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ARTICLE

A pan-cancer analysis of PD-L1 immunohistochemistry and gene amplification, tumor mutation burden and microsatellite instability in 48,782 cases

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Abstract

PD-L1 immunohistochemistry (IHC) currently has the most Food and Drug Administration (FDA) approvals as a companion diagnostic (CDx) for immunotherapies in specific tumor types; however, multiple other immunotherapy biomarkers exist. We performed this study to examine and report the prevalence of PD-L1 expression in a wide variety of tumor types and examine its relationship to microsatellite instability (MSI), tumor mutational burden (TMB), and *CD274* (PD-L1) gene amplification. We performed a retrospective analysis of all cases in which both PD-L1 IHC (using the DAKO 22C3 IHC assay with either tumor proportion score (TPS) or combined positive score (CPS); or the VENTANA SP142 assay with infiltrating immune cell score (IC)) and comprehensive genomic profiling (CGP) were tested at Foundation Medicine between January 2016 and November 2019. Of note, PD-L1 positivity is defined per the CDx indication and tumor proportion score (TPS ≥ 1) for indications without a CDx claim; and TMB positivity is defined as ≥10 mutations/Mb. A total of 48,782 cases were tested for PD-L1 IHC and CGP. Immune cell expression of PD-L1 was more frequently identified than tumor cell expression of PD-L1. We saw a high correlation between PD-L1 expression and *CD274* gene amplification ($p < 0.0001$), MSI and TMB ($p < 0.0001$), and PD-L1 and TMB ($p < 0.0001$). In addition, the combination of PD-L1 and TMB identified four unique disease subsets PD-L1$^-$/TMB$^-$, PD-L1$^+$/TMB$^-$, PD-L1$^-$/TMB$^+$, and PD-L1$^+$/TMB$^+$ with varying prevalence dependent on tumor type. Lastly, 50.3% (24527/48782) of the overall cohort was positive for at least one of the CDx or exploratory biomarkers described above. This is the largest pan-cancer analysis of relevant biomarkers associated with response to checkpoint inhibitors to date, including more than 48,000 cases. Additional clinical trials with treatment outcome data in individual tumor types are needed to determine whether the double positive PD-L1$^+$/TMB$^+$ disease subset would respond best to immunotherapy.

Introduction

Cancer care has been revolutionized by immunotherapy, leading to multiple regulatory approvals for program death-ligand 1 and programmed cell death protein 1 (PD-L1 and PD-1) immunotherapies in recent years due to the high efficacy and relatively low toxicity of immunotherapy [1]. PD-1 targeted immunotherapies such as pembrolizumab and nivolumab have been approved by the United States Food and Drug Administration (US-FDA) for multiple indications such as non-small cell lung cancer (NSCLC) and melanoma [2, 3]. Furthermore, PD-L1 immunotherapies such atezolizumab have been recently approved in difficult-to-treat cancer types such as urothelial bladder carcinoma,

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triple negative breast carcinoma (TNBC) and small cell lung cancer, diseases which have not benefited from new treatments with significant increased survival in decades [4–7]. Drug development for immunotherapies has included the investigation of multiple biomarkers as predictors of clinical benefit to checkpoint inhibitors (CPIs). As a result, many CPIs have been approved with a companion diagnostics (CDx) assay [8]. A companion diagnostic is a diagnostic test that provides required information that is essential for the safe and effective use of a therapy; a complementary diagnostic is a diagnostic test that may inform on the benefit/risk ratio of a therapy [9]. Currently, PD-L1 immunohistochemistry (IHC) has the most approvals from the US-FDA as a CDx for immunotherapies in specific tumor types and is currently widely used for this purpose [8]. In addition to PD-L1 IHC, microsatellite instability-high (MSI-H) has been approved by the US-FDA as a pan-tumor biomarker for pembrolizumab [10]. Tumor mutational burden (TMB) is promising as a predictive biomarker for PD-L1/PD-1 immunotherapies, and recently, studies with pembrolizumab monotherapy have shown clinical benefit in a pan-tumor setting with a TMB cutoff of 10 mutations/Mb [11, 12]. Lastly, one potential tumor cell intrinsic biomarker not as well studied is *CD274* (PD-L1) gene amplification, which has shown to identify patients with Hodgkin's lymphoma to derive benefit from monotherapy CPIs [13–15].

PD-L1 IHC identifies T-cell infiltrated or interferon-gamma (IFNγ) regulated tumors and can distinguish tumor cells (TC) and immune cells (ICs) positive for PD-L1 expression [16]. The current PD-L1 IHC biomarker landscape is complex. Multiple IHC assays with different scoring algorithms are approved for different therapies and associated tumor indications [8]. Two commonly used IHC assays used clinically to determine PD-L1 expression level are the DAKO PD-L1 IHC 22C3 pharmDx Assay (PD-L1 22C3) and the VENTANA PD-L1 SP142 Assay (PD-L1 SP142). Currently, the PD-L1 22C3 is an approved CDx for pembrolizumab in NSCLC, gastric or gastroesophageal junction adenocarcinoma, cervical cancer, urothelial carcinoma, head and neck squamous cell carcinoma (HNSCC), and esophageal squamous cell carcinoma with various scoring algorithm cutoffs (tumor proportion score (TPS) ≥ 1%, combined positive score (CPS) ≥ 1%, CPS ≥ 1%, CPS ≥ 10%, CPS ≥ 1%, and CPS ≥ 10%, respectively). PD-L1 SP142 is an approved CDx for atezolizumab in NSCLC, urothelial carcinoma, and TNBC, also with different scoring algorithms (≥50% TC or ≥10% IC, ≥5% IC, and ≥1% IC, respectively) [8].

MSI results from defective DNA mismatch repair proteins and causes genetic hypermutability in patients. MSI-H tumors have a higher probability of presenting tumor neoantigens, thus conferring an inflamed status or increasing the immunogenicity of a tumor, an immune phenotype amenable to immunotherapy response [17, 18]. Multiple methodologies are available to detect MSI in patients, including polymerase chain reaction, IHC, and most recently next generation sequencing [19]. Based on a US-FDA approval, patients with solid tumors that are MSI-H are eligible for pembrolizumab as mentioned above.

TMB is derived from the number of somatic, coding, base substitutions, and indel mutations per megabase of genome examined, and similar to MSI-H tumors, is thought to exhibit immunogenic tumor associated "neo-antigens" on the tumor cell surface, thus making TMB high tumors amenable to response to CPIs [17, 20–23]. TMB can be measured by comprehensive genomic profiling (CGP) or whole exome sequencing [22]. Some clinical studies have shown response to immunotherapy based on higher TMB levels [11, 12, 24]. In June 2020, TMB at a cutoff of ≥10 mutations/Mb as measured by FoundationOne®CDx was approved by the US-FDA as a CDx in solid tumors for pembrolizumab based on the KEYNOTE-158 clinical trial [25].

Another, less studied, potential tumor intrinsic biomarker for PD-L1 and PD-1 immunotherapy is examining *CD274* (PD-L1) amplification status as determined by CGP.

As a clinical laboratory with one of the largest PD-L1 IHC and CGP clinical testing volumes, we performed this study to investigate PD-L1 expression prevalence in a wide variety of tumor types and examine the relationship between the immunotherapy biomarkers of PD-L1 expression, TMB, MSI, and *CD274* gene amplification across a cohort of 48,782 solid tumor cases.

Materials and methods

Data collection

We performed an analysis of all cases that received both PD-L1 IHC and CGP testing (Western Institutional Review Board Protocol No. 20152817) at our Foundation Medicine clinical testing laboratories between January 2016 to November 2019. Formalin-fixed, paraffin-embedded (FFPE) tissue of either whole section samples, biopsies, or cytology specimens were received as a paraffin block or unstained slides from outside institutions.

All specimens received were assigned a diagnosis by one of our board-certified pathologists based on microscopic examination of a hematoxylin and eosin (H&E) stained slide from the FFPE tissue, pathology report, and clinical information provided by the ordering physician. These diagnoses were grouped into 17 major groups for the purposes of this study. For example, in this analysis, one major group is neuroendocrine tumors and included tumor types such as small cell carcinoma and neuroendocrine

carcinoma; and another major group is endocrine tumors and included tumor types such as pituitary tumor and thyroid carcinoma.

DAKO PD-L1 IHC 22C3 pharmDx assay

PD-L1 22C3 was run according to manufacturer instructions in a Clinical Laboratory Improvement Amendments (CLIA) certified and College of American Pathologists (CAP) accredited laboratory (Foundation Medicine, Inc, Morrisville, North Carolina) for all non-breast carcinoma cases in this study. All patient cases were tested with manufacturer-recommended system level controls, H&E stained slide, negative reagent control slide, and PD-L1 22C3 IHC slide. One unstained patient slide was stained with H&E using Leica AutoStainer XL. Another unstained patient slide was stained with DAKO's negative control reagent on the DAKO Autostainer Link 48. Lastly, an unstained slide was stained with the PD-L1 22C3 IHC following recommended manufacturing protocol on the DAKO Autostainer Link 48.

VENTANA PD-L1 SP142 CDx assay

PD-L1 SP142 testing was performed using the VENTANA SP142 CDx assay per manufacturer's instructions in a CLIA-certified and CAP-accredited reference laboratory (Foundation Medicine, Morrisville, NC) on all breast carcinoma cases in this study. In brief, the VENTANA SP142 CDx assay consists of the rabbit monoclonal anti-PD-L1 SP142 clone, the Opti-View DAB IHC detection kit, the Opti-View Amplification Kit stained on the VENTANA BenchMark ULTRA instrument using the staining protocol provided by the package insert and interpreted with the guidelines of the VENTANA interpretation guide [26, 27]. All cases have an accompanying H&E stained patient slide, negative regent control stained patient slide with an on-slide tonsil control, and a VENTANA PD-L1 SP142 stained patient slide with an on-slide tonsil control.

IHC slide pathologist interpretation

All controls were determined to be adequate before interpretation of the PD-L1 22C3 and PD-L1 SP142 IHC cases. If inadequate, testing was repeated. All stained IHC slides were interpreted by board-certified pathologists (American Board of Pathology).

All the pathologists were specifically trained on the DAKO TPS scoring method. We used DAKO's TPS scoring method, where TPS = # PD-L1 positive TC/(total # of PD-L1 positive + PD-L1 negative TC) for all NSCLC and tumor types that have no CDx claim, and hence were considered exploratory. Also, as per DAKO's interpretation guide, we also followed the following guidance: "Score partial or complete cell membrane staining (≥1+) that is perceived distinct from cytoplasmic staining. Exclude cytoplasmic staining from scoring. Score only viable TC. Exclude all other cells from scoring: infiltrating ICs, normal cells, necrotic cells, and debris." [28] The PD-L1 22C3 TPS staining result was stratified into a negative (<1%), low positive (1–49%), or high positive (≥50%) category for all the exploratory indications. NSCLC is the only indication scored with TPS that has a CDx cutoff, which is TPS ≥ 1. An example of TPS staining in a NSCLC case is in Fig. 1a, b.

All the pathologists were also specifically trained on the DAKO CPS scoring method. Five specific indications stained with DAKO 22C3 had a CDx claim using CPS scoring. The indications were gastric or gastroesophageal junction adenocarcinoma, cervical cancer, urothelial carcinoma, HNSCC, and esophageal squamous cell carcinoma with a CDx cutoff of CPS ≥ 1%, CPS ≥ 1%, CPS ≥ 10%, CPS ≥ 1%, and CPS ≥ 10%, respectively. CPS is the number of PD-L1 staining cells (TC, lymphocytes, and macrophages) divided by the total number of viable TC, multiplied by 100 [29]. The CPS score differs from the tumor proportion score (TPS) in that it accounts for certain IC (lymphocyte, macrophages) staining in addition to the tumor cell staining in the score. As per DAKO's interpretation guide, we scored any convincing partial or complete linear membrane staining (≥1+) of viable TC that is perceived as distinct from cytoplasmic staining and any convincing membrane and/or cytoplasmic staining (≥1+) of lymphocytes and macrophages (mononuclear inflammatory cells, MICs) within tumor nests and/or immediately adjacent supporting stroma [29]. Examples of CPS scoring in HNSCC casers is shown in Fig. 1c–f.

Breast carcinoma PD-L1 SP142 IHC slides were interpreted by board-certified pathologists using the tumor-infiltrating IC scoring method where IC = proportion of tumor area that is occupied by PD-L1 staining IC of any intensity. Tumor-infiltrating ICs consist of lymphocytes, macrophages, dendritic cells, and granulocytes. In general, IC stains with a dark, granular punctate pattern; however, different staining patterns such as membranous staining can also be present as explained in the VENTANA interpretation guide [27]. For the purposes of this assay, tumor area was defined as TC and associated peri-tumoral and intratumoral stroma. The CDx cutoff for atezolizumab plus nab-paclitaxel for TNBC is an IC score of ≥1%. TC usually stain with a linear pattern, but tumor cell staining percentage is not considered in the TNBC CDx cutoff for atezolizumab plus nab-paclitaxel. An example of IC staining in a TNBC case is in Fig. 1g, h.

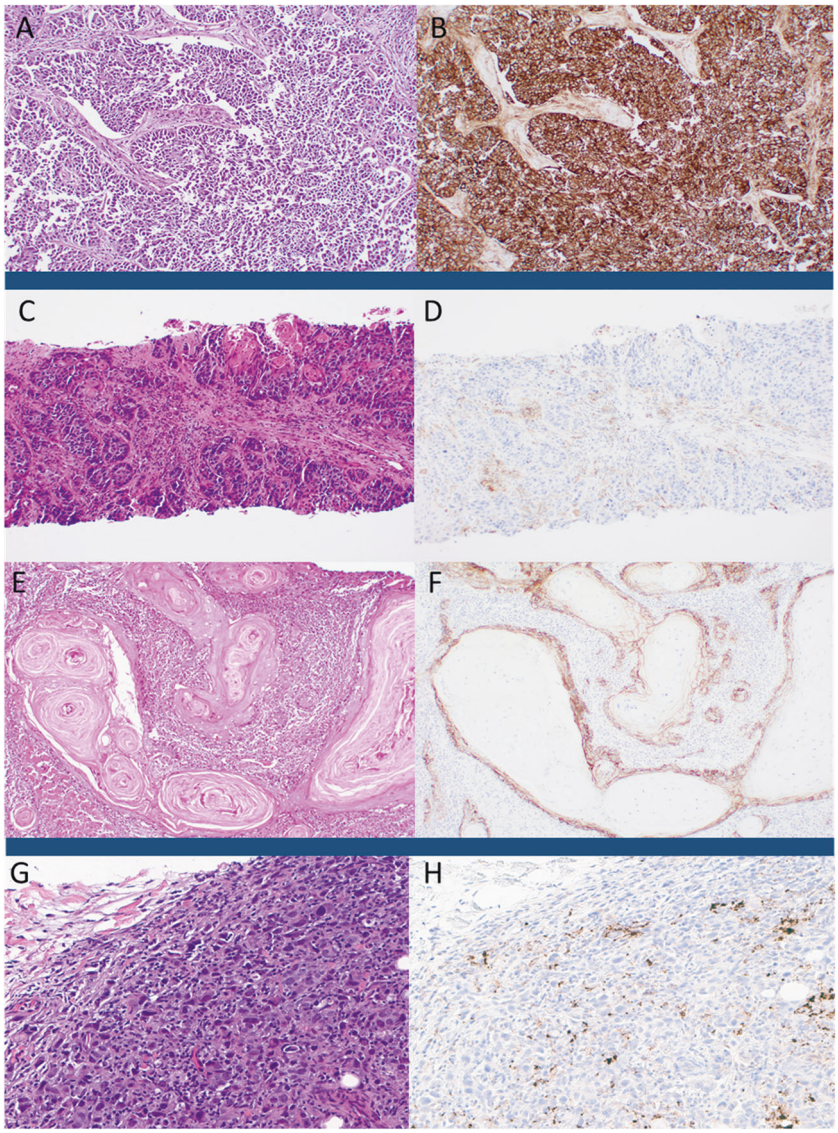

Fig. 1 Examples of a PD-L1 expression in different tumor types using different PD-L1 immunohistochemistry (IHC) assay. **a** Hematoxylin and Eosin (H&E) stain of a non-small cell lung carcinoma (NSCLC) case with all tumor cells staining in the corresponding. **b** DAKO 22C3 IHC giving it a tumor proportion score (TPS) of 100. Next are examples of two head and neck squamous cell carcinoma (HNSCC) with different biomarker status based on a tumor proportion score (TPS) cutoff of 1 and combined positive score (CPS) cutoff of 1. **c** H&E stain of a HNSCC case with no tumor cells but with immune staining in the corresponding. **d** DAKO 22C3 IHC giving it a negative status with the TPS score (TPS < 1) but positive status with the CPS score (CPS ≥ 1). **e** H&E stain of a HNSCC case with no immune cell but with tumor cell staining in the corresponding. **f** DAKO 22C3 IHC giving it a positive status for both the CPS score (CPS ≥ 1) and the TPS score (TPS ≥ 1). **g** The last H&E is of a triple negative breast carcinoma case that was stained with a (**h**) SP142 CDx IHC and shows the immune cells staining with a dark, granular punctate pattern. This case had a tumor-infiltrating immune cell (IC) score ≥ 1, which is considered positive with the SP142 CDx assay for TNBC. All digital images are at ×200 magnification.

Comprehensive genomic profiling for TMB, MSI, and CD274 amplification status

CGP was performed on hybridization-captured, adaptor ligation-based libraries using DNA extracted from FFPE tumor in a CLIA-certified and CAP-accredited laboratory (Foundation Medicine, Inc, Cambridge, Massachusetts). The samples were sequenced for up to 324 cancer related genes, and/or select gene rearrangements [30]. The two specific CGP assays used in this analysis were FoundationOne®CDx (324 genes and select gene rearrangements), and FoundationOne® (315 genes and introns from 28 genes involved in rearrangements). TMB was determined on 0.8–1.1 Mb of sequenced DNA using a mutation burden estimation algorithm that, based on the genomic alterations detected, extrapolates to the exome or the genome as a whole [22]. Assessment of MSI was performed from DNA sequencing across 114 loci as previously described to

determine microsatellite status [31]. MSI status was determined using a principle component analysis (PCA) algorithm that generates an MSI score for stratification as described in Trabucco et al. published paper [31]. The MSI cutoff were determined by the specific PCA trained. *CD274* amplification status was determined using statistical copy-number model to normalized coverage and allele frequencies as previously described [30]. Copy number ≥ 6 was considered positive for amplification.

Immunotherapy biomarker positivity definitions

For PD-L1 IHC, we considered a case for a tumor type with a CDx claim as positive based on the cutoff definition of the CDx claim. For all tumor types without a CDx claim, we considered tumor cell expression positive at a TPS ≥ 1. MSI positivity was defined as MSI-High (H) as per the pan-tumor approval for pembrolizumab. For TMB, we considered a TMB cutoff of 10 mutations/Mb as positive in our analysis based on the US-FDA pan-solid tumor CDx approval for pembrolizumab based on this cutoff. *CD274* (PD-L1) gene amplification was considered positive when determined to be amplified based on our bioinformatics pipeline.

Immunotherapy biomarker correlation

We examined the correlation between the different immunotherapy biomarkers by comparing these groups and performing a Fisher's Exact Test on each of these comparisons: PD-L1 IHC vs. *CD274* (PD-L1) gene amplification, MSI-H vs. TMB, MSI-H vs. PD-L1 IHC, and TMB vs. PD-L1 IHC.

In addition, we examined the prevalence of PD-L1$^-$/TMB$^-$, PD-L1$^+$/TMB$^-$, PD-L1$^-$/TMB$^+$, and PD-L1$^+$/TMB$^+$ disease subsets.

Lastly, we examined the impact of all four CPI biomarkers by determining how many patients had positivity in at least one of the biomarkers.

Results

Cohort demographics

A total of 48,782 cases were tested at Foundation Medicine with both PD-L1 IHC and CGP between January 2016 to November 2019. Approximately 34.2% (16,666/48,782) of the cases were represented by NSCLCs. A significant portion of the specimens received was from a metastatic site (35.9%, 17,497/48,782) or from the primary tumor site (46.1%, 22,482/48,782). It was either ambiguous or unknown in a portion of cases (18.0%, 8803/48,782) whether the specimen received was from a metastatic or primary tumor site. Of the cases with known gender data, 53.9% (26,289/48,782) were female and 46.1% (22,475/48,782) were male. We did not have the gender status on 18 cases. Mean age is 64.4 years old, median age is 65 years old, and age ranged from <1 year old to >89 years old.

PD-L1 protein expression

When examining PD-L1 tumor cell expression in NSCLC using the DAKO 22C3 CDx assay, we observed a 59.7% (9955/16,666) positive rate. Next, when examining both TC and ICs with the DAKO 22C3 and using the CPS scoring methodology, we had a wide range of positivity rates. For the CDx CPS 1 cutoffs, we had high positivity rates, with a positivity rate of 95.2% (297/312) in HNSCC, an 83.7% (431/515) in cervix cancer, and 79.0% (1156/1463) in gastric/esophageal adenocarcinoma. Purely for exploratory purposes, if we used a CPS 1 cutoff for ESCC and urothelial carcinoma, the positive prevalence would be 98.0% (100/102) and 83.6% (989/1183), respectively. In the indications with a CDx CPS cutoff of 10, we had a more moderate positivity rate, with ESCC having a 59.8% (61/102) positivity and urothelial carcinoma having a positivity of 46.5% (550/1183). Also, purely for exploratory purposes, if we used a CPS 10 cutoff for HNSCC, cervix cancer, and gastric/esophageal adenocarcinoma, the positive prevalence would be 64.1% (200/312), 46.8% (241/515), and 26.4% (386/1463), respectively. Lastly, when examining ICs only using the SP142 CDx assay in breast carcinoma samples, we had a 39.3% (900/2289) positive rate (Fig. 2a).

Next, we examined the PD-L1 tumor cell expression using the two exploratory cutoffs of TPS 1 and TPS 50 (Fig. 2b). Overall, most tumor samples (61.6% [26,457/42,918]) were negative for PD-L1 protein expression on TC, with 22.7% (9752/42,918) low positives [TPS 1–49], and 15.6% (6709/42,918) high positives [TPS ≥ 50]. Notably, all tumor types had cases that exhibited high positivity for PD-L1. The percent of high positive [TPS ≥ 50] per major tumor type varied from 2.2 to 31.1%. Of note, while the numbers are limited, the histology with a high percentage of high positivity [TPS ≥ 50] for PD-L1 tumor cell expression included sarcomatoid tumors (lung sarcomatoid carcinoma (59.3%, 70/118); unknown primary sarcomatoid carcinoma (50%, 14/22); kidney sarcomatoid carcinoma (61.5%, 8/13). The three major tumor types with highest percent of 22C3 high positives [TPS ≥ 50] were NSCLC (31.3%, 5189/16,666), endocrine tumors (22.3%, 100/448), and tumors that were not otherwise specified (17.3%, 533/3084), and the three tumor types with lowest percent of high positives [TPS ≥ 50] were gastrointestinal tumors (non-PD-L1 CDx indications) (3.3%, 330/9948), neuroendocrine tumors (3.1%, 60/1945), and gynecologic tumors (2.2%, 127/5771). The percent of low positives [TPS 1–49] per tumor type varied from 12.3 to 35.5%. The three major

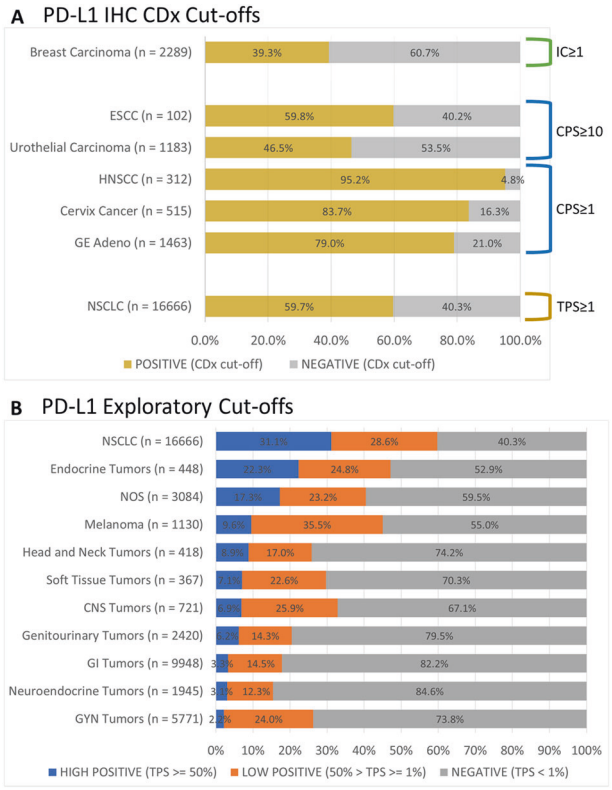

Fig. 2 Prevalence rates of various tumor types. a Shows all the prevalence rates based on the CDx cutoffs: DAKO 22C3 assay and tumor proportion scoring (TPS) method were used for NSCLC with a TPS cutoff of 1; DAKO 22C3 assay with combined positive scoring (CPS) method were used for gastric or gastroesophageal junction adenocarcinoma, cervical cancer, urothelial carcinoma, head and neck squamous cell carcinoma (HNSCC), and esophageal squamous cell carcinoma with a CDx cutoff of CPS ≥ 1%, CPS ≥ 1%, CPS ≥ 10%, CPS ≥ 1%, and CPS ≥ 10%, respectively; and SP142 CDx assay with tumor-infiltrating immune cell (IC) scoring method was used for breast carcinoma cases. **b** Shows the prevalence of all the tumor types without a CDx cutoff (except for NSCLC which has a CDx). DAKO 22C3 with the TPS scoring method was used for these cases and the results were stratified into a negative (<1%), low positive (1–49%), or high positive (≥50%) category for all the exploratory indications.

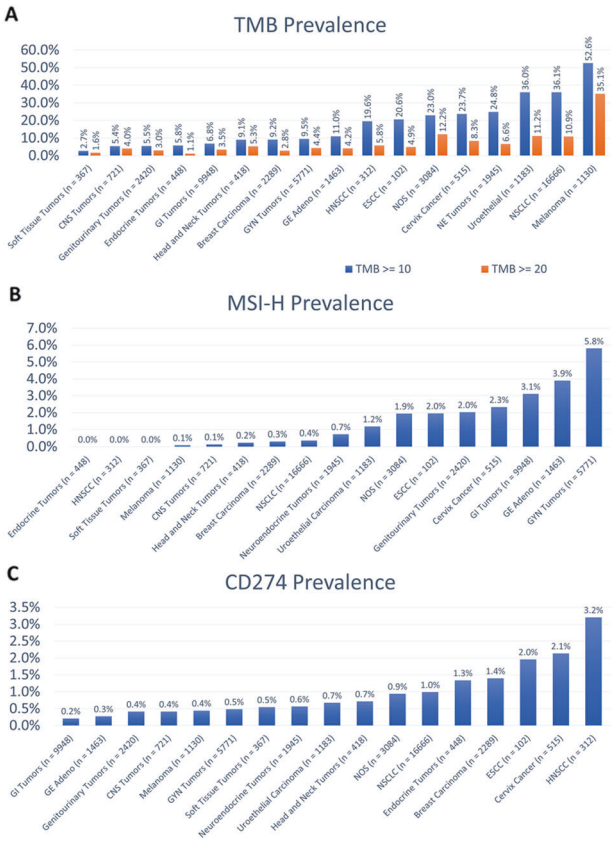

Fig. 3 The prevalence of rates of TMB, MSI-H, and *CD274* gene amplification in individual tumor types. a In the overall cohort TMB ≥ 10 mutations/Mb was 21.1% (10273/48,782), and TMB ≥ 20 mutations/Mb was 7.7% (3767/48,782). **b** Overall, for MSI, 1.9% (925/48,782) were MSI-H, 88.6% (43217/48,782) of cases were MSS, and 9.5% (4640/48,782) were MSI-unknown. **c** The overall prevalence of *CD274* amplification in the pan-tumor cohort is 0.72% (350/48,782).

tumor types with highest percent of 22C3 low positives [TPS 1–49] were melanoma (35.5%, 401/1130), NSCLC (28.6%, 4766/16,666), and central nervous system (CNS) tumors (25.9%, 187/721), and the three tumor types with lowest percent of low positives [TPS 1–49] were gastrointestinal tumors (non-PD-L1 CDx indications) (14.5%, 1445/9948), genitourinary tumors (14.3%, 347/2420) and neuroendocrine tumors (12.3%, 239/1945). Percent of negative [TPS < 1] per tumor type ranged from 40.3 to 84.6%.

TMB, MSI, and *CD274* (PD-L1) amplification results

In the overall cohort, mean TMB was 7.9 mutations/Mb, median TMB was 3.8 mutations/Mb, TMB range was <1–818 mutations/Mb, TMB ≥ 10 mutations/Mb was 21.1% (10,273/48,782), and TMB ≥ 20 mutations/Mb was 7.7% (3767/48,782). For individual tumor types, the TMB distribution based on a TMB cutoff of ≥10 and ≥20 mutation/Mb are shown in Fig. 3a. The three tumor types with the highest prevalence of TMB ≥ 10 mutations/Mb were melanoma (52.6%, 594/1130), NSCLC (36.1%, 6010/16,666), and urothelial carcinoma (36.0%, 426/1183); and the individual tumor types with lowest prevalence of TMB ≥ 10 mutations/Mb were non-urothelial genitourinary tumors (5.5%, 132/2420), CNS tumors (5.4%, 39/721), and soft tissue tumors (2.7%, 10/367).

For MSI, 1.9% (925/48,782) were MSI-H, 88.6% (43,217/48,782) of cases were MSS, and 9.5% (4640/48,782) were MSI-unknown. The three tumor types with the highest prevalence of MSI-H tumor types were gynecologic tumors (5.8%, 335/5771), gastric/esophageal adenocarcinoma (3.9%, 57/1463), and gastrointestinal tumors (non-PD-L1 CDx indications) (3.1%, 310/9948); and the tumor

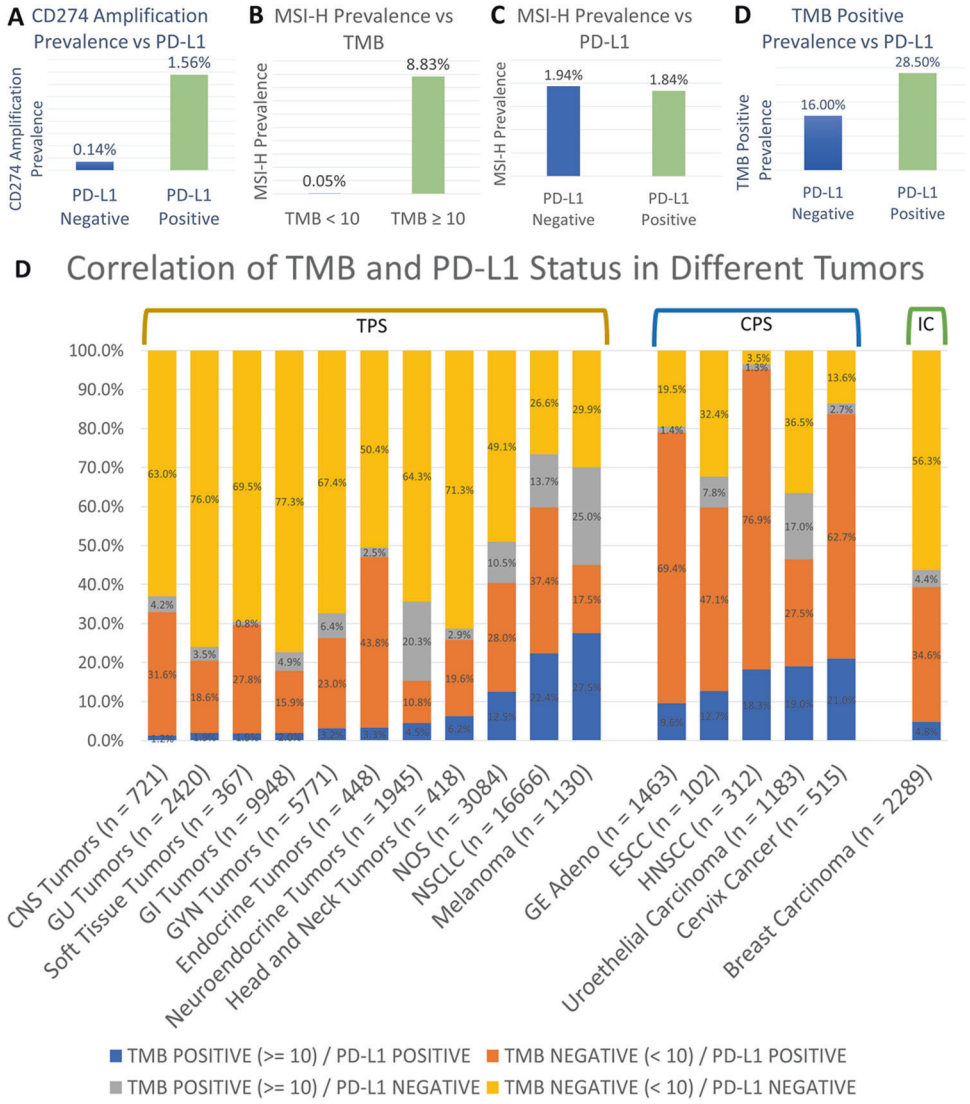

Fig. 4 Immunotherapy biomarker correlation of PD-L1 IHC, MSI, TMB, and *CD274* gene amplification. **a** When comparing *CD274* gene amplification with PD-L1 IHC, prevalence was significantly higher (1.56%, 310/19,856) in the PD-L1 positive cohort and lower in the PD-L1 negative cohort (0.14%, 40/28,926) (Fisher's exact test, $p < 0.0001$). **b** The prevalence of MSI-H was also significantly higher at 8.83% (907/10,273) in the TMB ≥ 10 mutations/Mb when compared to 0.05% (18/38,509) in the TMB < 10 mutations/Mb cohort (Fisher's exact test, $p < 0.0001$). **c** No significant difference was found in MSI-H prevalence between the PD-L1 IHC negative and positive groups (Fisher's exact test, $p = 0.457$). **d** A significantly higher TMB positive rate (28.5%, 5650/19,856) in the PD-L1 positive group when compared to the PD-L1 negative group was present (16.0%, 4623/28,926) (Fisher's exact test, $p < 0.0001$). **e** The prevalence of $TMB^-/PD\text{-}L1^-$, $TMB^+/PD\text{-}L1^-$, $TMB^-/PD\text{-}L1^+$, $TMB^+/PD\text{-}L1^+$ cohorts were dependent on tumor type.

types with no MSI-H cases in our cohort included soft tissue tumors, HNSCC, and endocrine tumors (Fig. 3b). Of note, gastrointestinal tumors (PD-L1 CDx and non-PD-L1 CDx indications) were the single largest contributor of MSI-H (39.7%, 367/925) cases in the overall cohort of MSI-H cases.

The overall prevalence of *CD274* (PD-L1) gene amplification in the pan-tumor cohort was 0.72% (350/48,782). The three tumor types with the highest prevalence of *CD274* (PD-L1) gene amplification were HNSCC (3.2%, 10/312), uterine cervix cancer (2.1%, 11/515), and ESCC (2.0%, 2/102); and the tumor types with the lowest prevalence of *CD274* (PD-L1) gene amplification in our cohort included genitourinary tumors (0.4%, 10/2420), gastric/esophageal adenocarcinoma (0.3%, 4/1463), and gastrointestinal tumors (non-PD-L1 CDx indications) (0.2%, 21/9948) (Fig. 3c).

Immunotherapy biomarker correlation: PD-L1 IHC, MSI, TMB, and *CD274* (PD-L1) amplification

When comparing *CD274* amplification with PD-L1 IHC, prevalence was significantly higher (1.56%, 310/19,856) in the overall PD-L1 positive cohort (defined in methods section Immunotherapy Biomarker Positivity Definitions) and lower in the PD-L1 negative cohort (0.14%, 40/28,926) (Fisher's exact test, $p < 0.0001$) (Fig. 4a). When examining *CD274* amplification with PD-L1 positivity based on TPS, CPS, and IC scoring methodologies, we saw a similar picture of having a higher prevalence of *CD274* (PD-L1) amplification in the PD-L1 positive cohort when compared to the PD-L1 negative cohort (Table 1). The prevalence of MSI-H was also significantly higher at 8.83% (907/10,273) in the TMB ≥ 10 mutations/Mb when compared to 0.05% (18/38,509) in the TMB < 10 mutations/Mb cohort (Fisher's

Table 1 *CD274* amplification prevalence vs. PD-L1 TPS, CPS, and IC positivity status.

PD-L1 cohort	*CD274* prevalence
TPS^+	1.53% (252/16,461)
TPS^-	0.12% (31/26,457)
CPS^+	1.36% (34/2495)
CPS^-	0.09% (1/1080)
IC^+	2.67% (24/900)
IC^-	0.58% (8/1388)

exact test, $p < 0.0001$) (Fig. 4b). No significant difference was found in MSI-H prevalence between the PD-L1 IHC negative and positive groups (Fisher's exact test, $p = 0.457$) (Fig. 4c). Lastly, when we examined the correlation between TMB and PD-L1 IHC, we also saw a significantly higher TMB positive rate (28.5%, 5650/19,856) in the PD-L1 positive group when compared to the PD-L1 negative group (16.0%, 4623/28,926) (Fisher's Exact test, $p < 0.0001$) (Fig. 4d). However, the prevalence of TMB^-/PD-$L1^-$, TMB^+/PD-$L1^-$, TMB^-/PD-$L1^+$, TMB^+/PD-$L1^+$ cohorts were dependent on tumor type (Fig. 4e). The tumor types with the highest prevalence of TMB^+/PD-$L1^+$ case were melanoma (27.5%, 311/1130), NSCLC (22.4%, 3728/16,666), and cervix cancer (21.0%, 108/515); and the tumor types with the lowest prevalence of TMB^+/PD-$L1^+$ cases were CNS tumors (1.2%, 9/721), non-urothelial genitourinary tumors (1.9%, 47/2420), and soft tissue tumors (1.9%, 7/367).

In addition, we examined the patients that had positivity in at least one CDx biomarker when combining PD-L1 IHC and CGP and found that 39.5% (19,260/48,782) had positivity in one CDx biomarker. Of the remaining patients, 17.8% (5267/29,522) were positive for one of the exploratory PD-L1 biomarkers. In sum, 50.3% (24,527/48,782) of the total cohort had positivity in at least one CDx or exploratory biomarker (Fig. 5).

Discussion

To the best of our knowledge, this is the largest cohort of cases with PD-L1 IHC, TMB, MSI, and *CD274* (PD-L1) gene amplification in the literature with 48,782 cases having these biomarkers tested on the same sample. Here, we present important data on PD-L1 IHC expression levels in ICs only, TC plus ICs, and TC only across a large variety of tumor types. What we found was that the positivity rate in both the tumor types with CDx cutoffs and exploratory cutoffs, the positivity prevalence varied between the different tumor types. When comparing the positivity prevalence of the tumor types with CDx cutoffs to the prevalence of the clinical trials in which the cutoff was derived, we saw some of our prevalence being extremely close to the clinical trial data while some not as close (Table 2). For example, in the IMPassion130 clinical trial, the positivity rate was 40.9% (369/902) TNBC patients, which was very similar to our positivity rate of 39.3% (900/2289) in our overall breast carcinoma cohort [32]. One caveat here is that since we are a referral laboratory, we do not know the ER and PR status of the patients. In cervical cancer, the KEYNOTE-158 study had a 83.7% (82/98) PD-L1 positivity rate, compared to our 83.7% (431/515) positivity rate [33]. For NSCLC, there were several clinical trials such as KEYNOTE-024, which had a low positive rate of 39.1% (646/1653) and high positive rate of 30.2% (500/1653); and KEYNOTE-010, with a low positive rate of 34.2% (344/1007) and high positive 22.8% (230/1007) [28]. These rates are similar to our rates of 28.6% (4766/16,666) low positives and 31.1% (5189/16,666) high positives. However, since there were many different clinical trials with specific enrollment criteria and smaller cohorts, comparisons to our "all comers" real-world patient dataset is challenging. For tumor types such as HNSCC, our prevalence rates are similar but not identical. In KEYNOTE-048, HNSCC had a positivity rate of 85.5% (754/882) compared to our PD-L1 positivity rate of 95.2% (297/312) [34]. Since this is a relatively newer CDx indication, we only had 312 cases compared to 882 cases in the clinical trial and so the differences are likely due to the smaller number of cases in our study.

IC expression of PD-L1 was the dominant pattern compared to tumor cell expression of PD-L1. Assessment of both tumor cell and IC expression of PD-L1 showed a higher prevalence of PD-L1 expression when using scoring algorithms that incorporate both tumor cell and IC expression of PD-L1 vs. tumor cell expression alone. Furthermore, the CPS algorithms have shown to be associated with clinical benefit to pembrolizumab in multiple cancer types including urothelial bladder cancer, HNSCC, and others [8]. This further highlights the importance of IC expression of PD-L1 in defining PD-L1 positivity. In addition, what we found in this analysis was that of the four biomarkers, PD-L1 IHC and TMB had the highest positive prevalence rates. In addition to PD-L1 IHC being highly correlated with *CD274* (PD-L1) amplification, TMB positivity was also highly correlated with MSI-H. It is interesting to note here that in our analysis, *CD274* (PD-L1) amplification was correlated with PD-L1 IHC positivity with all three scoring algorithms (TPS, CPS, and IC). Also, the clinical relevance of TMB and MSI is further described in the literature in a colorectal cancer study where they found that MSI-H patients with high TMB are likely to respond to immunotherapy and MSI-H patients with low TMB are not likely to benefit from immunotherapy [35]. In addition, we saw

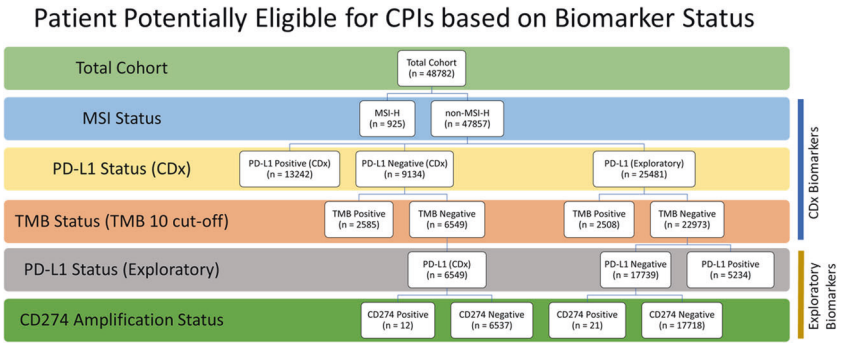

Fig. 5 Patients potentially eligible for checkpoint inhibitors (CPIs). By combining PD-L1 IHC and CGP, we found that 39.5% (19,260/48,782) had positivity in at least one CDx biomarker. Of the remaining patients, 17.8% (5267/29,522) were positive for at least one of the exploratory PD-L1 biomarkers. In sum, 50.3% (24,527/48,782) of the total cohort had positivity in at least one CDx or exploratory biomarker.

Table 2 Real world data PD-L1 positivity rate vs. clinical trial PD-L1 positivity rate.

PD-L1 IHC indication and cutoff	Positivity rate (real-world data)	Positivity rate (clinical trial data)	Clinical trial name
NSCLC (TPS1-49)	28.6% (4766/16,666)	39.1% (646/1653)	KEYNOTE-024
		34.2% (344/1007)	KEYNOTE-010
NSCLC (TPS 50)	31.1% (5189/16,666)	30.2% (500/1653)	KEYNOTE-024
		22.8% (230/1007)	KEYNOTE-010
ESCC (CPS 10)	59.8% (61/102)	42.8% (157/367)	KEYNOTE-181
Urothelial carcinoma (CPS 10)	46.5% (550/1183)	30.5% (110/361)	KEYNOTE-052
HNSCC (CPS 1)	95.2% (297/312)	85% (754/882)	KEYNOTE-048
Cervix cancer (CPS 1)	83.7% (431/515)	83.7% (82/98)	KEYNOTE-158
GE adenocarcinoma (CPS 1)	79.0% (1156/1463)	58.0% (148/257)	KEYNOTE-059
Breast carcinoma (IC 1)	39.3% (900/2289)	40.1% (369/902)	IMPassion130 (TNBC patients only)

that in the overall cohort, there was a correlation between PD-L1 IHC positivity and TMB positivity. However, when we looked at individual tumor types, we saw that there were four distinct populations, PD-L1$^-$/TMB$^-$, PD-L1$^+$/TMB$^-$, PD-L1$^-$/TMB$^+$, and PD-L1$^+$/TMB$^+$ and there were considerable differences in the prevalence of these three populations depending on the tumor type. Of interest to the authors is the PD-L1$^+$/TMB$^+$ cohort, whose prevalence varied from 1.2 to 27.5% in the individual tumor types. These findings highlight the need for clinical trials designed to examine clinical response to CPI in PD-L1$^+$/TMB$^+$ tumors. The combination of these two biomarkers may identify distinct patient populations (across multiple tumor types) who may have a superior response to CPI than would be predicted by each biomarker independently.

We also examined the prevalence rates of cases tested at our institution to the prevalence rates of other nonclinical trial studies. Some existing studies describe the prevalence of PD-L1 tumor cell expression with 22C3 with significantly smaller cohorts of cases [36–38]. For example, Viglar et al. examined PD-L1 expression in 193 samples of NSCLC, in which they found 62% of cases <1%, 17.6% of cases between 1 and 49%, and 19% case >50% TPS [37]. This study used a 22C3 laboratory-developed test, but our study used the 22C3 CDx assay, so these results might not be directly comparable. Consistent with the literature, in our cohort, in tumors with "sarcomatoid" histology, PD-L1 TC expression was high (high positives: 60.1%, 92/153) [39, 40]. This observation was notable because it has been shown in various studies that lung/kidney tumors with "sarcomatoid" histology respond well to CPIs [41–43]. For example, in a study by Hanif et al. they cohort of renal cell carcinoma with sarcomatoid component had a median overall survival of 33.8 months in the immunotherapy group compared to 8.8 months in the non-immunotherapy group [41]. We found only one study in the literature that had a large cohort of overall cases tested by PD-L1 IHC [44]. O'Malley et al. had characterized many of their patients' tumor types differently from our characterization, so direct comparison of some of the tumor types are difficult [44]. However, tumor types such as NSCLC using the DAKO 22C3 assay with TPS scoring method were similar,

while other tumor types such breast carcinoma using the SP142 assay were quite different. For the SP142 assay in breast carcinoma, they had a positivity rate of 12.2% (6/49) compared our prevalence rate of 40.9% (369/902). This difference is likely because at the time of the O'Malley publication, SP142 assay did not have a CDx claim in TNBC yet, and hence the scoring methodology in their study was likely not based on the CDx scoring methodology. This compares to our data where all the data presented for breast carcinoma cases with SP142 came after the CDx approval; hence, the pathologist at our institution scored the cases based on the CDx scoring methodology and cutoff, and therefore had a very similar prevalence rate as the IMPassion130 trial as mentioned above. Regardless of these differences, since our laboratory is a referral laboratory for CGP, our specimen cohort largely consisted of cases with advanced disease, in which the cases have already been treated with previous therapies. This is important to keep in mind when comparing the prevalence from studies in which the patient cohort has overall less advanced disease and has not been previously treated.

We chose TPS of 1% as the cutoff for the other tumor types that did not have a CDx cutoff and considered them to be exploratory in this study to provide maximum sensitivity when considering PD-L1 tumor cell expression in this analysis. At our institution, we have adjusted our methodology of scoring the PD-L1 IHC assays on indication as new CDx approvals have emerged. Pan-tumor, we have defaulted to using the DAKO 22C3 and TPS scoring for all tumor types without specific approvals, due to NSCLC first being approved with a TPS scoring methodology with DAKO 22C3. However, we currently score all the recently approved indications (gastric or gastroesophageal junction adenocarcinoma, cervical cancer, urothelial carcinoma, HNSCC, and esophageal squamous cell carcinoma) using the DAKO 22C3 with the CPS scoring methodology and breast carcinomas with SP142 with the IC scoring methodology. One of the limitations of this study when using the PD-L1 IHC TPS ≥ 1 in the tumor types without a CDx indications is that there is lack of clinical outcomes data of patients treated with immunotherapy using this PD-L1 cutoff and hence we consider it exploratory.

As shown in our data, the IHC assay and scoring methodology used for these predictive biomarkers are important, because different assays and different scoring methods will yield substantially different results. As shown in Fig. 1c–f of HNSCC cases, depending on the scoring methodology and cutoff used, a patient may be considered positive or negative for PD-L1 IHC. Considering a TPS cutoff of 1 and a CPS cutoff of 1 as an example, in Fig. 1c, d, no TC are staining for PD-L1 and there are ICs staining for PD-L1. In this case, with the TPS cutoff of 1, the case would be considered negative, but with a CPS cutoff of 1 it would be considered positive. However, in Fig. 1e, f, tumor cell staining is present with no IC staining. Here, the patient would be positive for both a TPS cutoff of 1 as well as the CPS cutoff of 1. The IHC assays can also look significantly different. As shown in Fig. 1g, h, the SP142 CDx assay accentuates ICs with a unique staining pattern (dark, granular, and punctate) due to the amplification step in the assay. The existence of tumor types such as TNBC, which do not readily express PD-L1 in TC, makes the clinical availability of an assay like the SP142 CDx important due to the unique staining pattern of ICs rendering it easy to distinguish true IC staining vs. background blush staining of ICs that is seen in some of the other PD-L1 IHC assays. In many of the tumor types with low prevalence of tumor cell expression, as shown in Fig. 2, all the new approvals include IC PD-L1 expression in addition to TC expression, or only consider IC PD-L1 expression. This further illustrates the importance of PD-L1 IC expression as a predictive biomarker for immunotherapy. Lastly, these examples illustrate the complexity of PD-L1 IHC and exemplify the concept that different assays with different scoring methods and cutoffs can yield significantly different results.

In this study, in addition to presenting the prevalence of PD-L1 expression in a large cohort of cases, we presented data on the relationship of immunotherapy biomarkers of PD-L1 protein expression and gene amplification, MSI, and TMB. These data not only demonstrate the relationship and landscape of these biomarkers, but also demonstrate the complexity of these biomarkers. In addition, this data is valuable for the design of future investigative and clinical trials to inform clinical utility of these biomarkers for CPIs. Importantly, more clinical trials need to be designed to examine the clinical outcomes based on combined PD-L1 expression, *CD274* amplification, MSI, and/or TMB, which when combined can identify distinct populations that are not identifiable when used alone. Also, it is important to keep abreast of the evolving nature of cutoffs for these biomarkers, as significant changes have already occurred in the past few years as new clinical trial data have been released. For example, the CDx for pembrolizumab as a first-line treatment in metastatic NSCLC was originally based on a TPS cutoff of 50, but now has been updated to a TPS cutoff of 1 based on the KEYNOTE-042 trial [45]. For atezolizumab in urothelial carcinoma, the SP142 assay was originally approved as a complementary assay, but has since been changed to a companion diagnostic assay. As more clinical trials with associated biomarkers yield new findings, we will gain greater understanding of whether single biomarkers or a combination of biomarkers are the best predictor for CPI response for each tumor type.

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Compliance with ethical standards

Conflict of interest All authors are Foundation Medicine employees and receive a salary and/or stock equity from Foundation Medicine.

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