Clinical Cancer Research

# PD-L2 Expression in Human Tumors: Relevance to Anti-PD-1 Therapy in Cancer

Jennifer H. Yearley[1], Christopher Gibson[2], Ni Yu[1], Christina Moon[1], Erin Murphy[1], Jonathan Juco[1], Jared Lunceford[1], Jonathan Cheng[1], Laura Q.M. Chow[3], Tanguy Y. Seiwert[4], Masahisa Handa[1], Joanne E. Tomassini[1], and Terrill McClanahan[1]

## Abstract

**Purpose:** Tumor-associated PD-L1 expression is predictive of clinical response to PD-1–directed immunotherapy. However, PD-L1–negative patients may also respond to PD-1 checkpoint blockade, suggesting that other PD-1 ligands may be relevant to the clinical activity of these therapies. The prevalence of PD-L2, the other known ligand of PD-1, and its relationship to response to anti-PD-1 therapy were evaluated.

**Experimental Design:** PD-L2 expression was assessed in archival tumor tissue from seven indications using a novel immunohistochemical assay. In addition, relationships between clinical response and PD-L2 status were evaluated in tumor tissues from patients with head and neck squamous cell carcinoma (HNSCC) with recurrent or metastatic disease, treated with pembrolizumab.

**Results:** PD-L2 expression was observed in all tumor types and present in stromal, tumor, and endothelial cells. The prevalence and distribution of PD-L2 correlated significantly with PD-L1 ($P =$ 0.0012–<0.0001); however, PD-L2 was detected in the absence of PD-L1 in some tumor types. Both PD-L1 and PD-L2 positivity significantly predicted clinical response to pembrolizumab on combined tumor, stromal and immune cells, with PD-L2 predictive independent of PD-L1. Response was greater in patients positive for both PD-L1 and PD-L2 (27.5%) than those positive only for PD-L1 (11.4%). PD-L2 status was also a significant predictor of progression-free survival (PFS) with pembrolizumab independent of PD-L1 status. Longer median times for PFS and overall survival were observed for PD-L2–positive than PD-L2–negative patients.

**Conclusions:** Clinical response to pembrolizumab in patients with HNSCC may be related partly to blockade of PD-1/PD-L2 interactions. Therapy targeting both PD-1 ligands may provide clinical benefit in these patients. *Clin Cancer Res; 23(12); 3158–67. ©2017 AACR.*



## Introduction

Immune checkpoint therapies targeting the programmed cell death protein 1 (PD-1) axis have resulted in groundbreaking improvements in clinical response in multiple human cancers (1–9). The interaction of the PD-1 receptor on T cells with its ligands, PD-L1 and PD-L2, on tumor and immune-infiltrating cells regulates T-cell–mediated immune responses and may play a role in immune escape by human tumors (10). Immune therapies targeting the PD-1 axis include monoclonal antibodies directed at PD-1 (nivolumab, pembrolizumab), blocking receptor interaction with both PD-L1 and PD-L2 (11–13), as well as antibodies which bind PD-L1 (atezolizumab), blocking ligand interaction with PD-1 (14). Both therapeutic approaches have demonstrated antitumor effects in several cancer types.

The clinical response to anti-PD-1 targeted therapies can vary in different tumor types, and much effort has been directed toward finding predictive biomarkers to help identify patients who will derive the most benefit from these therapies. Screening of patients eligible for PD-1 axis targeted treatments has primarily focused on the evaluation of PD-L1 expression in tumors, as detected by immunohistochemistry (IHC). Although PD-L1 has demonstrated significant utility as a predictive biomarker in some tumor types, subsets of PD-L1–positive patients have responded poorly to anti-PD-1 axis therapies, whereas some PD-L1–negative patients have shown favorable responses (2, 5, 6, 15, 16). This suggests that molecular interactions with PD-1 other than PD-L1, including PD-L2, may be relevant toward predicting clinical responsiveness to these treatments.

The expression of PD-L2 in tumor tissue and its correlation with response to PD-1 axis targeted therapy has been less well-studied than PD-L1. Similar to PD-L1, PD-1 interaction with PD-L2 inhibits T-cell proliferation, cytokine production, and T-cell cytolysis (17, 18). Previous studies have found PD-L1 to be expressed in T and B cells, dendritic cells, and macrophages as well as non-immune cells, whereas PD-L2 expression has been reported to be more restricted to antigen-presenting cells, although inducible in other immune and non-immune cells by various microenvironmental stimuli (17, 19–21). In limited studies, PD-L2 expression has been demonstrated in human tumors from several indications examined, with expression detected in the absence of PD-L1 in some samples, and varied results regarding its relationship with clinical response (15, 22, 23).

[1]Merck & Co., Inc., Kenilworth, New Jersey. [2]University of Pennsylvania, Philadelphia, Pennsylvania. [3]University of Washington, Seattle, Washington. [4]University of Chicago, Chicago, Illinois.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

**Corresponding Author:** Jennifer H. Yearley, Merck Research Laboratories, 901 S. California Ave, Palo Alto, CA 94304. Phone: 650-496-1177; Fax: 267-305-6529; E-mail: jennifer.yearley@merck.com

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## Translational Relevance

Tumor-associated PD-L1 expression has been shown to be a predictive marker for response to anti-PD-1 axis targeted therapies. Nonetheless, not all PD-L1–positive patients show clinical responses to such therapies, and some PD-L1–negative patients do respond. This suggests that other molecular interactions with PD-1, such as interactions with PD-L2, may also be important in predicting patient responses. However, studies that assess the prevalence and distribution of PD-L2 in human tumors have been limited. In this analysis of more than 400 archival tumor samples, PD-L2 expression was observed in seven different tumor types and was expressed in the absence of PD-L1 in subsets of patient samples. Moreover, PD-L2 expression was independently associated with clinical response in pembrolizumab-treated patients, indicating that presence or absence of PD-L2 expression may also play a role in response to PD-1 axis targeted therapies.

Given that expression of PD-L2 either alone or in combination with PD-L1 could impact the efficacy of therapies targeting the PD-1 axis, this study assessed the prevalence and distribution of PD-L2 in more than 400 archival human tumor samples across 7 cancer indications using a novel PD-L2 IHC assay. The potential relevance of PD-L2 status in patient responsiveness to inhibition of the PD-1 checkpoint with the anti-PD-1 antibody, pembrolizumab, was also evaluated in 172 patients with head and neck squamous cell carcinoma (HNSCC).

## Materials and Methods

A novel IHC assay for PD-L2 protein detection was developed and applied to formalin-fixed, paraffin-embedded (FFPE) sections of archival human tumor tissue from the Merck Palo Alto tissue bank. Results of PD-L2 IHC staining were compared to results of PD-L1 IHC staining (Merck clone 22C3) and to PD-L2 mRNA levels as determined using the NanoString platform. Tissue specimens were obtained with the approval of the institutional review boards and patients provided informed consent.

### IHC assay development

***Antibody generation and specificity.*** The primary antibody for PD-L2 IHC in this study, clone MEB123.3G2.038 (3G2), was generated through immunization of mice with a combination of human PD-L2-Fc (amino acids 20-219) and human PD-L2-His (amino acids 1-219) fusion proteins and was identified by screening supernatants from 446 hybridomas (Merck Research Laboratories). Binding of clone 3G2 to PD-L2 but not PD-L1 was assessed by ELISA. Recombinant PD-L2 and PD-L1 proteins were coated on a plate at 1 μg/mL, and MEB123.3G2.038.14E (3G2) was incubated with the proteins starting at 3 μg/mL and serially diluted 1:3 in an 11-point titration curve. Following binding with 3G2, a goat anti-mouse-HRP detection antibody was incubated in the wells and binding detected using 3,3′,5,5′-tetramethylbenzidine (TMB; Supplementary Fig. S1). Clone 3G2 was evaluated on a panel of 37 normal human tissues, where abundant expression of PD-L2 was detected in placenta, in hepatocytes in liver, as well as in multiple lymphoid tissues. Appropriateness of IHC signal distribution was assessed by comparison to PD-L2 mRNA distribution as detected by *in situ* hybridization (ISH; RNAscope, Advanced Cellular Diagnostics; Supplementary Fig. S2). ISH mRNA distribution corroborated IHC signal appropriateness for placenta and lymphoid tissues but did not support the hepatocyte signal as specific (strong cytoplasmic staining by IHC, no PD-L2 mRNA by ISH). Because of the finding of off-target binding in normal hepatocytes, a conservative approach was taken in assessing appropriateness of labeling in tumor tissues, where routine cross-checking by ISH was performed to ensure that mRNA patterns matched those of the protein. No off-target binding was identified in the tumor tissues evaluated. Further assessment of signal specificity was performed by conducting a blocking study on PD-L2–positive normal human tissues, in which pairs of slides from each tissue were evaluated: one with 3G2 pre-adsorbed with immunogen and one with 3G2 alone (Supplementary Fig. S3). The appropriateness of dynamic range detection was assessed by staining FFPE pellets from 12 cell lines with widely varying PD-L2 mRNA content, including NCIH226, HOP92, and SKBR3, and then correlating IHC staining with mRNA content (Supplementary Fig. S4). Staining was also assessed for reproducibility using 3 normal tonsils stained on 3 successive days with pathologist assessment of output comparability. The PD-L2 IHC signal also significantly correlated with PD-L2 mRNA levels quantitated by NanoString methodology in human tumor samples ($P < 0.0001$ to $P = 0.0037$; Supplementary Fig. S5). Overall, these data demonstrate the high specificity of anti-PD-L2 antibody for binding to PD-L2.

***Staining.*** FFPE tissue sections were routinely deparaffinized and rehydrated for PD-L2 and PD-L1 IHC. All slides were subjected to heat-induced epitope retrieval in a PT Link unit (PT10027, Dako) at 97°C for 20 minutes using FLEX high pH target retrieval solution (K8012, Dako). Slides were stained on Dako Autostainers using the Envision FLEX Kit, High pH (Plus) with mouse linker (K8012, Dako) according to manufacturer's instructions. Primary antibodies (anti-PD-L2 clone 3G2 at 0.8 μg/mL or anti-PD-L1 clone 22C3, Merck Research Laboratories, at 2 μg/mL) were incubated on slides for 60 minutes. Antigen–antibody binding was visualized with 3,3′-diaminobenzidine (DAB) chromogen (K8012, Dako), and slides were counterstained with Mayer hematoxylin (S216-1GL, Polyscientific).

***Scoring of archival tumor specimens.*** Archival FFPE tumor specimens were sourced from the Merck Palo Alto tissue bank. Scoring was conducted by a pathologist, with scores incorporating prevalence of both tumor cell and non-tumor cell labeling. A semiquantitative 0–5 scoring system was applied such that 0 = no staining; 1 = rare individuated positive cells or only very small focus within or directly adjacent to tumor tissue; 2 = infrequent small clusters of positive cells within or directly adjacent to tumor tissue; 3 = single large cluster, multiple smaller clusters, or moderately dense diffuse infiltration, within or directly adjacent to tumor tissue; 4 = single very large dense cluster, multiple large clusters or dense diffuse infiltration; 5 = coalescing clusters, dense infiltration throughout the tumor tissue. Presence or absence of endothelial cell expression was evaluated specifically as a separate value.

***In situ hybridization.*** Cellular distribution of PD-L2 mRNA was evaluated by ISH using the RNAscope platform (RNAscope 2.0

High Definition Kit, Advanced Cell Diagnostics) according to manufacturer's instructions. Hybridization was conducted using anti-sense and sense DNA probes for human PD-L2 (test probe and negative control, respectively) and anti-sense probe for PPIB (positive control), all designed by Advanced Cell Diagnostics (catalog numbers 316291, 551891, and 313901, respectively).

### Gene expression analysis

***Quantitative RT-PCR.*** For real-time, quantitative PCR analysis, DNase-treated total RNA was reverse-transcribed using QuantiTect Reverse Transcription (Qiagen) according to manufacturer's instructions. Primers specific for PDCD1LG2 (PD-L2, CD273) were obtained commercially from Applied Biosystems. Real-time quantitative PCR was performed on the Fluidigm Biomark using specific probe/primer mix with TaqMan Universal PCR Master Mix with uracil-DNA glycosylase. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the $\Delta C_t$ method.

***NanoString methodology.*** Tissue lysates were generated from sectioned FFPE tissue according to the manufacturer's protocol (NanoString). Cellular lysate (50 ng per sample) was mixed with a barcoded 3′-biotinylated capture probe and a fluorescently tagged 5′ reporter probe from the desired gene expression codeset. Probes and target transcripts were hybridized overnight as per manufacturers' recommendations. Hybridized samples were run on the NanoString nCounter instrument and then samples were scanned at maximum scan resolution using the nCounter Digital Analyzer.

Data analysis was performed using quantile normalization in which relative ranks of genes (across all genes on the NanoString codeset) within each sample were replaced by values having the same relative rank from the pooled distribution (from all samples and genes in the dataset). All quantile-normalized data underwent subsequent $\log_{10}$ transformation.

### Correlation of PD-L2 expression and clinical response to anti-PD-1 therapy

The relationship between PD-L1 and PD-L2 expression and clinical response to pembrolizumab therapy was explored in tumor tissue samples from 172 PD-L1-positive and -unselected patients with HNSCC from the KEYNOTE-12 trial (24, 25). Pretreatment samples were included from patients with HNSCC with recurrent or metastatic disease measurable per RECIST 1.1, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, treated with 200 mg pembrolizumab every 3 weeks or 10/mg/kg every 2 weeks, with PD-L1 and PD-L2 IHC scoring data available. Expression for both analytes was scored using a 1% positivity cutoff (positive $\geq$ 1%; negative < 1%) that included evaluation of both tumor- and immune-infiltrating cells. PD-L2 expression was also assessed in tumor cells alone at a later time by a different pathologist, as was previously reported for PD-L1 expression (24). Overall response rate (ORR) was assessed in 146 of these patients in the full analysis set population defined as those who had received $\geq$1 dose of study drug, had a baseline disease measurement and $\geq$1 post-baseline scan, or who had discontinued drug due to a drug-related adverse experience or clinical progressive disease. Progression-free survival (PFS) and overall survival (OS) were assessed in the 172 all-patients-as-treated population, defined as those who had received $\geq$1 dose of study drug. Relationships with PD-L2 expression were explored by logistic (ORR) or Cox (PFS, OS) regression analyses with or without adjustment for variation in clinical response explained by PD-L1 expression (i.e., including a term in the regression model for PD-L1 positivity status in addition to the term for PD-L2 positivity status). *P* values reported are one-sided in the direction of the hypothesis of improved clinical outcome in patients positive for PD-L1 or PD-L2 expression. Kaplan–Meier curves were used to estimate the median survival times for PFS and OS in PD-L2–positive and -negative patients.

## Results

### PD-L2 expression in tumor and immune cells

The expression of PD-L2 protein was assessed in cohorts of several tumor types including renal cell carcinoma (RCC; $n = 71$), bladder carcinoma ($n = 34$), melanoma ($n = 83$), non–small cell lung cancer (NSCLC; $n = 94$), HNSCC ($n = 40$), triple-negative breast cancer (TNBC; $n = 22$), and gastric carcinoma ($n = 73$) by IHC staining with 3G2 anti-PD-L2 antibody. As shown in Fig. 1A, PD-L2 protein was expressed to varying degrees on stromal cells (including immune cell infiltrate), endothelium, and tumor cells.

Each cohort was evaluated for the overall prevalence of PD-L2 expression, with stromal, tumor, and endothelial cells evaluated together. Although PD-L2 expression was observed in all tumor types assessed, the overall prevalence of PD-L2 expression differed by indication (Fig. 1B). RCC was noteworthy for a predominance of low overall levels of PD-L2 expression, whereas gastric cancer and TNBC demonstrated moderate-to-high expression. Expression in other tumor types distributed more broadly from low to high across evaluated samples.

When the presence or absence (scores $\geq$1 and <1, respectively, on a 0–5 scale) of PD-L2 protein expression was evaluated by IHC staining in the 3 categories of stromal, tumor, and endothelial cells for each tumor type, several patterns emerged (Fig. 1C). The presence of PD-L2 expression in stromal cells, including immune cell infiltrate, was generally the most common and was observed across all tumor types with relatively minimal variation. In contrast, PD-L2 expression in tumor cells varied quite significantly across tumor types, with none of the RCCs and few of the melanoma samples demonstrating tumor cell expression, whereas more than half of the HNSCC samples expressed PD-L2. Finally, while endothelial cell expression was present in a minority of samples for most of the tumor types assessed, the prevalence of samples with endothelial expression was notably higher in RCC and gastric carcinomas.

The relative prevalence and distribution of PD-L2 protein in tumor tissues in these cohorts was compared with that of PD-L1 in additional sections of the same samples, using Merck's 22C3 anti-PD-L1 IHC antibody (Fig. 2). In general, distributional patterns and prevalence of PD-L2 closely mirrored those of PD-L1, as illustrated in Fig. 2A and B. At higher magnification (Fig. 2I and J), intratumoral and peripheral expression of both PD-L1 and PD-L2 were evident. However, a significant number of samples exhibited discordance between PD-L2 and PD-L1 with some showing PD-L1 signal in the absence of PD-L2, as observed in Fig. 2C and D, and other samples displaying PD-L2 expression in the absence of PD-L1, as seen in Fig.2E–H. The percentage of samples in each cohort that showed PD-L1 and PD-L2 expression scores differing by greater than or equal to 2 increments on the 0–5 scale employed is presented in Fig. 2K.

When the overall expression of PD-L1 and PD-L2 was compared across all samples (using the same 0 to 5 scoring system and

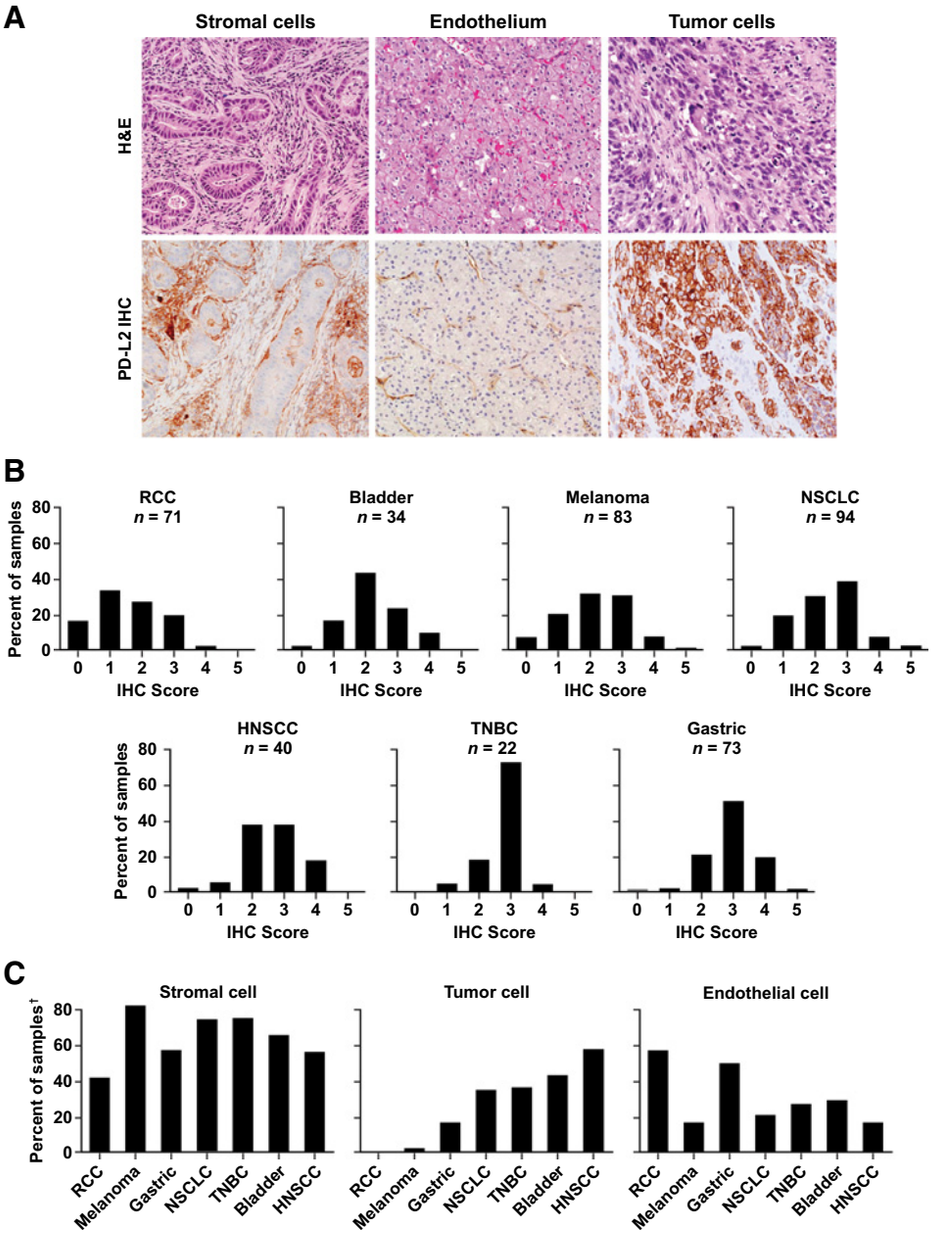


**Figure 1.**

Distribution and expression of PD-L2 in human tumors. IHC staining of PD-L2 protein in tumor samples with 3G2 anti-PD-L2 monoclonal antibody. **A,** Distribution patterns. 3G2 anti-PD-L2 in bottom panels and hematoxylin and eosin (H&E) in top panels for gastric cancer stromal cells (includes immune cell infiltrate), RCC, endothelium, and melanoma tumor cells. **B,** PD-L2 expression in various tumor types. Prevalence of stromal, endothelial, and tumor cell expression is taken together. IHC scoring on a 0–5 semiquantitative scale: 0 = negative, 1 = rare, 2 = low, 3 = moderate, 4 = high, 5 = very high. **C,** PD-L2 expression in stromal (includes immune cell infiltrate), tumor, and endothelial cells of various tumor types. Presence (≥1) or absence (<1) of IHC staining on a 0–5 scale. †, Percentage of sample with PD-L2 expression score of ≥1 on a 0–5 scale.

evaluating combined expression by both tumor and non-tumor cells for both biomarkers), the scores were found to be significantly correlated ($P = 0.0012$ to $P < 0.0001$) for all indications examined (Fig. 3). The strongest relationship between PD-L1 and PD-L2 ($R^2 = 0.6238$) was observed for TNBC, with no significant discordance between PD-L2 and PD-L1 expression for any of the samples. For all other indications, while PD-L2 and PD-L1 expression scores were significantly correlated, discordant expression was observed in some samples. Bidirectional discordant expression was observed for melanoma and RCC, with some samples showing PD-L1 expression well in excess of PD-L2, and others displaying PD-L2 expression well in excess of PD-L1. Primarily unidirectional discordance was observed in other indications examined, with PD-L1 expressed in excess of PD-L2 in a subset of NSCLC and bladder tumor samples, but PD-L2 expressed in excess of PD-L1 in a subset of HNSCC and gastric tumor samples.

### Relationship of PD-L2 expression and clinical response to anti-PD-1 therapy

The clinical relevance of PD-L2 expression was evaluated in tumor tissue samples derived from 172 pembrolizumab-treated patients with HNSCC with recurrent or metastatic disease in the KEYNOTE-12 trial who had PD-L2 and PD-L1 IHC scoring data available. The median age of the patients sampled was 60 years (range, 37–84 years), most were male (83.1%) and a large proportion were human papillomavirus (HPV)-negative (65.7%; Table 1). The majority of patients were ECOG status 1 (71.5%) with metastatic staging of M1 (84.9%) and many (60.4%) had received ≥2 prior therapies for recurrent or

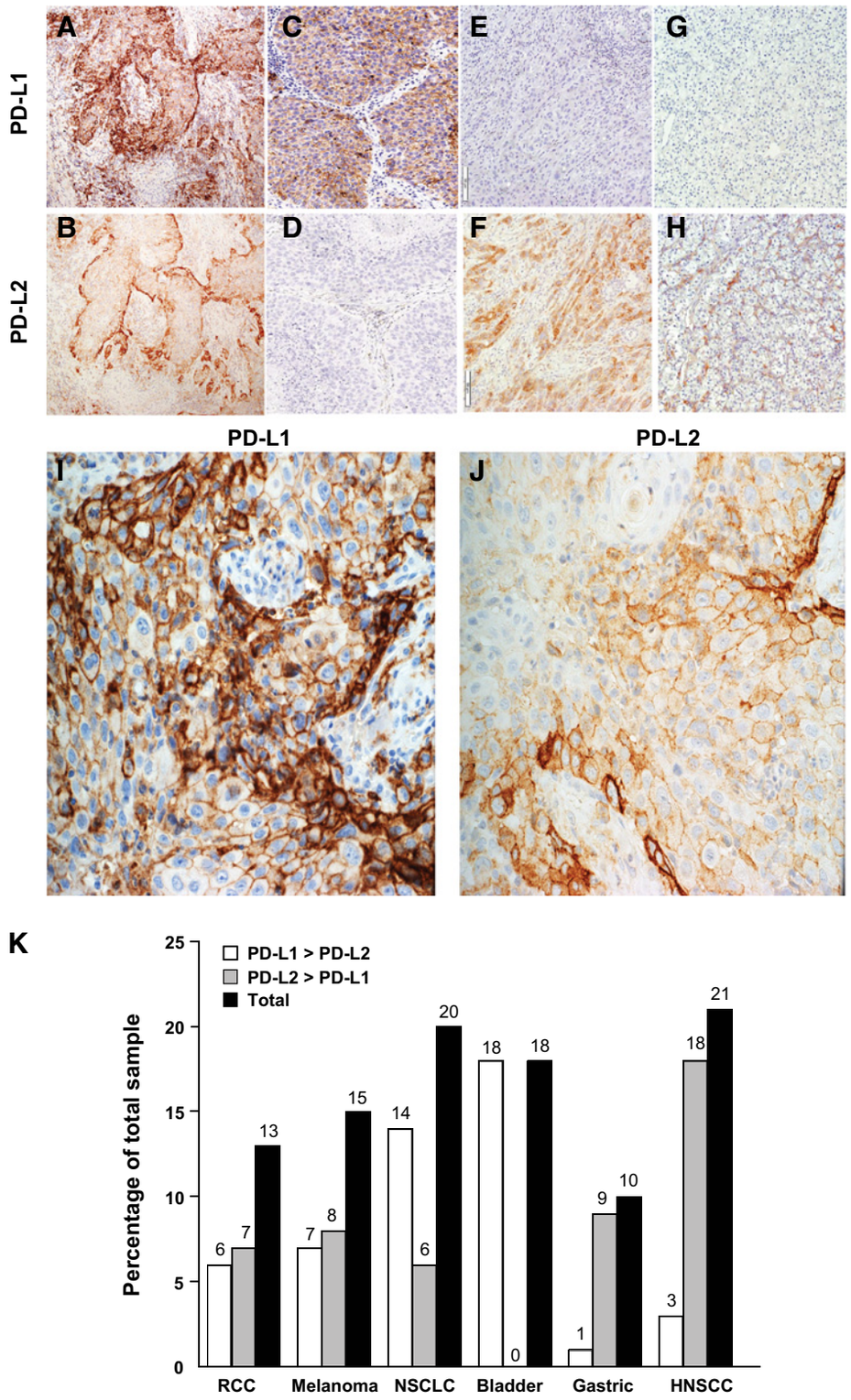


**Figure 2.**
Concordance of PD-L1 and PD-L2 expression in tumors. PD-L1 (top) and PD-L2 (bottom) IHC staining of the same tumor samples showing comparable distribution and positive cell prevalence (**A** and **B**), PD-L1 expression in the absence of PD-L2 (**C** and **D**), PD-L2 expression in the absence of PD-L1 (**E–H**), and higher magnification of **A** and **B** displaying intratumoral staining of PD-L1 and PD-L2 (**I** and **J**; anti-PD-L1 clone 22C3; anti-PD-L2 clone 3G2). Percentage of total samples where PD-L1 and PD-L2 expression differed by $\geq$2 IHC scores evaluated in combined tumor and non-tumor cells on 0–5 scale (**K**).

metastatic disease. In these 172 patients, PD-L2 positivity was significantly associated with PD-L1 positivity ($P < 0.001$), with 108 of 147 (73.5%) PD-L1–positive tumors being PD-L2–positive, whereas only 3 of 25 (12%) of PD-L1–negative tumors were PD-L2–positive. It should be noted that in 105 of these patients who had both IHC data and HPV status, neither PD-L1 nor PD-L2

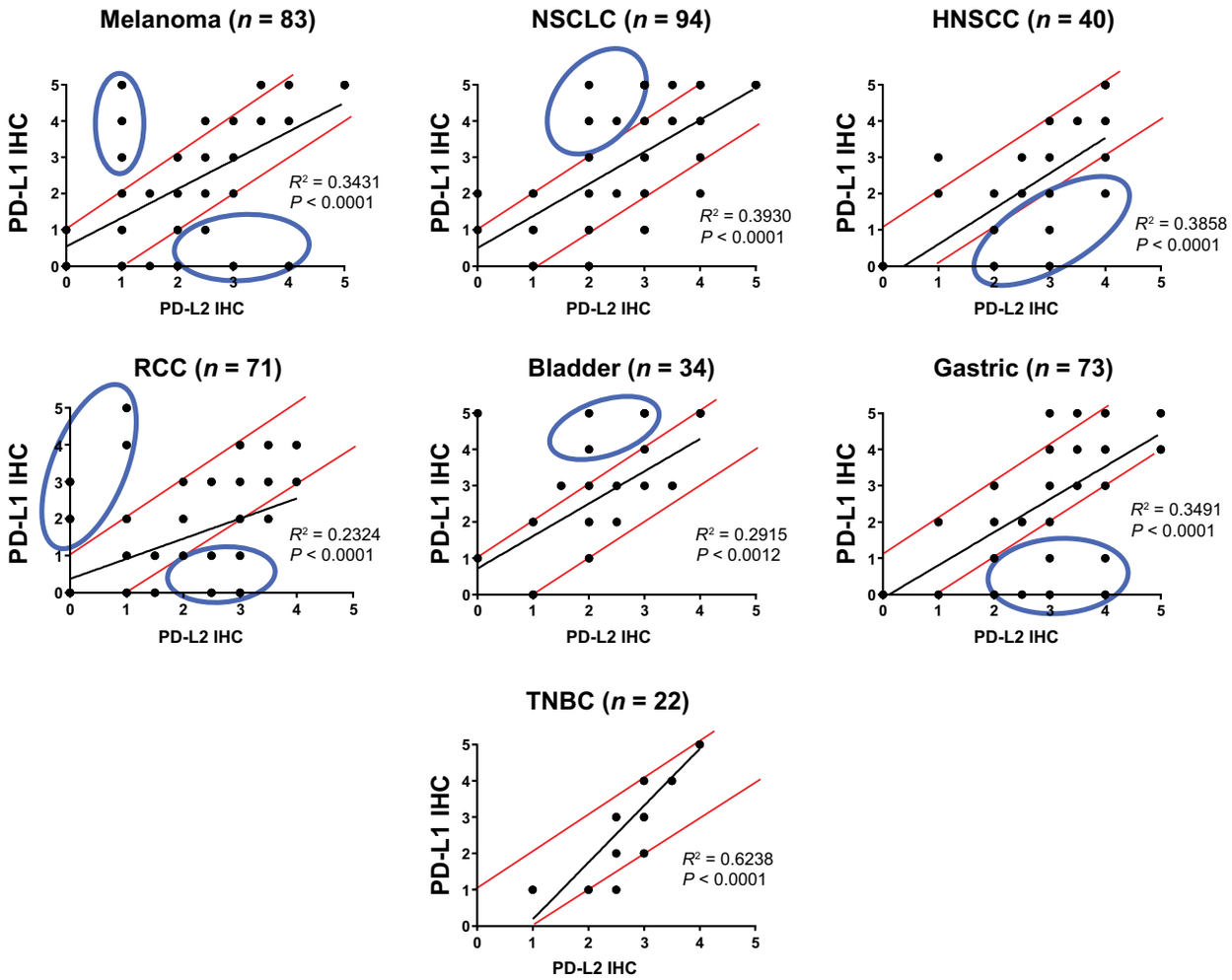


**Figure 3.**

Relationship of PD-L1 and PD-L2 in tumor types. Correlation plots of overall expression between PD-L1 and PD-L2 across all samples using the same 0–5 scoring system for both analytes. Numbers of evaluated tissues range from 22 (TNBC) to 94 (NSCLC). Dots for samples with identical scores in both assays overlap. Scores were significantly correlated in all indications ($P = 0.0012$ to $P < 0.0001$). Blue circles highlight samples where scores for PD-L1 and PD-L2 substantially differed.

positivity was significantly associated with HPV status at the 0.05 significance level.

ORRs were assessed as a function of PD-L1 and PD-L2 status (positivity cutoff $\geq$ 1%) in 146 patients in the full analysis set population by IHC staining in combined tumor- and immune-infiltrating cells. Of these, 126 (86.3%) patients had tumors that were scored as PD-L1–positive, 94 (64.3%) as PD-L2–positive, 20 (13.6%) as PD-L1–negative, and 52 (35.6%) as PD-L2–negative (Table 2). The response rates in the PD-L1 [23.0%; 95% confidence interval (CI), 16.0–31.4] and PD-L2 (26.6%; 95% CI, 18.0–36.7)–positive patients were both numerically higher than the response rates in the PD-L1- and PD-L2–negative patients (5.9%; 95% CI, 0.1–28.7; Table 2). Further evaluation of PD-L2 status in a logistic regression model adjusting for PD-L1 status suggested that PD-L2 positivity provided additional predictive value for determining response ($P = 0.038$). The ORR was greatest in patients who were positive for both PD-L1 and PD-L2 and was 2-fold higher (27.5%; 95% CI, 18.6–37.8) than in patients whose tumors were positive only for PD-L1 (11.4%; 95% CI, 3.2–26.7). When PD-L2 expression was evaluated in tumor cells only, PD-L2–positive (cutoff $\geq$ 1%) patients showed an ORR of 26.5% (95% CI, 14.9–41.1) and PD-L2–negative patients showed an ORR of 16.7% (95% CI, 9.8–25.6), the latter reflecting the poorer sensitivity to detect responders when PD-L2 was scored in tumor alone (44.8%) than when scored in both tumor and inflammatory cells (83.3%). Logistic regression testing adjusting for PD-L1 status did not show statistically significant additional predictive value for determining response when PD-L2 was scored on tumor alone ($P = 0.188$). These results are consistent with previous findings in this cohort showing that PD-L1 expression in combined tumor and immune cells was significantly associated with response to pembrolizumab, whereas expression in tumor cells alone was not (24).

In the overall cohort of all 172 patients (all-patients-as-treated population), the relationships between PFS and PD-L1 and PD-L2 status were each assessed individually. PD-L1–positive versus

**Table 1.** Baseline characteristics of patients in HNSCC cohort

| Characteristic | Total $N = 172$ |
|---|---|
| Age, median (range), y | 60 (37–84) |
| Male | 143 (83.1) |
| Race | |
| White | 129 (75.0) |
| Asian | 27 (15.7) |
| Other | 16 (9.3) |
| ECOG performance status | |
| 0 | 49 (28.5) |
| 1 | 123 (71.5) |
| Metastatic staging | |
| MX | 1 (0.6) |
| M0 | 25 (14.5) |
| M1 | 146 (84.9) |
| HPV status | |
| Positive | 57 (33.1) |
| Negative | 113 (65.7) |
| Unknown | 2 (1.2) |
| Sum of target lesions at baseline, median (range),[a] mm | 99.2 (10–664) |
| Previous adjuvant and/or neoadjuvant therapy | |
| Yes | 81 (47.1) |
| No. of previous lines of therapy for recurrent or metastatic disease | |
| 0 | 32 (18.6) |
| 1 | 36 (20.9) |
| 2 | 40 (23.3) |
| 3 | 30 (17.4) |
| 4 | 19 (11.0) |
| ≥5 | 15 (8.7) |

NOTE: All-patients-as-treated population.
[a]$n = 157$.

-negative status was not significantly associated with PFS at the 0.05 level ($P = 0.080$). However, PD-L2 positivity was a statistically significant predictor of PFS ($P = 0.005$) and remained significantly associated with PFS after adjustment for PD-L1 positivity status ($P = 0.013$). The relationships between OS and PD-L1 and PD-L2 were similarly assessed. A statistically significant association of PD-L1 status with OS ($P = 0.033$) was observed. PD-L2 status was also significantly associated with OS (0.030), and in a model that included terms for both measures, after adjustment for PD-L1 status, PD-L2 status was no longer significant ($P = 0.112$). The median PFS times for PD-L2–negative and PD-L2–positive patients were 59 and 65 days, respectively, and median OS times were 199 and 303 days, respectively (Fig. 4).

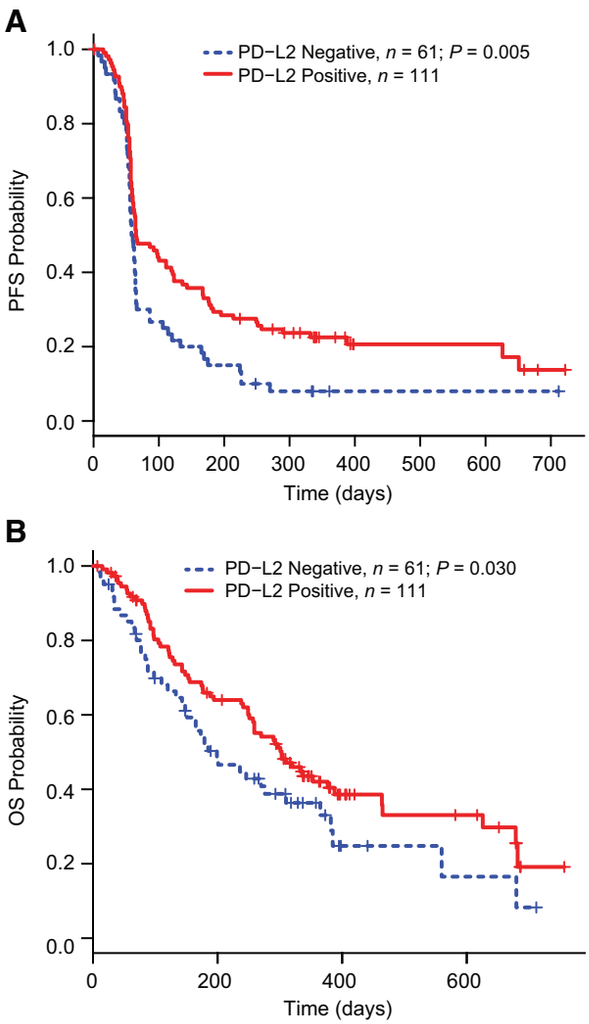


**Figure 4.**
PFS and OS by PD-L2 status. Kaplan–Meier curve showing PFS (**A**) and OS (**B**) for PD-L2–positive ($n = 111$) and PD-L2–negative ($n = 61$) tumor samples (tumor and immune cells) from 172 all-patients-as-treated population in KEYNOTE-12.

## Discussion

In this study, PD-L2 expression was assessed across more than 400 archival samples from 7 tumor types using a novel IHC assay. PD-L2 expression generally correlated with that of PD-L1; however, PD-L2 expression was also present in the absence of PD-L1 in subsets of patient samples. In a cohort of pembrolizumab-treated patients with HNSCC, PD-L2 positivity was significantly associated with ORR regardless of PD-L1 status, and ORR was greatest in patients expressing both PD-L1 and PD-L2 ligands. PD-L2 expression was a significant predictor of PFS and was associated with longer median survival times for both PFS and OS. These findings suggest that PD-L2 may play a role in clinical responses observed with anti-PD-1 therapy, consistent with the ability of PD-1 antibodies like pembrolizumab to block the interaction of PD-1 with both PD-L1 and PD-L2.

PD-L1 expression has been shown to be related to clinical response to anti-PD-1 axis therapies in NSCLC (2, 26–28), metastatic urothelial cancer (29–31), and melanoma (4–6,

**Table 2.** PD-L1 and PD-L2 status and overall clinical response

| Status | Total | Non-responder | Responder | Response, % (CI) |
|---|---|---|---|---|
| PD-L1$^-$ | 20 | 19 | 1 | 5.0 (0.1–24.9) |
| PD-L1$^+$ | 126 | 97 | 29 | 23.0 (16.0–31.4) |
| PD-L2$^-$ | 52 | 47 | 5 | 9.6 (3.2–21.0) |
| PD-L2$^+$ | 94 | 69 | 25 | 26.6 (18.0–36.7) |
| PD-L1$^-$/PD-L2$^-$ | 17 | 16 | 1 | 5.9 (0.1–28.7) |
| PD-L1$^+$/PD-L2$^-$ | 35 | 31 | 4 | 11.4 (3.2–26.7) |
| PD-L1$^-$/PD-L2$^+$ | 3 | 3 | 0 | 0.0 (0.0–70.8) |
| PD-L1$^+$/PD-L2$^+$ | 91 | 66 | 25 | 27.5 (18.6–37.8) |

NOTE: Full analysis set population. Both PD-L1 and PD-L2 expression were evaluated by IHC staining in combined tumor and inflammatory cells. Positive (+), ≥1% staining; negative (−), <1% staining.

32–34). While PD-L1 is predictive of response in all of these tumor types, low response rates are still observed in PD-L1–negative patients. Several possible explanations have been proposed for these findings, including intratumoral heterogeneity of PD-L1 expression, the dynamic nature of PD-L1 expression in the tumor microenvironment, and variation in detection methods as well as differing cell types evaluated (35). PD-L1 expression is often found in regions of active T-cell inflammation within the tumor environment, driven by signals such as IFNγ in response to immune-mediated attack, thus its presence may be indicative of an immune active milieu engaged in an antitumor response (34–37). However, some oncogenic signals also induce PD-L1 expression, and further studies are needed to better understand the underlying mechanisms involved in induction of PD-L1 expression and their relationship to clinical response to PD-1 checkpoint blockade (38, 39).

Limited studies have assessed a potential role for PD-L2 in predicting patient response to anti-PD-1 axis therapy (15, 22, 23). PD-L2 has been found to be highly upregulated on certain B-cell lymphomas, including primary mediastinal and follicular lymphomas, as well as Hodgkin lymphoma (40, 41). In a recent study of 38 pretreatment tumor specimens from patients with advanced and refractory cancers using a different PD-L2 IHC assay from that employed here, 8 (21%) of the evaluated specimens demonstrated PD-L2 expression, including RCC ($n = 1$), melanoma ($n = 5$), and NSCLC ($n = 2$; ref. 22). PD-L2 protein was observed in tumor cells or infiltrating immune cells and was associated with PD-L1 expression in all cases but one; however, correlation of PD-L2 expression with response to PD-1 axis targeted therapy was not reported. In another analysis of tumor samples from patients with advanced cancer (NSCLC, melanoma, RCC, colorectal, gastric, and HNSCC), PD-L2 expression did not appear to be associated with resistance to anti-PD-L1 therapy, and some patients with PD-L2–positive tumors showed objective clinical responses (15). Recently, PD-L2 RNA expression was detected in RCC, melanoma, metastatic urothelial, and NSCLC tumors in immune-infiltrating cells and generally correlated with that of PD-L1. PD-L1 and PD-L2 protein expression were not evaluated in this study (23). Higher levels of both PD-L1 and PD-L2 RNA were associated with improved OS to anti-PD-L1 therapy with atezolizumab across the 4 tumor types. However, these studies did not assess whether PD-L2 was predictive of response independent of PD-L1 status and did not assess responses in patients who were discordant for PD-L1 and PD-L2 expression.

In our study, PD-L2 expression was detected by IHC staining to some extent in all 7 tumor types assessed, with the highest expression levels in TNBC and gastric carcinoma, rare-to-low expression in RCC, and moderate expression in bladder, NSCLC, HNSCC, and melanoma. PD-L2 expression was detected with the highest frequency in stromal cells including immune cell infiltrate and was also found in endothelial cells and in tumor cells with more variability across tumor types. The finding of endothelial PD-L2 expression in many tumors is of particular interest given the high potential for interaction of PD-L2 with PD-1 on T cells exiting the vasculature and trafficking into tumor tissue. The fact that this interaction may be physiologically relevant is supported by evidence which has demonstrated the capacity of endothelial PD-L2 to downregulate CD8 T-cell activation and cytolysis.(18) However, an analysis of the relationship between endothelial cell PD-L2 expression and response in patients with HNSCC in our study did not show significant predictive value (data not shown). Although the expression of PD-L1 and PD-L2 was strongly correlated in all the tumor types evaluated in our analysis, PD-L2 was expressed within some tumors in the absence of PD-L1 and was independently associated with clinical response in a cohort of pembrolizumab-treated patients with HNSCC when assessed in combined tumor and immune cells. The correlation of PD-L1 and PD-L2 expression detected by IHC across tumor types and the higher ORR to pembrolizumab observed in patients expressing both ligands in our study is consistent with the known upregulation of both PD-L1 and PD-L2 in the INFγ pathway (17, 22, 42). The differential expression of PD-L1 and PD-L2 observed in some tumor types may be related to other additional inducers of PD-L2 expression (17, 22, 42).

Strengths of this study include the evaluation of PD-L2 expression in a large number of samples across 7 indications and the ability to assess the relationship between PD-L2 status and clinical response to anti-PD-1 therapy with pembrolizumab. Although a highly selective IHC assay was validated and optimized for PD-L2 detection in this study, direct comparison of our results to other studies is limited by technical differences in antibodies, staining methods, and scoring methods. A positivity cutoff for PD-L2 expression was not designated in this exploratory analysis of archival samples, and as such, the expression data are described in relative terms. We were also unable to assess PD-L2 expression in relation to patient clinical characteristics for the 400 archival samples, due to heterogeneous and sometimes sparse clinical annotation, nor the relationship of PD-L2 to other components of the immune milieu in the patients with HNSCC due to limitations on tissue availability. Thus, future studies are needed to address these relationships. It should be noted that although PD-L2 was present to some extent in all tumor indications evaluated, assessment of clinical response to pembrolizumab therapy was conducted only in patients with HNSCC. Although the expression of PD-L2 in combined tumor- and immune-infiltrating cells appeared to be more sensitive for detecting responders than expression in tumor cells alone, these results are considered exploratory because the study was not designed to make a formal comparison between these 2 methods. Nonetheless, these results are consistent with previous observations showing that PD-L1 expression in combined tumor- and immune-infiltrating cells is more predictive of response than expression in tumor cells alone in patients with HNSCC (24).

In summary, our study showed that PD-L2 expression is present in many tumor types, and while generally associated with PD-L1, can also occur in the absence of PD-L1, despite the fact that both ligands are generally upregulated in T-cell–inflamed microenvironments in the presence of IFNγ. Moreover, PD-L2 expression was independently associated with improved clinical outcomes including high ORRs and longer PFS in patients with HNSCC. This suggests that PD-L2 expression may provide information beyond that of PD-L1 in predicting clinical response to anti-PD-1 targeted agents, which block the interactions of both PD-L1 and PD-L2 with PD-1 and may help in identifying patients who may derive benefit from these therapies. Further studies are needed to more fully understand the clinical relevance and predictive value of PD-L2 in cancer immunotherapy.

## Disclosure of Potential Conflicts of Interest

L.Q.M. Chow is a consultant/advisory board member for Merck. T. Y. Seiwert reports receiving speakers bureau honoraria from Merck/MSD and is a

consultant/advisory board member for Amgen, Astra Zeneca, Bristol-Myers Squibb, Celgene, Eli Lilly, Innate, Jounce, Merck/MSD, and Merck-Serono. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** J.H. Yearley, C. Gibson, J. Cheng
**Development of methodology:** J.H. Yearley, N. Yu, C. Moon, M. Handa
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.H. Yearley, N. Yu, C. Moon, E. Murphy, J. Cheng, L.Q.M. Chow, T. Y. Seiwert, M. Handa
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.H. Yearley, C. Gibson, E. Murphy, J. Lunceford, J. Cheng, L.Q.M. Chow, T. Y. Seiwert, M. Handa, J.E. Tomassini
**Writing, review, and/or revision of the manuscript:** J.H. Yearley, C. Gibson, N. Yu, C. Moon, E. Murphy, J. Juco, J. Lunceford, J. Cheng, L.Q.M. Chow, T. Y. Seiwert, M. Handa, J.E. Tomassini, T. McClanahan
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.H. Yearley, C. Moon
**Study supervision:** J.H. Yearley, T. McClanahan
**Other (*submission of manuscript to the journal*):** J.E. Tomassini

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