Programmed Death-Ligand 1 Copy Number Loss in NSCLC Associates With Reduced Programmed Death-Ligand 1 Tumor Staining and a Cold Immunophenotype

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Received 8 December 2021; revised 18 January 2022; accepted 20 January 2022
Available online - 4 February 2022

ABSTRACT

Introduction: Programmed death-ligand 1 (PD-L1) copy number gains may be predictive of clinical response to immunotherapy in NSCLC. This study investigated PD-L1 copy number variations in tumor resection and bronchoscopy biopsies and its relationship with PD-L1 tumor cell staining and inflammatory gene expression.

Methods: PD-L1 gene copy number and mRNA expression were evaluated by real-time polymerase chain reaction in surgically resected NSCLC tumor biopsies (n = 87) and control biopsies (n = 20). A second cohort (n = 15) of bronchoscopy-derived tumor biopsies was analyzed, including multiple biopsies from the same patient across different anatomical sites.

Results: PD-L1 mRNA levels strongly correlated with PD-L1 tumor staining (r = 0.55, p < 0.0001). Interferon-γ mRNA expression associated with PD-L1 immune cell staining, but not PD-L1 tumor cell staining. In contrast, PD-L1 copy number positively associated PD-L1 tumor staining, but not PD-L1 immune cell staining. PD-L1 copy number analysis detected loss (15 of 87 = 17%) and gain (5 of 87 = 7%) of copy number. Tumors with low PD-L1 copy number expressed significantly reduced levels of inflammatory (interferon-γ, interleukin [IL]-6, IL-1β, MMP-9) and immunosuppressive (IL-10, transforming growth factor β) mediators. Analysis of bronchoscopy-derived biopsies revealed low heterogeneity in copy number values across different anatomical sites, in contrast to more variable PD-L1 mRNA expression.

Conclusions: Low PD-L1 copy number tumors display reduced PD-L1 expression, reduced PD-L1 tumor cell staining, and an immunologic cold tumor microenvironment. Because PD-L1 copy number values are highly stable across different tumor regions, its evaluation may represent a robust and complimentary biomarker for predicting response to immunotherapy, where low copy number may predict lack of response.

Keywords: NSCLC; Immunotherapy; Biomarkers; PD-L1; Copy number

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Disclosure: Dr Bozinovski received research funding from NHMRC Australia and CSL Ltd. Australia. Dr Steinfort received honoraria from AstraZeneca, Broncus Medical, and GlaxoSmithKline research funding from Broncus Medical, Zidan Medical, and Morair MedTech. The remaining authors declare no conflict of interest.

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ISSN: 1556-0864
https://doi.org/10.1016/j.jtho.2022.01.013
Introduction

Although durable clinical responses to immunotherapy can occur in a broad range of human cancers, there is a substantial portion of patients with cancer who fail to respond to this treatment. Approximately 20% to 30% of patients with advanced NSCLC respond to immunotherapeutic agents that target the programmed cell death protein-1 and programmed death-ligand 1 (PD-L1) pathway, and prescreening for PD-L1-high tumors can double the response rate. Accordingly, PD-L1 tumor staining is routinely used as an immunotherapy biomarker in solid tumors, including NSCLC, and pembrolizumab is approved as a first-line therapy for patients with metastatic NSCLC whose tumors express PD-L1 (≥1%), as determined by a test approved by the Food and Drug Administration. Important safety issues including immune-related adverse events also necessitate the need for using biomarkers as companion diagnostics for immunotherapy.

Because PD-L1 tumor staining by immunohistochemistry (IHC) represents an imperfect test, there is a need to establish better immunotherapy biomarkers. PD-L1 tumor staining can be challenging in the diagnostic setting where access to tumor is typically limited to small bronchoscopy cytology or biopsy specimens in patients with advanced NSCLC. Although PD-L1 staining of the primary nodule and metastatic lymph nodes is feasible, it is dependent on tissue availability after a suite of histologic and molecular tests. In addition, spatial heterogeneity in PD-L1 tumor staining within and across tumor sites has been detected after multisite biopsy of patients with NSCLC with sufficient variation to result in alteration in clinical management. This spatial heterogeneity may necessitate the need for assessment of multiple biopsies, which is not routinely performed.

An alternative biomarker approach is to investigate the mechanisms that regulate PD-L1 levels within the tumor microenvironment. Interferon-γ (IFNγ) is a cytokine that regulates the expression of PD-L1 levels through the JAK-STAT signaling pathway, which is critical for innate and adaptive immunity. IFNγ can influence tumor immunophenotypes where an “inflamed or hot” gene expression profile in tumor samples was related to more favorable immunotherapy responses, whereas “cold” tumors exhibited lower response rates. Alternative immunotherapy biomarkers that harness molecular technology are also emerging that may be less heterogeneous than PD-L1 tumor staining. A high tumor mutational burden is associated with increased neoantigen burden and may be more predictive of immunotherapy response in advanced NSCLC when compared with PD-L1 tumor proportion score (TPS).

PD-L1 copy number is also increasingly recognized as an important molecular determinant of PD-L1 tumor levels and response to immunotherapy. The PD-L1 gene is located on chromosome 9p24.1, and amplification of this region was frequently observed in patients with Hodgkin lymphoma who responded well to nivolumab therapy in heavily pretreated patients with relapsed or refractory disease. This genetic marker of immunotherapy resistance may not be limited to Hodgkin lymphoma as PD-L1 copy number and protein levels were found to be highly correlated in multiple tumor types. A randomized phase 2 trial has found that PD-L1 amplification and copy number gains were predictive of durvalumab response in metastatic breast cancer. PD-L1 copy number alterations have also been detected in resected tumors from patients with NSCLC, where tumors with PD-L1 genomic gains displayed markedly higher levels of PD-L1 expression. Furthermore, PD-L1 copy number gains were identified as a predictive biomarker for response to nivolumab monotherapy in patients with advanced NSCLC. On the basis of this background, we evaluated how two distinct mechanisms, IFNγ and PD-L1 copy number variations, influence PD-L1 levels on tumor and immune cells, respectively, in NSCLC. Our main findings were also validated in a second cohort of bronchoscopy-derived tumors samples, where we identify a “cold” immunophenotype associated with a loss of PD-L1 copy number.

Materials and Methods

Resection Biopsy Cohort and PD-L1 IHC

Tumor tissue excess to diagnostic requirements was obtained from patients with NSCLC undergoing resection surgery at the Royal Melbourne Hospital and included adenocarcinoma and squamous cell carcinoma (SCC) subtypes, as summarized in Table 1. The biospecimens were acquired as means of the Victorian Cancer Biobank, and the ethics approval was obtained from RMIT University, Australia (Ethics identification: SEHAPP 09-17). Macrodissection of a 1- to 2-cm tumor sample was performed by a pathologist to enrich for tumorous material, with a minimum of at least 50% tumor content as determined by microscopic analysis. The tumor sample was bisected to generate a snap-frozen specimen and a matching formalin-fixed, paraffin-embedded tumor block as per standard laboratory protocols. The control samples included biopsies that were collected from patients undergoing bronchoscopy for nonmalignant causes.
EBUS Cohort

Linear endobronchial ultrasound (EBUS)-guided needle aspirates were obtained from 14 patients with NSCLC at The Royal Melbourne Hospital. EBUS bronchoscopy was combined with rapid on-site examination to confirm the malignant nature of the lesion site by a medically trained cytopathologist, before sampling, as previously described.16,17 This was immediately followed by additional sampling of the same lesion site for collection of diagnostic analysis, including PD-L1 IHC.20,21 An additional biopsy for PD-L1 gene expression and copy number analysis was collected and snap frozen. To evaluate tumor heterogeneity, multiple biopsies were collected from the same patient, including the primary lesion and the surrounding lymph nodes.17 All EBUS-biospecimens were immediately snap frozen and cryobanked by the Victorian Cancer Biobank.

DNA/RNA Isolation, Quantitative Reverse Transcription Polymerase Chain Reaction, and KRAS Genotyping

Total RNA and DNA were isolated from snap-frozen biopsy specimens using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. Briefly, the surgically resected and EBUS biopsies were suspended in RLT lysis buffer and homogenized using the TissueLyser LT (Qiagen) bead mill protocol. The NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to measure DNA/RNA yield and purity. cDNA was prepared from RNA with a High-Capacity cDNA kit (Thermo Fisher Scientific, Waltham, MA), and quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed using the QuantStudio 7 PCR system (Thermo Fisher Scientific, Waltham, MA). The following TaqMan primers were used: PD-L1 (Hs00204257), MMP-9 (Hs00957562), interleukin (IL) IL-6 (Hs00174131), IL-10 (Hs00961622), IL-1β (Hs01555410), IFNγ (Hs00989291), and TGF-β1 (Hs00210887). Gene expression was normalized to the geometric mean of TBP (Hs00427620) and PUM1 (Hs00472881), which are reported to be stable in NSCLC tissue specimens.16 The relative fold changes were determined using the ΔΔCt method, where samples were expressed as a fold change relative to a control biospecimen. KRAS mutation status was evaluated using the ddPCR KRAS screening kit in conjunction with the QX200 Droplet Digital PCR System (BioRad, Hercules, CA). This kit screens for seven KRAS mutations including G12A, G12C, G12D, G12R, G12S, G12V, and G13D, and a cutoff of 0.2% mutant allele frequency was used for this assay, as previously described.22

Assessment of PD-L1 Copy Number Variation

The frozen biopsies from the resected tissue and EBUS-transbronchial needle aspiration samples were analyzed for PD-L1 copy number using the TaqMan Copy Number Assay kit specific to PD-L1 (Hs03704252). PCR was performed utilizing the TaqMan Genotyping Pro-Amp Master Mix (Thermo Fisher Scientific, Waltham, MA) on the QuantStudio 7 platform (Thermo Fisher Scientific, Waltham, MA). Single-well reactions were run in quadruplicate per sample in a 384-microplate layout following the manufacturer’s instructions. In brief, each single-well reaction contained 2 ng of genomic DNA, Master Mix, and PD-L1 TaqMan primer that was duplexed with the TaqMan Copy Number Reference Assay (RNase P). Copy numbers were calculated with the CopyCaller v2.0 Software (Thermo Fisher Scientific, Waltham, MA) using the ΔΔCt relative quantification

Table 1. Patient Characteristics and Tumor Stage

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls Nonmalignant</th>
<th>NSCLC Adeno</th>
<th>SCC</th>
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<tr>
<td>Patients, n</td>
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<td>48</td>
<td>39</td>
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<td>Age in y, mean ± SD</td>
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<td>66.92 ± 8.55</td>
<td>68.25 ± 9.33</td>
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<td></td>
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<td>Ex-smoker</td>
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<td>Never smoked</td>
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<td>10</td>
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<tr>
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<td>II</td>
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</tr>
<tr>
<td></td>
<td>III</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
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Adeno, adenocarcinoma; SCC, squamous cell carcinoma.

(n = 10) as previously described.16 In addition, matching tissue from the adjacent tumor-free region of patients with adenocarcinoma (n = 10) was used when available, as an alternative control sample source as previously described.16 The control samples were combined unless a pairwise analysis was performed, where the adjacent sample was only used. PD-L1 IHC staining was performed on 5-μm-thick sections by the Anatomical Pathology Laboratory at The Royal Melbourne Hospital, Australia. Slides were stained with the human PD-L1 (SP263) rabbit monoclonal antibody (Ventana, Tuscan, AZ) using the Benchmark Ultra automatic staining instrument (Ventana). Scoring of PD-L1 TPS and immune cell staining was blindly performed by an experienced pathologist as previously described.16
method, which calculates the relative copy number of a target gene normalized to RNase P. On the basis of the range values within the nonmalignant control tissue, A PD-L1 copy number of 2.5 or greater was defined as a gain of copy number, whereas a PD-L1 gene copy number less than 1.5 was defined as loss of copy number.

Statistical Analysis

All graphs were generated using GraphPad Prism version 9 (GraphPad Software Inc., San Diego, CA). Two-tailed t tests were performed for the comparison of gene expression changes across groups. Paired groups were evaluated using nonparametric Wilcoxon test. The fold change between multiple groups was compared using the Kruskal-Wallis test. A p value of less than 0.05 was considered statistically significant.

Results

PD-L1 Tumor Staining Does Not Associate With IFNγ Expression in NSCLC

Matching NSCLC resection blocks and frozen biopsies were used to concurrently stain for PD-L1 levels by IHC and quantify PD-L1 mRNA expression by RT-qPCR. Representative images of PD-L1 stained sections are found in Figure 1A, where approximately 50% of the tumors were negative for PD-L1 tumor staining and 9 of 87 (10%) displayed high (>50% TPS) PD-L1 tumor staining (Fig. 1B). A total of 16 of 78 (18%) displayed low PD-L1 levels (1%–9% TPS) and 17 of 87 (19%) displayed moderate PD-L1 levels (10%–49% TPS). The expression of PD-L1 mRNA was significantly higher in tumors that had moderate (10%–49%) tumor staining and expression levels were further increased in tumors with a high (>50%) TPS (Fig. 1C). Consistent with PD-L1 tumor levels being regulated at the transcriptional level, there was a strong positive association between PD-L1 tumor staining and PD-L1 mRNA expression (Fig. 1D).

In addition, PD-L1 immune cell staining was analyzed and scored by an experienced pathologist, where low to moderate PD-L1 immune cell staining was observed in most tumors (71 of 87) and fewer samples display no PD-L1 (16 of 87)–positive immune cells (Fig. 1E). PD-L1 expression levels were highest in tumors with moderate immune cell staining; however, this fell short of significance (Fig. 1F). There was a modest positive association between PD-L1 immune cell staining and PD-L1 expression levels (Fig. 1G).

Because PD-L1 transcript levels can be regulated in a manner that is dependent on IFNγ signaling,23 IFNγ expression in tumor biopsies was next evaluated by RT-qPCR. PD-L1 mRNA expression levels positively correlated with IFNγ expression, and tumors that expressed the highest IFNγ levels also expressed significantly higher levels of PD-L1 mRNA (Fig. 2A and B). Further comparative analyses revealed that IFNγ expression levels did not associate with PD-L1 tumor staining but did positively correlate with PD-L1 immune cell staining (Fig. 2C and D).

PD-L1 Tumor Staining Associates With PD-L1 Copy Number in NSCLC

DNA extracted from the same tumor samples used for the PD-L1 and IFNγ expression analysis was also used for assessment of PD-L1 copy number. As a reference, PD-L1 copy number was determined in control biopsies (n = 20), where values ranged from 1.7 to 2.4. A subanalysis revealed that there was no difference in copy number between the nonmalignant control samples (2.26 ± 0.21) and adjacent tissue from the tumor region (2.3 ± 0.19). On the basis of this range, a copy number value of less than 1.5 was scored as a loss of copy number and a value of greater than 2.5 was scored as a gain of copy number. Loss of PD-L1 copy number was detected in 23% of adenocarcinomas, whereas only 1 of 40 had a gain of copy number in this subtype (Fig. 3A and B). Copy number gains were more frequent in SCC, with 13% SCC tumor biopsies having a copy number above 2.5, and copy number losses were detected but less frequent in this subtype (10%). Comparison of PD-L1 copy number values across histologic subtypes confirmed that copy number levels are significantly lower in lung adenocarcinomas compared with either control or SCC biopsies (Fig. 3C). In addition, paired analysis of lung adenocarcinoma biopsies with their respective adjacent tissue (histologically graded as nonmalignant) revealed a significant reduction in PD-L1 copy number in tumor biopsies (Fig. 3D).

We next investigated whether tumors with a low PD-L1 copy number were associated with smoking or KRAS mutations. The proportion of tumors with a low copy number were similar in nonsmokers (30%), ex-smokers (20%), and current smokers (43%), to indicate that smoking does not drive this genetic alternation (Fig. 3E). As expected, KRAS genotyping detected a higher frequency of KRAS mutations in ex and current smokers compared with nonsmokers with lung adenocarcinoma (Fig. 3F). Analysis of PD-L1 copy number levels according to KRAS status revealed that copy number variations were more frequent in KRAS wild-type tumors (10 of 31 = 32%), compared with KRAS mutated tumors (1 of 16 = 6%) (Fig. 3G).

Comparison of PD-L1 copy number with PD-L1 mRNA expression revealed a positive correlation between these two parameters in NSCLC tumor biopsies (Fig. 4A). Tumors that displayed a loss of copy number (<1.5) expressed significantly lower PD-L1 mRNA expression (Fig. 4B). Further comparative analyses also identified a
position correlation between PD-L1 copy number and PD-L1 tumor staining (Fig. 4C). PD-L1 tumor staining was significantly lower in tumors with loss of copy number, whereas tumors with a gain of copy number displayed the highest levels of PD-L1 tumor staining (Fig. 4D). In contrast, there was no association between
PD-L1 copy number and PD-L1 immune cell staining (Fig. 4E and F).

**PD-L1 Copy Number Associates With a “Cold” Immunophenotype in Resection and Bronchoscopy-Derived Samples**

A panel of inflammatory markers including IFN-γ, IL-6, IL-1β, and MMP-9 was evaluated by RT-qPCR using the same tumor biopsies used for PD-L1 mRNA expression and copy number determination. Gene expression levels for IFN-γ, IL-6, IL-1β, and MMP-9 were significantly lower in tumors that harbored a loss of PD-L1 copy number compared with tumors with a normal copy number value (Fig. 5A–D). In contrast, gene expression levels were not significantly altered in tumors that acquired a gain in copy number when compared with tumors with a normal copy number value. A similar pattern was observed for two immunosuppressive genes, where IL-10 and transforming growth factor β levels were reduced in tumors with a loss of PD-L1 copy number (Fig. 5E and F).

A second cohort of tumor biopsies was collected using EBUS-derived bronchoscopy samples and analyzed using a similar approach to the tumor resection cohort. Assessment of PD-L1 tumor staining identified positive staining in approximately 60% of the EBUS-derived biopsies, including 4 of 15 (26%) that displayed high levels of PD-L1 tumor staining (Fig. 6A). Consistent with the resection cohort biospecimens, PD-L1 tumor staining strongly associated with PD-L1 mRNA expression in EBUS-derived biopsies (Fig. 6B). Assessment of PD-L1 expression across different biopsy sites including the primary mass and different lymph node regions revealed that there was a spectrum of PD-L1 expression levels in biopsies, although they were not significantly different between the primary mass or regional lymph nodes (Fig. 6C). A similar outcome was observed for PD-L1 copy number, where there was variability in copy number values, but values did not significantly differ across different sampling sites including the primary lesion and adjacent lymph nodes (Fig. 6D).

A paired subanalysis of matching tumor biopsies from the same patient revealed that there is very little heterogeneity in PD-L1 copy number across different tumor sampling sites that included comparison between...
Figure 3. *PD-L1* copy number variations in NSCLC histologic subtypes. (A) *PD-L1* copy number values across three cohorts including nonmalignant biopsies, ADENO, and SCC. Red bar graphs depict tumors with a copy number below the CON range (loss) and the black bars depict tumors with a copy number above the CON range (gain). (B) The proportional of tumors with a gain or loss of *PD-L1* copy number grouped according to the main subtypes of NSCLC. (C) Individual *PD-L1* copy number values grouped accordingly to histology. (D) Paired analysis comparing adjacent CON and tumor tissue of biopsies obtained from the same resection patient. (E) The proportional of normal and low copy number tumors grouped according to smoking status. (F)
the primary mass and adjacent lymph node or two different lymph node regions (Fig. 6E). In contrast, there was greater heterogeneity of PD-L1 mRNA expression when comparing two different tumor sampling sites within the same patient (Fig. 6E). In addition, IFNγ expression levels were quantified in the same EBUS-derived bronchoscopy samples and a positive association was observed between IFNγ and PD-L1 copy number (Fig. 6F). This graph also highlights three samples whose copy number value was less than or equal to 1.5, which also expressed much lower IFNγ levels. This observation is consistent with PD-L1 copy number influencing the immunophenotype of lung tumors.

Discussion

PD-L1 levels within the tumor microenvironment are regulated at the transcriptional level, but differential mechanisms may control expression on tumor cells and immune cells. In this study, we observed a strong association between PD-L1 mRNA expression and PD-L1 tumor staining, whereas there was a weaker association with PD-L1 immune cell staining. These data suggest that both tumor and immune cells contribute to increased PD-L1 mRNA expression; however, tumor cells are a more dominant cellular source in NSCLC biopsies. Furthermore, we investigated the relationship between IFNγ and PD-L1 tumor and immune cell staining, as IFNγ can increase PD-L1 levels in a JAK/STAT/IRF1-dependent manner. PD-L1 mRNA expression was much higher in tumors that expressed high levels of IFNγ, and importantly, IFNγ levels correlated with PD-L1 staining on immune cells, but not tumor cells.

Our findings are consistent with a previous study that investigated differential expression of PD-L1 on tumor and immune cells, where high PD-L1 immune cell staining was associated with an IFNγ-mediated adaptive response and increased tumor-infiltrating lymphocytes. Durable clinical responses to atezolizumab (anti-PD-L1) were observed in patients expressing high PD-L1 levels on either tumor or immune cells in this study. A subsequent clinical trial has revealed improved overall survival with atezolizumab in patients who were selected on the basis of PD-L1 expression (<1%) on tumor cells or immune cells. Our finding that IFNγ levels closely associated with PD-L1 immune cell staining but not tumor cell staining may be exploited for clinical benefit. For example, the evaluation of IFNγ and PD-L1 mRNA expression in tumor biopsies may complement existing immunotherapy biomarkers, particularly where the evaluation of differential PD-L1 tumor and immune cell staining by IHC is challenging.

Because IFNγ levels were not associated with PD-L1 tumor staining in NSCLC biopsies, we focused on cell-intrinsic genetic events that can influence PD-L1 tumor levels. We observed that PD-L1 copy number positively associated with PD-L1 mRNA expression and PD-L1 tumor staining in NSCLC. It is now recognized that PD-L1 copy number alterations including deletions and gains occur in solid tumors, such as melanoma and lung cancer. PD-L1 copy number gains and losses are associated with poor prognosis, and tumors with PD-L1 gains displayed a higher tumor mutation burden compared with nonamplified tumors. The association with tumor mutation burden is intriguing as a high mutation load can be predictive of better responses to immunotherapy agents in NSCLC. From a technical perspective, the analysis of copy number using TaqMan PCR-based assays represents a more rapid, simple, and affordable platform relative to next generation sequencing platforms needed for determination of tumor mutation burden.

Our study also revealed that the concurrent assessment of PD-L1 copy number and PD-L1 or IFNγ mRNA expression is highly feasible using EBUS bronchoscopy-derived samples. Paired investigation across distinct tumor sites including the primary node and surrounding lymph nodes reveals that PD-L1 mRNA expression is heterogeneous, whereas PD-L1 copy number is less variable across sampling sites. Our findings are consistent with a previous study that found less spatial heterogeneity in PD-L1 copy number when compared with PD-L1 tumor staining in EBUS-derived NSCLC specimens. Heterogeneity in PD-L1 expression can also be attributed to variations in different NSCLC histotypes, where solid areas may increase the PD-L1 TPS, in contrast to lepidic areas with reduced PD-L1 tumor staining. Immunotherapy is also increasingly used as an adjuvant treatment after surgery or platinum-based chemotherapy, and increased heterogeneity in PD-L1 staining has been reported after adjuvant therapy. Because cell-intrinsic genetic alterations such as copy number are more stable within and across tumor sites, they may represent a more reliable immunotherapy biomarker then PD-L1 tumor staining.

PD-L1 copy number losses have been reported to be more frequent then gains in melanoma and NSCLC, whereas copy number gains were more frequent in ovarian, head and neck, bladder, cervical, and colorectal
Figure 4. **PD-L1** copy number correlates with PD-L1 TPS. (A) Spearman correlation was performed to analyze the relationship between **PD-L1** expression and **PD-L1** copy number. (B) analysis of **PD-L1** mRNA expression was presented in groups on the basis of copy number ranges including loss (<1.5), normal (1.5–2.49), and gain (>2.5). (C) Spearman correlation was performed to analyze the relationship between **PD-L1** copy number and PD-L1 TPS. (D) PD-L1 TPS levels in tumors with a loss, normal, or gain in copy number. E Spearman correlation was performed to analyze the relationship between **PD-L1** copy number and PD-L1 immune cell staining. (F) PD-L1 immune cell staining levels in tumors with a loss, normal, or gain in copy number. *p < 0.05, ***p < 0.005. IHC, immunohistochemistry; ns, not significant; PD-L1, programmed death-ligand 1; RT-qPCR, quantitative reverse transcription polymerase chain reaction; TPS, tumor proportion score.
We observed that PD-L1 copy number losses were more frequent in lung adenocarcinomas compared with SCC. Comparison of tumor samples and their respective nonmalignant tissue adjacent to the tumor revealed that copy number losses were confined within the tumor region. Of importance, loss of PD-L1 copy number was associated with reduced PD-L1 tumor staining and a poorer clinical response to immunotherapy treatment. Conversely, patients with PD-L1 copy number amplifications were found to have improved survival outcomes in response to nivolumab monotherapy in advanced NSCLC, and we found that PD-L1 copy number gains were more frequent in SCC. We also observed that tumors with a loss of PD-L1 copy number expressed lower levels of immunoregulatory genes including IFNγ, IL-6, IL-1β, MMP9, IL-10, and transforming growth factor β. The PD-L1 gene resides within the chromosome 9p.24.1 region, which is in very close proximity to the JAK2 gene. JAK2 is a signaling molecule that is activated by IFNγ signaling, which in turn activates the STAT transcription factors to increase expression of a suite of immunoregulatory genes including PD-L1. Simultaneous gains in JAK2 and PD-L1 copy number can co-exist in lung cancer specimens, which is proposed to cooperatively enhance PD-L1 tumor levels. Consistent with this finding, co-amplification of PD-L1 and JAK2 copy number was associated with higher PD-L1 TPSs in NSCLC. Conversely, PD-L1 copy number losses may result in loss of JAK/STAT signaling within the tumor microenvironment. Consistent with this concept, we found that tumors with a low PD-L1 copy number were phenotypically "cold" as multiple immune modulators were downregulated.

Our study has some limitations, which include a relatively small sample size in the EBUS bronchoscopy-derived cohort. Our study also did not investigate how PD-L1 copy number influenced response to immunotherapy. In addition, our resection cohort is limited to patients with stages I to III disease. Future studies should investigate PD-L1 copy number variations in advanced patients using bronchoscopy-derived biopsies from the primary mass and lymph nodes and determine whether copy number loss translates to immunotherapy resistance in this important group. In summary, this study clearly reveals that real-time PCR-based TaqMan assays can accurately and rapidly quantify PD-L1 copy number levels in small bronchoscopy-derived tumor samples. Future studies should be designed to establish whether a specific cutoff for PD-L1 copy number can be
predictive of response to immunotherapy agents. Our findings also reveal that PD-L1 copy number losses are more frequent in lung adenocarcinomas. Furthermore, PD-L1 copy number losses are representative of a “cold” immunophenotype previously associated with a reduction in tumor-infiltrating lymphocytes and poor response to immunotherapeutic agents. Given the relative simplicity and accuracy of TaqMan-based copy number assays, this approach may complement existing immunotherapy biomarkers to better differentiate responders from nonresponders.

CRediT Authorship Contribution Statement

Savreet Aujla: Investigation, Methodology, Formal analysis, Writing - original draft.

Christian Aloe: Investigation, Methodology, Formal analysis, Writing - review and editing.

Amanda Vannitamby, Shona Hendry, Hao Wang: Investigation, Formal analysis, Writing - review & editing.

Kanishka Rangamuwa: Investigation, Writing - review & editing.

Ross Vlahos, Stavros Selemidis: Formal analysis, Writing - review & editing, Supervision.

Tracy Leong: Investigations, Formal analysis, Writing - review & editing.

Daniel Steinfort: Conceptualization, Investigation, Formal analysis, Project administration, Writing - original draft.

Steven Bozinovski: Conceptualization, Formal analysis, Writing - original draft, Supervision, Funding acquisition, Project administration.
Acknowledgments
The study was partially supported by a research grant from the National Health & Medical Research Council (NHMRC) of Australia (APP1142013).

References


