4 Survival of Filipinos with EGFR-mutant NSCLC treated with different TKIs and correlation with PD-L1 expression. The Kaplan-Meier method was used to analyze the (A) median PFS and (B) median OS of Filipinos with EGFR-mutant NSCLC. The P values of the curves were determined using the log-rank test. ns, not significant. (C) Pearson's correlation of EGFR-TKIs with EGFR mutations and PD-L1 expression. The heatmap score (range, –1 to 1) represents the Pearson's coefficient. (D) Proteome network of proteins associated with signaling pathways involved in EGFR-TKI resistance and PD-L1 expression. The protein interaction network was analyzed using STRING interaction network. EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TKIs, tyrosine kinase inhibitors; PD-L1, programmed death-ligand 1; PFS, progression-free survival; OS, overall survival.

Consistent with the immune-regulation activity of PD-1, higher expression leads to shorter mOS (*Figure 5A*).

Lastly, we correlated the expression of PD-1 with the observed occurrence of EGFR mutation and PD-L1 positivity to identify if PD-1 may act as independent prognostic marker. PD-1 expression positively correlates with PD-L1 expression (r=0.17) and the occurrence of exon21 L858R (r=0.16), whereas, PD-1 expression negatively correlates with the occurrence of exon19del (*Figure 5B*). These findings may suggest the differential clinical value of PD-L1 and its receptor, PD-1, in NSCLC with different EGFR mutations.

Discussion

To our knowledge, this is the first study to report the prognostic value of PD-L1 in Filipinos with EGFR-mutant NSCLC, and verified the result using TCGA

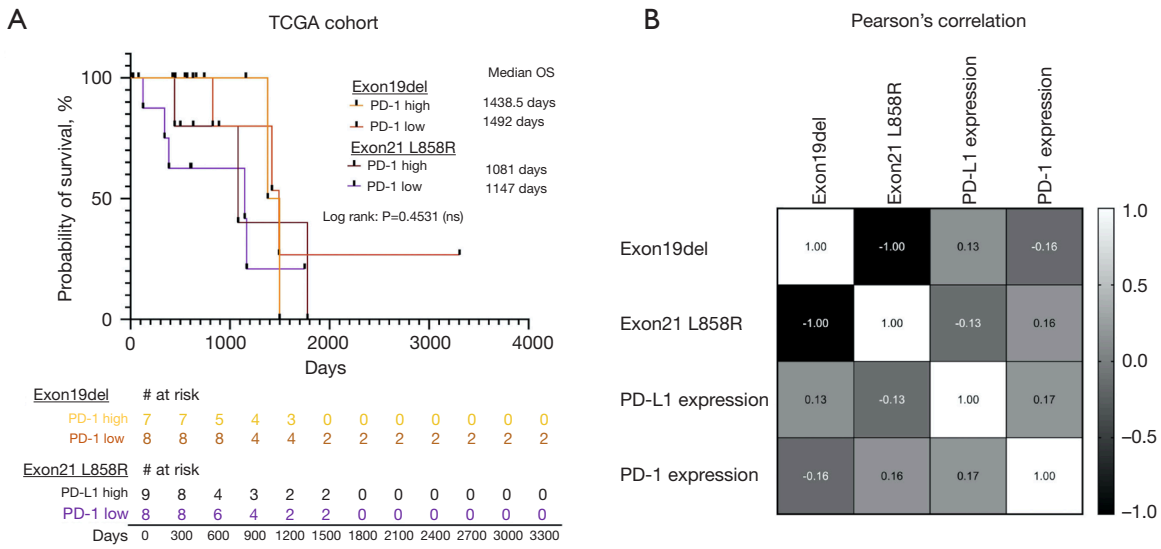

Figure 5 The influence of PD-1 RNA expression levels (low and high) in the survival of TCGA cohort with PD-L1 positive EGFR-mutant NSCLC. PD-1 RNA expression levels were stratified using the mean cut off value of PD-1 RNA-seq abundance reads in FPKM. (A) The Kaplan-Meier method was used to analyze the median OS of patients with exon19del and exon21 L858R mutants positive for PD-L1. The P values of the curves were determined using the log-rank test. ns, not significant. (B) Pearson's correlation of PD-1 with the occurrence of certain EGFR-sensitizing mutation, and PD-L1 expression. The heatmap score (range, –1 to 1) represents the Pearson's coefficient. PD-1, programmed cell death protein 1; TCGA, The Cancer Genome Atlas; PD-L1, programmed death-ligand 1; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; FPKM, fragments per kilobase of exon per million reads mapped; OS, overall survival.

cohort. Previous studies have shown that NSCLC from Asian population have high EGFR mutation prevalence of up to 62%, whereas, 23% was found among Indians, 10–15% in Caucasians, North American and Europeans, and 19% in African Americans (32). Filipinos were reported to have about 49.4% mutation frequency, with exon19del and exon21 L858R to be the most commonly occurring mutations with frequencies of 54.7% and 27.4%, respectively (33). Out of the 42 recruited patients in our study, 54.8% had exon19del, 35.7% had exon2 1L858R mutation, 4.75% had exon19del or exon21 L858R in tandem with exon20 T790M, and 4.75% had exon21 L861Q alone or in tandem with exon18 G719X (unpublished data). Females have been found to have higher occurrence of exon19del (4), which may explain higher frequency of this mutation in our study that consisted of 80% female. Previous studies demonstrated the positive correlation of PD-L1 expression with the presence of EGFR mutation (20-22). In addition, about 55.6% of Filipinos with NSCLC tested positive for PD-L1 (34). In our cohort analysis of 30 patients with complete EGFR and PD-L1 profiles, more than half (60%) expressed PD-L1.

The prognostic significance of PD-L1 in EGFR mutant NSLC is currently confronted by conflicting results (19,20,24,27,28) and prognostic data among Filipinos is currently unknown. Therefore, we were generally interested in determining the prognostic implications of PD-L1 among Filipino patients with NSCLC. In our analysis, we found that PD-L1 positivity were associated with significantly longer PFS. This correlation has been found among other Asian populations (20), and in bigger population size study (15).

Previous reports showed that patients harboring different types (i.e., exon19 *vs.* exon21) and variations (i.e., 21L858R *vs.* 21L861Q) of EGFR mutations exhibited differential survival outcomes (24,35-37). Our initial results showed that PD-L1 differentially influenced the OS of patients with different EGFR-sensitizing mutation in both Filipinos and TCGA cohorts. In exon19del, PD-L1 positivity was associated with shorter OS, whereas, positivity to PD-L1 in exon21 L858R was associated with longer OS.

The counterintuitive correlation of PD-L1 status in the outcomes of NSCLC with exon19del and exon21 L858R could be partially explained by the distinct concomitant mutations and inherent differences in gene expression pattern between these two EGFR mutational variants (38,39).

For example, a higher frequency of KRAS mutation was observed in exon21 L858R and without PD-L1 expression (38,40). Consequently, KRAS mutation was associated with higher risk of acquired resistance to TKIs than wildtype (41). These findings could explain the differential survival of EGFR-mutant NSCLC patients with varying PD-L1 status, and they justify the shorter PFS of exon21 L858R/PD-L1 negative group in our cohort. Additionally, we recently identified the potential involvement of PI3K-induced signaling in the cross-talk between EGFR and PD-L1 pathways through TTF-1 upregulation (39). Indeed, PI3K-mediated signaling has been found to downregulate PD-L1 in NSCLC (42). Consequently, gene amplification of PIK3CA, a component of the PI3K complex, has been found to positively correlate with PIK3CA activating mutations (43). PIK3CA mutations have been found to be enriched in non-responsive NSCLC with exon21 L858R compared to exon19del (38). PIK3CA mutations were associated with shorter OS (43), which may further explain the differential OS observed among exon19del and exon21 L858R with different PD-L1 status in our cohort. These observations highlight the importance of investigating concomitant mutations and aberrant signaling pathways to better understand disease outcomes in EGFR-mutant NSCLC.

Previous studies found that high PD-L1 expression was associated with poor prognoses (9,19,24), accompanied by increased frequency of EGFR mutations (20), and primary resistance to EGFR-TKIs (18,21). Meanwhile, low PD-L1 expression has been correlated with the best prognoses for PFS (44). In our analysis, stratification of patients based on different PD-L1 expression (high, low, none) at the protein and RNA levels did not produce significant prognostic value in Filipinos and TCGA cohorts. However, we observed the good prognostic value of low PD-L1 expression in exon21 L858R. In fact, EGFR-mutant NSCLC with low PD-L1 has been found to bear the least tumor mutational burden (3.4 Mut/Mb) compared to those with negative or high PD-L1 (4.0 and 6.0 Mut/Mb, respectively) (40). Additionally, exon21 L858R has been found to bear lesser driver mutations than exon19del (77.8% *vs.* 83.3%, respectively) (38). Lower tumor mutational burden and fewer driver mutations were associated with better survival (45,46). Additionally, for the first time, we retrospectively unraveled and reported evidences that PD-L1 expression of different NSCLC subtypes at various disease stages varies among NSCLC which may also explain the conflicting results in previous prognostic studies. Therefore, examining the prognostic value of PD-L1 expression levels in different subtype of NSCLC and assessing tumor mutational burden may provide a more comprehensive result.

The rate of acquired resistance to EGFR-TKIs by NSCLC is alarmingly high (28,47,48). In Filipino cohort, Erlotinib treatment produced the longest PFS compared to other EGFR-TKIs. Although a number of studies reported the differential occurrence of T790M mutations among patients treated with first- and second-line therapy (49). Erlotinib has remained its efficacy as monotherapy (50-52) or in combination with other drugs for NSCLC (53-55). Previous reviews enumerated the significance of targeting other driver mutations in NSCLC which include KRAS, ALK, ROS-1 and MET (56). Meanwhile, the presence of EGFR co-mutations has been shown to decrease TKI efficacy by 51% (57). Therefore, tumor mutational burden (TMB) analysis and EGFR co-mutation detection have become recommended practices in NSCLC pathology that could aid in improving the treatment plan for patients.

A number of studies report the positive correlation of PD-L1 expression with the occurrence of EGFR mutation, but not EGFR-TKI response (19,44). In our analysis, we found that the responses we observed in exon19del and exon21 L858R were strongly correlated with gefitinib and erlotinib, respectively. However, we did not find any sufficient correlation between EGFR-TKIs and PD-L1 status, confirming previously published data. Protein network analysis revealed that signaling pathways associated with EGFR-TKI resistance did not enrich for PD-L1 expression pathway in NSCLC, providing evidence on the lack of direct correlation between these two pathways.

At the molecular level, NSCLC tumors are heterogenous which influence the differential responses of patients to TKIs. In addition to EGFR mutations, several biomarkers have been previously associated with decreased survival rates among patients with advanced NSCLC. These biomarkers include the upregulation of growth factors such as insulin-like growth factor (IGF) (58), the occurrence of oncogenic fusions such as EML4-ALK rearrangement (59), alteration of cell cycle regulators such as the upregulation of cyclin D (60), activation of oncogenes such as KRAS (61), and the deletion of tumor suppressor genes such as PTEN (62). Identifying the degree of tumor heterogeneity in NSCLC may help better understand survival outcomes of patients receiving TKIs.

Several models of EGFR-TKI resistance have been proposed (16,18,48,63). A number of molecular culprits have been identified that confer NSCLC resistant to TKIs.

Some of these mechanisms include the over-expression of SPP1 (18), and HGF (16), constitutive activation of the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways (48), occurrence of EGFR co-mutations (T790M and C797S), including the suppression of pro-apoptotic proteins such as BIM (16), and the development of other driver mutations such as alterations in HER2, MET, BRAF, and PI3K (59). For most NSCLC patients who developed resistance to third-generation TKIs, treatment options normally become limited. Thus, interest for immunotherapy targeting immune checkpoint proteins, such as PD-L1, has been growing recently (64). Although the high expression level of PD-L1 was associated with poor prognoses in TKI-based management, it becomes a valuable marker in immunotherapy to predict the likelihood of patients responding to immune checkpoint inhibitors such as atezolizumab (65).

PD-L1 is an immune checkpoint protein that binds PD-1 receptor expressed on the surface of immune cells. The activation of PD-1/PD-L1 axis results in the downregulation of immune functions. This study provides the first evidence about the influence of PD-1 expression in the survival of PD-L1 positive NSCLC with EGFR-sensitizing mutations. Consistent with PD-1 function, high expression is associated with shorter survival while low expression may prognose longer survival. Additionally, PD-1 positively correlates with the occurrence PD-L1 positive exon21 L858R, which highlights the independent prognostic potential of PD-1 in EGFR-mutant NSCLC.

There is currently limited data on the expression patterns of PD-L1 and PD-1 proteins in Filipinos with EGFR-mutant NSCLC. Thus, the prognostic value of PD-1/PD-L1 pathway in Filipino cohort treated with TKIs is largely unknown to this date. However, previous clinical findings seem to suggest that the PD-1/PD-L1 pathway could render insufficient in describing the real contribution of immune checkpoint pathways in the treatment outcomes due to a significantly lower PD-L1 expression in Asian cohort with EGFR-mutant NSCLC than in wildtype (66). Indeed, PD-L1 negative NSCLC tend to be more prevalent than those with low and high PD-L1 expression in Filipino cohort (67). This lack of tumor cell-expressed PD-L1 could partly explain the inadequate findings about the predictive value of the PD-1/PD-L1 pathway, which necessitates the need to identify other immune checkpoint targets such as LAG-3, TIM-3, and TIGIT (68). Alterations in other oncogenes such as BRAF, ROS1, RET, HER2, and MET may also confound with treatment outcomes associated with the expression of PD-1 and PD-L1 (69).

Despite the lack of correlation between sex and tumor cell-expressed PD-L1, females with low PD-L1 expression have higher risk of having <50% PD-L1 expression on infiltrating lymphocytes (67), which further complicates immunohistochemical scoring, patient stratification, and assessment of treatment outcome. Additionally, Asian females were found to have higher soluble and membrane-bound PD-1 than men (70), which may influence the findings of clinical studies with a high variation of male and female participants. Thus, highlighting the sex-specific subgrouping in PD-1/PD-L1-related prognostic studies in NSCLC is also vital.

There were certain limitations to our study. First, the sample size was relatively small for both cohorts which may hindered some survival curves to reach statistical significance. Further validation is recommended to confirm consistency of the observed trends. Second, half of the Filipinos participants were given Gefitinib as first-line therapy which means that the survival trends of those receiving other TKIs maybe less represented. Third, many of the patients did not adhere to the follow-up schedules, and some left the study within weeks and months after enrolling, which affected the sample size and the results of analyses. Fourth, the observation period was not long enough which hindered median survival estimation. Fifth, tumor heterogeneity and tumor mutational burden were not determined in this study. EGFR and PD-L1 were preferentially analyzed due to availability of commercial kits. More sophisticated analytical techniques such as next generation sequencing (NGS) can identify more biomarkers. Sixth, many studies demonstrated variations in the results of PD-L1 IHC kits (71,72). Our PD-L1 testing lacks harmonization with other kits or protocols from other studies which may affect future reproducibility in TPS. Lastly, some clinical data (such as drug name, progression days, ethnicity or nationality) were lacking from TCGA annotations.

Conclusions

Despite preliminary, this is the first study to assess the prognostic significance of PD-L1 in Filipinos with EGFR-mutant NSCLC, and retrospectively correlated the data to TCGA cohort. We showed that PD-L1 positivity was associated with better PFS, but may influence OS differentially based on type of EGFR mutation. Additionally, NSCLC subtypes, disease stage, and PD-1 expression may

impact the collective outcomes associated with PD-L1 and EGFR-sensitizing mutations.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-118/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-118/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-118/coif). The authors have no conflicts of interest to declare.

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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Review Board (protocol number LCP-CS-001-2019) of the Lung Center of the Philippines. All participating hospitals/institutions were informed and agreed the study. All patients provided written informed consent for genetic testing, as well as use of their clinical data.

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