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ORIGINAL ARTICLE

Clinicopathological and genomic correlates of programmed cell death ligand 1 (PD-L1) expression in nonsquamous non-small-cell lung cancer

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Background: Programmed cell death ligand 1 (PD-L1) tumor proportion score (TPS) is the primary clinically-available biomarker of response to immunotherapy in non-small-cell lung cancer (NSCLC), but factors associated with PD-L1 expression are not well understood.

Materials and methods: Consecutive nonsquamous NSCLCs with successful PD-L1 assessment and targeted next-generation sequencing were included in this retrospective study. Clinicopathological characteristics, gene mutations, and copy number changes in gene and chromosomal arms were compared among three PD-L1 expression groups: negative (TPS $<$ 1%), low (TPS 1%—49%), and high (TPS $\geq$ 50%). A *Q*-value $<$0.25 was considered significant after multiple comparisons correction.

Results: A total of 909 nonsquamous NSCLCs were included. High PD-L1 expression compared with low and negative PD-L1 expression was associated with increased tobacco exposure (median pack-years: 25 versus 20 versus 20, respectively; $P = 0.01$), advanced stage at diagnosis (76% versus 67% versus 61% with advanced stage of disease, respectively; $P < 0.001$), and higher tumor mutational burden (TMB) (median 12.2 versus 10.6 versus 10.6 mutations/megabase, respectively; $P < 0.001$). Negative PD-L1 expression when compared with high PD-L1 expression was associated with: mutations in *STK11* (19% versus 5%; $Q < 0.001$), *EGFR* (22% versus 11%; $Q < 0.001$), *CTNNB1* (4.3% versus 0.4%; $Q = 0.04$), *APC* (5% versus 1%; $Q = 0.17$), and *SMARCA4* (9% versus 4%; $Q = 0.20$); copy number loss of *CD274* (PD-L1, 28% versus 6%; $Q < 0.001$), *PDCD1LG2* (PD-L2, 28% versus 6%; $Q < 0.001$), and *JAK2* genes (27% versus 7%; $Q < 0.001$), loss of chromosomal arm 9p (23% versus 10%; $Q = 0.04$), and gain of 1q (46% versus 21%; $Q < 0.001$). High PD-L1 expression compared with negative PD-L1 expression was associated with copy number gain of *CD274* (11% versus 3%; $Q = 0.01$) and *PDCD1LG2* (11% versus 3%; $Q = 0.01$). NSCLCs with *CD274* loss, compared with those without loss, had a lower response rate (23% versus 9%; $P = 0.006$) and shorter progression-free survival (3.3 versus 2.0 months; $P = 0.002$) on immunotherapy.

Conclusions: PD-L1 expression is associated with specific genomic alterations and clinicopathologic characteristics in nonsquamous NSCLC.

Key words: biomarker, immunotherapy, NSCLC, PD-L1, PD-L1 expression

INTRODUCTION

Treatment with immune checkpoint inhibitors (ICIs) directed against programmed cell death 1 (PD-1) or its ligand (PD-L1) improves survival in metastatic non-small-cell lung cancer (NSCLC), but only a subset of patients benefit from treatment, and biomarkers of response to immunotherapy are limited.[1] The PD-L1 tumor proportion score (TPS), defined as the percent of PD-L1-positive tumor cells by immunohistochemistry, is the primary clinically-available predictive factor of response to immunotherapy in NSCLC, and increasing PD-L1 expression levels are associated with improved survival in patients treated with immunotherapy.[1,2] Despite the important impact of PD-L1 on immunotherapy efficacy, the factors associated with its expression are not well understood.

In various cancers, PD-L1 expression can be stimulated by extrinsic factors such as interferon-gamma,[3] or tumor-intrinsic factors, including activation of the mammalian target of rapamycin (mTOR) and mitogen-activated protein

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kinase pathways,[4,5] and transcriptional regulation of the *CD274* gene, which is located at chromosome 9p24.1 and encodes PD-L1.[6,7] In NSCLC, activating genomic alterations in *KRAS*, *EGFR*, and *ALK*, and loss of *PTEN* can influence PD-L1 expression.[4,8–10] *CD274* amplification in NSCLC may be associated with increased PD-L1 expression,[11,12] while *STK11* mutations are associated with absence of PD-L1 expression and decreased response to immunotherapy.[13]

A deeper characterization of the factors associated with PD-L1 expression can help elucidate mechanisms of primary response or resistance to immunotherapy. We sought to analyze clinicopathological and genomic factors associated with PD-L1 expression in nonsquamous NSCLC.

MATERIALS AND METHODS

Clinicopathological data were collected from all consecutive patients with nonsquamous NSCLC with successful PD-L1 assessment and targeted next generation sequencing (NGS) through OncoPanel at the Dana-Farber Cancer Institute. Gene mutations and copy number changes in genes and chromosomal arms were compared among PD-L1 expression groups. Gene mutations were filtered by considering only alterations annotated as oncogenic by OncoKB. A Q-value <0.25 was considered significant after multiple comparisons correction. Significant genomic alterations were tested as predictive factors in a cohort of immunotherapy-treated patients. Detailed methods are reported in the supplementary Materials, available at *Annals of Oncology* online.

RESULTS

Clinicopathologic correlates of PD-L1 expression

A total of 909 patients with nonsquamous NSCLC with PD-L1 assessment and successful targeted NGS were identified and grouped into the following categories: PD-L1-negative with a TPS <1% ($N = 304$, 33%), PD-L1-low with a TPS 1%–49% ($N = 326$, 36%), and PD-L1-high with a TPS ≥50% ($N = 279$, 31%) (Table 1). High PD-L1 tumors, compared with low and negative PD-L1 tumors, were more commonly diagnosed at an advanced stage at diagnosis (stage IIIB–IV: 76% versus 67% versus 61%, respectively; $P < 0.001$) and

Table 1. Patient characteristics by PD-L1 expression groups

	PD-L1 <1%	PD-L1 1%–49%	PD-L1 ≥50%	*P*
N = 909	304 (33%)	326 (36%)	279 (31%)	
Sex (*n*)				
Male (360)	125 (41%)	130 (40%)	105 (38%)	0.69
Female (549)	179 (59%)	196 (60%)	174 (62%)	
Age at diagnosis (years)				
Median (range)	66 (35–91)	64 (22–88)	67 (29–92)	0.21
Smoking status (*n*)				
Never (185)	68 (22%)	68 (21%)	49 (18%)	0.34
Current/former (724)	236 (78%)	258 (79%)	230 (82%)	
Pack-years				
Median (IQR)	20 (1.2–40.0)	20 (3.5–37.0)	25 (7.5–40.5)	***0.01***
ECOG PS (*n*)				
0–1 (693)	228 (84%)	255 (84%)	212 (83%)	0.95
≥2 (133)	42 (16%)	49 (16%)	42 (17%)	
Stage at diagnosis (*n*)				
I-IIIA (297)	120 (39%)	109 (33%)	68 (24%)	***<0.001***
IIIB-IV (612)	184 (61%)	217 (67%)	211 (76%)	
Histology (*n*)				
Adenocarcinoma (851)	287 (94%)	311 (95%)	253 (91%)	***0.047***
Other (58)	17 (6%)	15 (5%)	26 (9%)	
Biopsy tumor site (*n*)				
Primary (424)	150 (49%)	148 (45%)	126 (45%)	0.51
Metastasis (485)	154 (51%)	178 (55%)	153 (55%)	
Tissue of origin (*n*)				
Lung (446)	152 (50%)	164 (50%)	130 (48%)	0.55
Lymph node (175)	57 (19%)	61 (19%)	57 (20%)	
Pleural/pericardium (90)	24 (8%)	34 (10%)	32 (11%)	
Bone (41)	14 (5%)	15 (5%)	12 (4%)	
Liver (40)	16 (5%)	14 (4%)	10 (4%)	
Brain (64)	26 (8%)	17 (5%)	21 (7%)	
Adrenal gland (18)	7 (2%)	9 (3%)	2 (1%)	
Other (35)	8 (3%)	12 (4%)	15 (5%)	
Timing of biopsy				
At diagnosis	253 (83%)	279 (86%)	247 (88%)	0.31
After first line of treatment	28 (9%)	24 (7%)	21 (8%)	
After second or further line of treatment	23 (8%)	23 (7%)	11 (4%)	
TMB (Mut/Mb)				
Median (IQR)	10.6 (7.3–14.5)	10.6 (6.8–15.2)	12.2 (8.4–18.2)	***<0.001***

ECOG PS, Eastern Cooperative Oncology Group Performance Status; IQR, interquartile range; Mut/Mb, mutations per megabase; PD-L1, programmed-death ligand 1; TMB, tumor mutational burden.
P values <0.05 are indicated in bold and italics.

had greater tobacco exposure [median pack-years (range): 25 (7.5—40.5) versus 20 (3.5—37.0) versus 20 (1.2—40.0), respectively; $P = 0.01$, Table 1], with a slight correlation between tobacco pack-years and PD-L1 TPS {$\rho = 0.08$ [95% confidence interval (CI): 0.01—0.14]; $P = 0.02$; supplementary Figure S1, available at *Annals of Oncology* online}. Nonsquamous tumors with non-adenocarcinoma histology were more common in the high PD-L1 group compared with the low and negative PD-L1 groups (non-adenocarcinoma: 9% versus 5% versus 6%, respectively; $P = 0.04$). Pleomorphic carcinoma histology was enriched in the PD-L1-high group compared with the PD-L1-low and -negative groups (3% versus 1% versus 0%, respectively; $P = 0.006$) (supplementary Table S1, available at *Annals of Oncology* online).

Mutational correlates of PD-L1 expression

The 25 most frequently altered genes in the overall cohort (including oncogenic mutations, deep deletions in tumor suppressors, and high amplification in oncogenes) grouped by PD-L1 expression levels, are summarized in supplementary Figure S2, available at *Annals of Oncology* online. The median TMB was significantly higher in the PD-L1-high group than in the PD-L1-low and -negative groups [median (interquartile range) 12.2 mut/Mb (8.4—18.2) versus 10.6 mut/Mb (6.8—15.2) versus 10.6 mut/Mb (7.3—14.5), respectively; $P < 0.001$] (supplementary Figure S3A, available at *Annals of Oncology* online), with a modest but significant linear correlation between TMB and PD-L1 TPS [$\rho = 0.12$ (95% CI: 0.05—0.18); $P < 0.001$] (supplementary Figure S3B, available at *Annals of Oncology* online). Because the higher TMB in the PD-L1-high group could introduce a bias in our mutational frequency analysis, a permutation test was performed to control for the confounding difference in mutation rate observed between different PD-L1 expression groups to minimize this bias (see detailed Methods, available at *Annals of Oncology online*).

The PD-L1-negative group, compared with the PD-L1-high group, was, respectively, enriched for mutations in *STK11* (19% versus 6%; $P < 0.001$), *EGFR* (22% versus 11%; $P < 0.001$), *CTNNB1* (4% versus 0.4%; $P < 0.001$), *APC* (5% versus 1%; $P = 0.005$), *SMARCA4* (9% versus 4%; $P = 0.007$), and *RB1* (4% versus 1%; $P = 0.018$) (Figure 1A and B). After correcting for multiple comparisons, *STK11* ($Q < 0.001$), *EGFR* ($Q < 0.001$), *CTNNB1* ($Q = 0.04$), *APC* ($Q = 0.17$), and *SMARCA4* ($Q = 0.20$) retained a significant association with PD-L1 negativity (Figure 1A). By contrast, the PD-L1 high group, compared with the PD-L1-negative group, was associated with mutations in *TP53* (48% versus 33%; $P = 0.009$), *EP300* (3% versus 0.3%; $P = 0.01$), *MET* (7% versus 2%; $P = 0.01$), and *CDKN2A* (9% versus 3%; $P = 0.02$) (Figure 1A and B). After correcting for multiple comparisons, none of these enrichments retained significance (Figure 1A).

PD-L1 expression in oncogenic driver subgroups

Because there is an interest in understanding whether NSCLCs with specific oncogenic driver mutations respond differently to immunotherapy,[14] we also conducted a PD-L1 expression analysis focused on oncogenes, without performing permutation testing and correction for multiple comparisons. The PD-L1-high group, when compared with the PD-L1-low and PD-L1-negative groups, was, respectively, associated with *KRAS* mutations (44% versus 32% versus 33%; $P = 0.004$), *BRAF* V600E mutations (3% versus 1% versus 0.3%; $P = 0.023$), and *MET* exon 14 skipping alterations (7% versus 4% versus 2%; $P = 0.018$). Conversely, *EGFR* mutations were associated with PD-L1 negativity

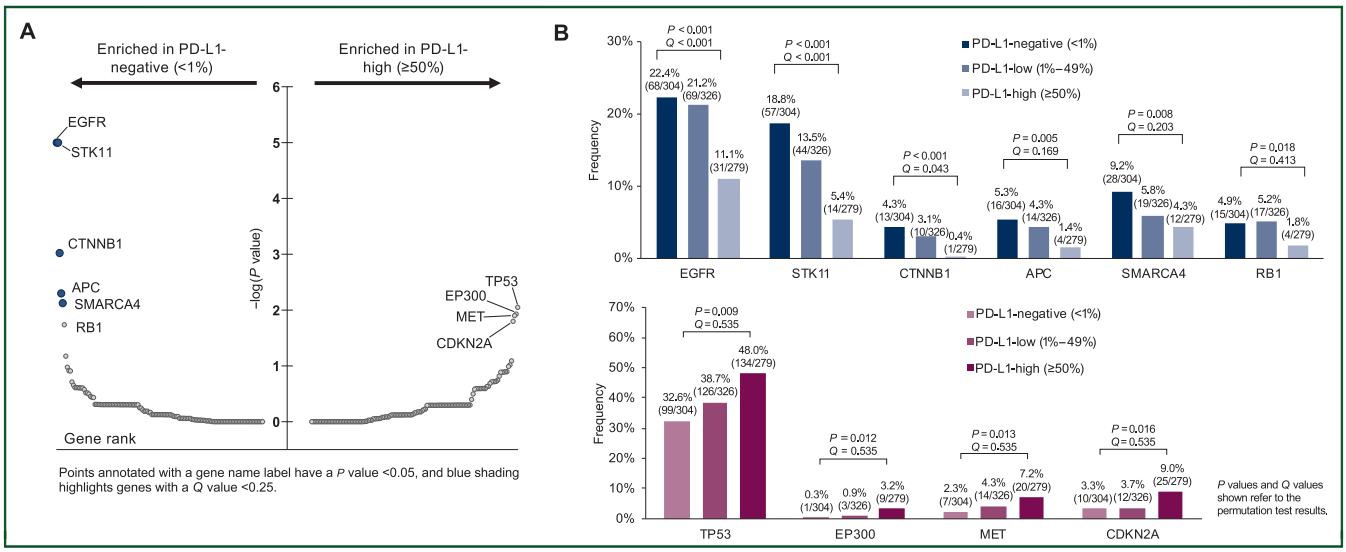

Figure 1. Gene mutations associated with programmed cell death ligand 1 (PD-L1) expression levels.
(A) Oncogenic mutations associated with programmed cell death ligand 1 (PD-L1) expression levels by permutation test (by *P* value and by *Q* value after correction for multiple comparisons). Points annotated with a gene name label have a *P*-value <0.05, and blue shading highlights genes with a *Q*-value <0.25. (B) Frequency of oncogenic gene mutations significantly associated ($P < 0.05$) with PD-L1 <1% (upper row) and with PD-L1 ≥50% (lower row), ranked by decreasing significance of association.

when compared with the PD-L1-low and PD-L1-high groups (22% versus 21% versus 11%; $P < 0.001$) (supplementary Figure S4, available at *Annals of Oncology* online).

Co-occurring mutation analysis

We next examined whether commonly co-occurring mutations impacted PD-L1 expression levels. Since *KRAS* mutations often co-occur with *STK11* and *TP53* mutations in nonsquamous NSCLC[15] and each of these genes was associated with PD-L1 expression in our analysis, we evaluated the association between co-occurring mutations and PD-L1 expression levels (supplementary Table S2, available at *Annals of Oncology* online). In *KRAS*-mutant tumors, a concurrent *STK11* mutation was associated with PD-L1 negativity (PD-L1 negativity: 37% in *STK11* wild-type versus 80% *STK11*-mutant; $P < 0.001$). By contrast, in *STK11*-mutant NSCLC, *KRAS* mutational status did not impact PD-L1 expression (PD-L1 negativity: 81% in *KRAS* wild-type versus 80% in *KRAS*-mutant; $P = 1.000$). However, among *STK11* wild-type tumors, *KRAS* mutations were associated with high PD-L1 expression (high PD-L1: 63% in *KRAS*-mutant versus 45% in *KRAS* wild-type; $P < 0.001$). This suggests that *STK11* mutation is associated with PD-L1 negativity regardless of *KRAS* mutation status, while *KRAS* mutation impacts PD-L1 expression mainly in *STK11*-wild-type tumors. Similarly, among *KRAS*-mutant tumors, *TP53* co-mutation was associated with high PD-L1 expression (high PD-L1: 77% in *TP53* mutant versus 45% in *TP53*-wild-type; $P < 0.001$).

Since *EGFR* mutation was associated with low PD-L1 and *TP53* was associated with high PD-L1, we tested the interaction between these commonly co-occurring mutations.[15] Among *EGFR*-mutant tumors, there was no difference in the proportion of PD-L1-high samples with or without concurrent *TP53* (40% versus 23%, respectively; $P = 0.083$, supplementary Table S2, available at *Annals of Oncology* online).

Gene copy number variations

We next examined gene copy number variations (CNVs) including shallow deletions, deep deletions, low gains, and amplifications by PD-L1 expression level (detailed in the supplementary Figure S5, available at *Annals of Oncology* online). Because CNV assessment is limited in samples with low tumor purity,[16] cases with <20% tumor content were excluded, leaving 873 of the 909 sequenced cases (96%) available for CNV analysis. There were no significant differences in tumor content among the PD-L1 expression groups (supplementary Figure S6A, available at *Annals of Oncology* online). PD-L1-negative tumors were more likely to have a greater number of CNVs than PD-L1-high tumors [median (interquartile range) number of CNVs per tumor 37 (6—64) versus 20 (4—53), respectively, $P = 0.003$, supplementary Figure S6B, available at *Annals of Oncology* online]. The PD-L1-negative group, compared with the PD-L1 high group was, respectively, associated with copy number loss of *CD274* (28% versus 6%; $Q < 0.001$), *PDCD1LG2* (28% versus 6%; $Q < 0.001$), and *JAK2* (27% versus 7%; $Q < 0.001$) (Figure 2). Loss of the 9p24.1 chromosomal locus (defined as concurrent loss of *CD274*, *PDCD1LG2*, and *JAK2*) was significantly associated with the PD-L1-negative group compared with the PD-L1-high group

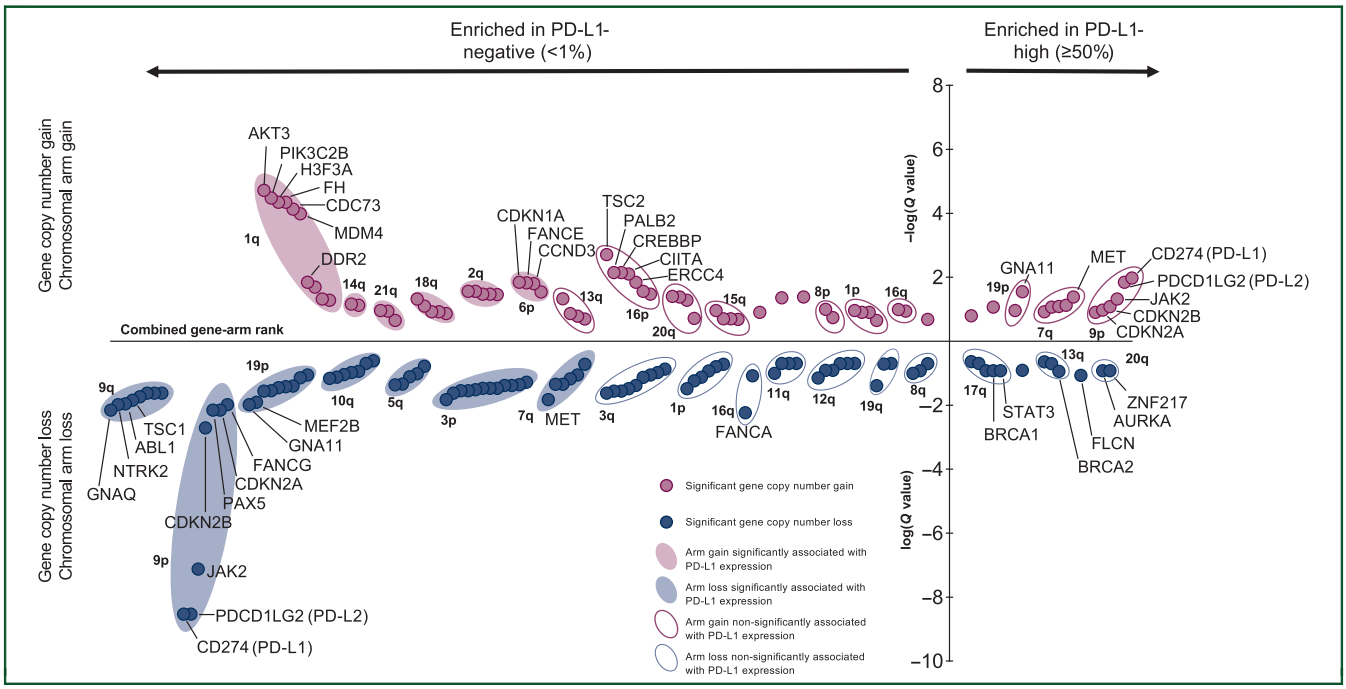

Figure 2. Chromosomal arm and gene copy number variations associated with programmed cell death ligand 1 (PD-L1) expression levels ($Q < 0.25$).
Chromosomal arm copy changes associated with PD-L1 expression levels are listed in rank order of significance. Within each chromosomal arm, individual genes with copy number variations (CNVs) associated with PD-L1 expression are then ranked by their level of significance. Only chromosomal arms with at least two significantly enriched genes are displayed.

(25% versus 6%, respectively; $P < 0.001$) (supplementary Figure S7A, available at *Annals of Oncology* online).

Among the gene copy gains most significantly enriched in PD-L1-high tumors compared with PD-L1-negative tumors there were *CD274* (11% versus 3%; $Q = 0.01$), *PDCD1LG2* (11% versus 3%; $Q = 0.01$), and *JAK2* (9% versus 3%; $Q = 0.05$) (Figure 2). Copy number gain of the 9p24.1 chromosomal locus (defined as co-gain of *CD274*, *PDCD1LG2*, and *JAK2*) was more commonly detected in PD-L1-high NSCLC compared with PD-L1-negative NSCLC (9% versus 3%, respectively; $P = 0.004$) (supplementary Figure S7B, available at *Annals of Oncology* online). When using more stringent criteria to include only those genes with either two-copy deletion or high-level amplification call, only *CDKN2A* and *CDKN2B* two-copy deletion were significantly associated with absence of PD-L1 expression (*CDKN2A* deleted in 13% of PD-L1-negative versus 5% of PD-L1-high tumors, $Q = 0.05$; *CDKN2B* deleted in 11% of PD-L1-negative versus 4% of PD-L1-high tumors; $Q = 0.05$) (supplementary Figure S8, available at *Annals of Oncology* online).

Arm-level CNVs

We also analyzed the association of chromosomal arm-level copy number changes and PD-L1 expression levels. Data were available for 446 samples (49%) for this analysis. The absence of PD-L1 expression, compared with high PD-L1 expression, was associated with loss of the 9q (24% versus 10%; $Q = 0.04$), 9p (23% versus 10.1%; $Q = 0.05$), 6q (22% versus 10%; $Q = 0.07$), and 19p arms (26% versus 13%; $Q = 0.07$), and gain of the 1q (46% versus 21%; $Q < 0.001$) (supplementary Figure S9, available at *Annals of Oncology* online). Only 18q arm-level loss was associated with the PD-L1-high group when compared with the PD-L1-negative group (36% versus 24%; $Q = 0.15$), but this result may be limited by a relative paucity of gene coverage on this particular gene arm. Frequencies of the significantly associated arm-level copy number changes are shown in supplementary Figure S10, available at *Annals of Oncology* online.

When considering whole chromosome copy number changes, we found that PD-L1-negative tumors compared with the PD-L1 high tumors were associated with gains of chromosome 20 (20% versus 5%; $Q = 0.006$) and loss of chromosome 9 (15% versus 4%; $Q = 0.05$) (supplementary Figure S11, available at *Annals of Oncology* online), while there were no significant enrichments in the PD-L1-high group.

Multivariable analysis

We then fitted clinicopathological characteristics and mutations significantly associated with PD-L1 expression by a permutation test in a multivariable model. High PD-L1 expression was associated with advanced stage at diagnosis [odds ratio (OR): 2.48 (95% CI: 1.67—3.67); $P < 0.001$] and higher TMB [OR: 1.02 (95% CI: 1.01—1.05); $P = 0.04$], while the absence of PD-L1 expression was associated with *STK11* mutation [OR: 0.19 (95% CI: 0.10—0.37); $P < 0.001$], *EGFR* mutation [OR: 0.30 (95% CI: 0.18—0.49); $P < 0.001$], *CTNNB1* mutation [OR: 0.10 (95% CI: 0.01—0.83); $P = 0.03$], *APC* mutation [OR: 0.28 (95% CI: 0.09—0.90); $P = 0.03$], and *SMARCA4* mutation [OR: 0.35 (95% CI: 0.16—0.76); $P = 0.008$] (supplementary Table S3, available at *Annals of Oncology* online).

Because NSCLCs with very high PD-L1 expression (TPS $\geq$ 90%) might be more responsive to treatment with PD-1 inhibitors than NSCLC with TPS 50%—89%,[2] we also compared clinicopathological and genomic features in NSCLC with PD-L1 TPS $<$ 1% versus $\geq$90% ($N = 118$, supplementary Table S4, available at *Annals of Oncology* online). Overall, we noted that associations in terms of gene mutations (supplementary Figure S12A, available at *Annals of Oncology* online), gene CNVs (supplementary Figure S12B, available at *Annals of Oncology* online), and arm-level CNVs (supplementary Figure S12C, available at *Annals of Oncology* online), and their fit in a multivariable analysis (supplementary Table S5, available at *Annals of Oncology* online), were similar to the analysis comparing PD-L1 TPS $<$ 1% versus $\geq$50% tumors (supplementary text, available at *Annals of Oncology* online).

Impact on outcomes to immunotherapy

Among a cohort of 486 patients with nonsquamous NSCLC treated with ICIs (supplementary Table S6, available at *Annals of Oncology* online), we evaluated the predictive value of the genomic alterations we identified that were associated with PD-L1 expression. Patients whose tumors had *CD274* copy loss compared with those without loss, respectively, showed significantly lower objective response rates [ORR 9% (95% CI: 3—18) versus 23% (95% CI: 19—27); $P = 0.006$] and significantly shorter median progression-free survival [mPFS 2.0 versus 3.3 months, HR: 1.52 (95% CI: 1.17—1.99); $P = 0.002$], while there was no difference in median overall survival (mOS) (Figure 3). Similar results were seen in NSCLCs with 9p24.1 loss and chromosomal arm 9p loss (supplementary Figure S13A and B, available at *Annals of Oncology* online). By contrast, ORR was similar in tumors with and without *CD274* copy number gain [ORR: 24% (95% CI: 11—42) versus 20% (95% CI: 17—25), respectively; $P = 0.656$]. Patients with *EGFR*-mutant compared with *EGFR*-wild-type tumors treated with ICIs had, respectively, significantly shorter mPFS [1.5 versus 3.4 months, HR: 2.20 (95% CI: 1.59—3.05); $P < 0.001$] and mOS [3.8 versus 12.0 months, hazard ratio (HR): 1.82 (95% CI: 1.29—2.55); $P < 0.001$] (supplementary Figure S13C, available at *Annals of Oncology* online), despite a similar ORR. Similarly, patients with *STK11*-mutant compared with those with *STK11*-wild-type tumors had a significantly shorter mPFS [2.1 versus 3.0 months, HR: 1.41 (95% CI: 1.06—1.87); $P = 0.02$] and mOS [8.9 versus 11.7, HR: 1.37 (95% CI: 1.00—1.87); $P = 0.05$] (supplementary Figure S13D, available at *Annals of Oncology* online), while ORR was not significantly different. We found no statistically significant difference in mPFS or mOS among patients with or without

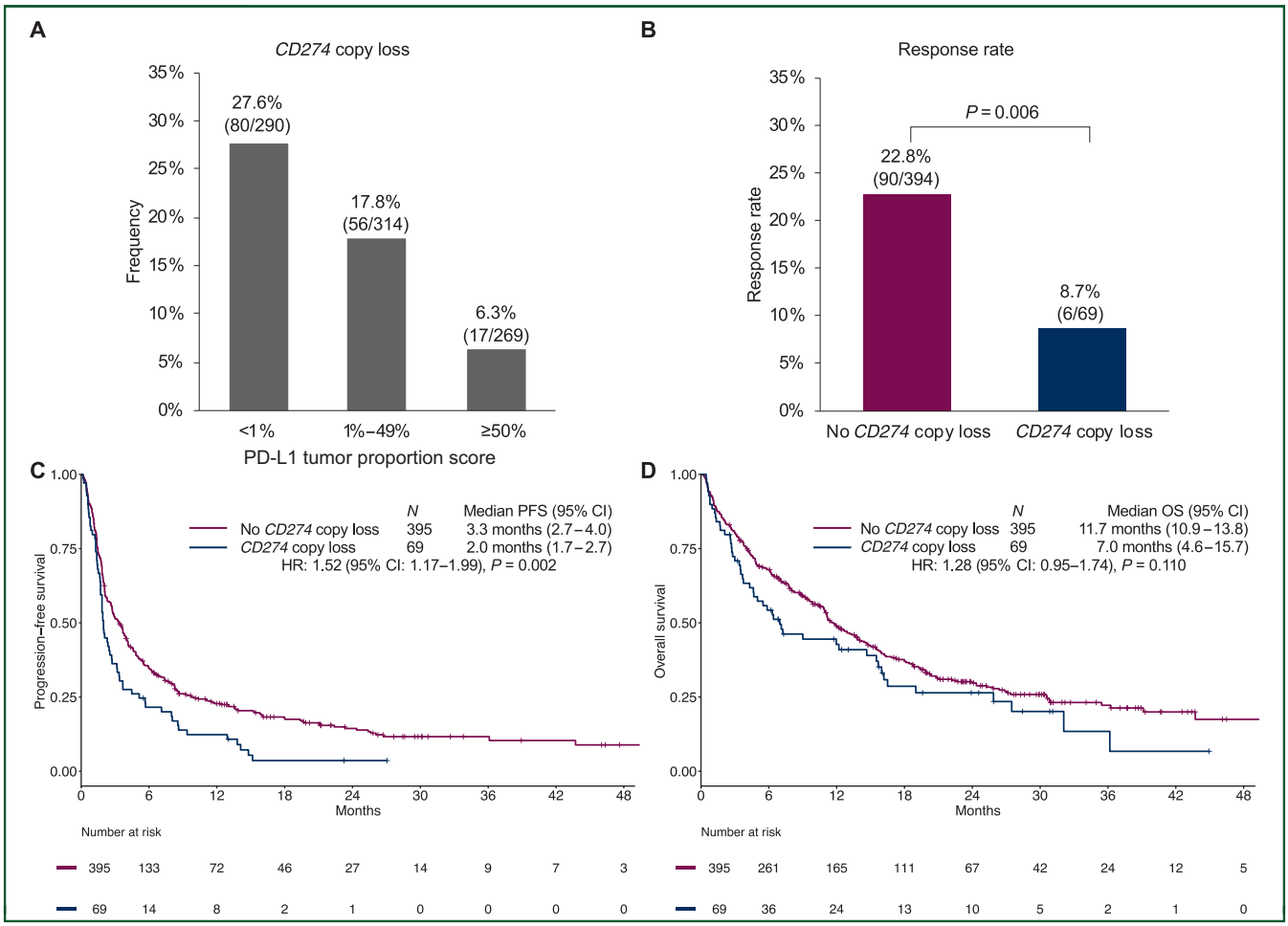

Figure 3. Correlation of CD274 gene copy loss with programmed cell death ligand 1 (PD-L1) expression and outcomes to immune checkpoint inhibition in non-small cell lung cancer.

(A) Frequency of *CD274* copy loss across PD-L1 expression levels, (B) response rate, (C) Kaplan-Meier estimates of progression-free survival, and (D) overall survival in the groups without and with *CD274* copy loss.

CI, confidence interval; HR, hazard ratio; OS, overall survival.

other aforementioned genomic alterations, although these analyses may be limited by small sample sizes for these molecular subsets (supplementary Figure S14, available at *Annals of Oncology* online).

After adjusting for potential confounding factors, an objective response to ICIs was significantly associated with higher PD-L1 expression and TMB, but not with *CD274* copy loss (supplementary Table S7, available at *Annals of Oncology* online). PFS was significantly associated with *EGFR* mutation [HR: 2.03 (95% CI: 1.29—3.19); $P = 0.002$], PD-L1 expression [HR: 0.41 (95% CI: 0.28—0.62); $P < 0.001$], and TMB [HR: 0.97 (95% CI: 0.95—0.99); $P = 0.002$] (supplementary Table S8, available at *Annals of Oncology* online), while OS was significantly associated with PD-L1 expression [HR:0.59 (95% CI: 0.38—0.93); $P = 0.02$] (supplementary Table S9, available at *Annals of Oncology* online).

DISCUSSION

Expression of PD-L1 in NSCLC is highly variable, but our understanding of factors related to different PD-L1 levels is very limited. We took a comprehensive, unbiased approach, to examine the clinical factors and genomic alterations, including mutations and CNVs, that are associated with different levels of PD-L1 expression. We found that high PD-L1 expression is associated with tobacco exposure, advanced stage at diagnosis, a higher TMB, and gain of *CD274* chromosomal locus 9p24.1. Conversely, PD-L1 negativity is associated with mutations in *EGFR*, *STK11*, *CTNNB1*, *APC*, and *SMARCA4*, as well as gene copy loss of *CD274*, *PDCD1LG2*, and *JAK2*, loss of the chromosomal locus 9p24.1, loss of chromosomal arm 9p, and gain of the chromosomal arm 1q.

In addition to confirming previously-reported findings that NSCLCs with *STK11* or *EGFR* mutations are more likely to have absent PD-L1 expression and are less likely to respond to immunotherapy,[13,17] our study identified a novel genomic association, to our knowledge, of oncogenic mutations in *CTNNB1* and *APC* with absent PD-L1 expression in NSCLC. *CTNNB1* and *APC*, which encode β-catenin and adenomatous polyposis coli, respectively, are key components of the Wnt signaling pathway, which has been implicated in hampering the antitumor immune response[18] and promoting an immunologically 'cold' tumor microenvironment, characterized by T cell exclusion and low PD-L1

expression.[19] *CTNNB1* mutations can drive primary and acquired resistance to ICIs,[20,21] while β-catenin inhibition promotes T cell infiltration, suggesting that Wnt pathway inhibitors might enhance the efficacy of ICIs by promoting T cell infiltration into tumors.[22] Although we did not see an association of *CTNNB1* or *APC* mutations with immunotherapy efficacy, the sample size in these two groups was small ($N \leq 15$).

Amplification of *CD274* (≥6 gene copies), was previously reported to occur in 0.6% of lung adenocarcinomas,[11] while in another study, 9p24.1 chromosomal locus amplification (≥3 copies) was found in 5.7% ($N = 5/94$) of resected NSCLCs.[12] In our study, the prevalence of copy number gain of *CD274* and 9p24.1 was 6% and 5%, respectively, and both alterations were associated with high PD-L1 expression. Similarly, PD-L1 negativity was significantly associated with *CD274* copy loss (18%) and 9p24.1 loss (16%). Loss of *CD274* in our study was associated with impaired efficacy of immunotherapy in NSCLC, and, if confirmed in larger studies, this might serve as a genomic biomarker to identify patients who are less likely to benefit from PD-1 inhibition.

Our study has several limitations. First, CNV analysis can be challenging using targeted NGS, since NSCLC samples are often obtained with low tumor cellularity.[16] The absence of significant difference in tumor content in the three PD-L1 expression groups makes it unlikely for tumor content to affect CNV analysis. Because NGS covers only a small fraction of the genome, we cannot affirm with certainty if specific CNV associations are biologically relevant or are simply passenger alterations adjacent to genomic regions with actual significance. Furthermore, arm-level CNV analysis may be limited for chromosomal arms with low coverage on each NGS panel. The probability of including germline variants in the analysis, given the lack of matched germline DNA testing in the OncoPanel NGS platform, was greatly reduced by filtering only for oncogenic mutations.

In conclusion, we report that PD-L1 expression is associated with well-defined clinicopathological and genomic characteristics. Additional work exploring the mechanistic links between tumor genotype and PD-L1 immunophenotype is warranted. These findings will help design strategies to improve outcomes on immunotherapy in NSCLC. Larger studies correlating immunotherapy response with less common NSCLC genotypes, such as mutation of *CTNNB1* and *APC*, and loss of *CD274*, among others, will be necessary to determine their relevance as predictive biomarkers for ICIs.

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