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Novel PD-L1 mAb HC16 reveals upregulation of PD-L1 in BAC subtype

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Abstract
Programmed death-ligand 1 (PD-L1) is an inhibitory transmembrane protein that can prevent autoimmune response. Upregulated PD-L1 serves as a predictive biomarker for patients who may respond well to immune checkpoint therapies. However, variable associations of PD-L1 level with prognoses have been reported. In this study, a short peptide sequence corresponding to PD-L1 amino acids 172-187 (from the extracellular Ig-like C-type domain, and with high predicted antigenicity and hydrophilicity) was used to generate a monoclonal antibody (mAb). The resultant PD-L1 mAb, clone HC16, was examined for binding specificity and reactivity in cancer cell-lines, as assessed by immunocytochemical, immunoblotting, and co-immunoprecipitation. The potential diagnostic and clinical applicability of clone HC16 was further tested using malignant tissue arrays derived from various cancer types analyzed with an automated immunohistochemical (IHC) staining platform. Additionally, tumor samples from patients diagnosed with non-small cell lung cancer (NSCLC) were analyzed by western blotting. Clone HC16 showed obvious staining activity in lung and breast cancer tissues. Interestingly, we observed that PD-L1 level was negatively associated with clinical stage in NSCLC. Strong PD-L1 expression tended to be found in patients diagnosed with bronchioloalveolar carcinoma (BAC). These results demonstrate that clone HC16 harbors good target specificity and is suitable for further development in diagnostic tools to assess PD-L1 expression in human tissues. In addition, our findings also suggest a role for PD-L1 in a non-invasive subtype of lung cancer.

Keywords: Programmed death-ligand 1; Monoclonal antibody; Non-small cell lung cancer (NSCLC)
Introduction

Programmed death-ligand 1 (PD-L1, also called B7-H1) is encoded by the cluster of differentiation 274 (CD274) gene and functions as an immune inhibitory receptor ligand expressed by lymphocytes (Dong et al., 1999) and various cancer cells (Dong et al., 2002). Interaction of PD-L1 with its receptor, PD-1, regulates the adaptive immune response to danger signals by preventing excessive activation of CD8+ T cells and/or CD4+ helper cells as well as cytokine production. PD-1 knockout mice exhibit enhanced autoimmunity, indicating a negative regulatory role for the receptor and its ligand. (Nishimura et al., 1999; Nishimura et al., 2001). PD-L1/PD-1-mediated actions require the phosphatases, Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2, which bind to the immunoreceptor tyrosine-based switch motif (ITSM) and subsequently trigger immunosuppression, prevent autoimmunity, and maintain immune system homeostasis (Freeman et al., 2000; Chemnitz et al., 2004; Keir et al., 2007).

Cancer cells express tumor antigens that are recognized and stimulate cell lysis by specific CD8+ cytotoxic T lymphocytes (CTLs) (Urban and Schreiber, 1992; van der Bruggen and Van den Eynde, 1992). However, tumor rejection may be inactivated by negative regulatory signals in the tumor and its microenvironment (Gajewski et al., 2006). The silencing of tumor-infiltrating lymphocytes (TILs) may be at least partially induced by PD-L1-expressing cells, such as tumor-associated macrophages (TAMs) (Hartley et al., 2018), stromal cells (O'Malley et al., 2018), or tumor cells (Freeman et al., 2000; Keir et al., 2007; Pardoll, 2012). The immune checkpoint inhibitors (ICIs), such as PD-1 and PD-L1 antibodies, were developed to counteract these immune escape mechanisms by disrupting immune checkpoint ligand-receptor interactions and have shown efficacy in many clinical trials (Pardoll, 2012; Gonzalez-Cao et al., 2015). Many studies have indicated that expression of PD-1 or PD-L1 may be valuable prognostic markers (Ghebeh et al., 2006; Thompson et al., 2006; Pyo et al., 2017; Chen et al., 2018; Shen and Zhao, 2018; Kahlmeyer et al., 2019; Saito et al., 2019; Shigemori et al., 2019). Indeed, some PD-L1 mAbs, including clones 28-8, 22C3, SP142 and SP263, are commonly used in clinical diagnostic tests (Shukuya and Carbone, 2016). However, heterogenic and variable intra-tumor expression of PD-L1 have been observed (Ilie et al., 2016; Parra et al., 2018). As a result, the prognostic value of PD-L1 is still uncertain. A meta-analysis of six studies revealed that PD-L1 expression was significantly higher in poorly differentiated tumors and in patients with poor overall survival (OS) in non-small cell lung cancer (NSCLC) (Wang et al., 2015). On the other hand, PD-L1 expression was analyzed with the 22C3 antibody in a tissue microarray representing a large cohort of NSCLC patients; the study revealed that higher PD-L1 expression is associated with longer OS, indicating that PD-L1 may be a favorable prognostic factor in early-stage NSCLC (Cooper et al., 2015). Clone 22C3 was epitope mapped and recognizes the extracellular domain of PD-L1 at amino acid residues 156-178 and 196-206 (Schats et al., 2017). Another report indicated that PD-L1 expression was associated with poorer OS and Progression Free Survival (PFS) in stage III NSCLC patients receiving chemo-radiotherapy (Adam et al., 2015). This study was conducted using the clone E1L3N antibody, which targets the intracellular domain of PD-L1 (Schats et al., 2017). Yet another study was conducted to compare the immunohistochemical (IHC) staining of different PD-L1 clones (E1L2N, E1J2J, SP142, SP263, 28-8, and 22C3) in NSCLC (Parra et al., 2018). The results showed similar membrane staining patterns for all antibodies, but clone SP263 identified more PD-L1-positive cases (Parra et al., 2018). Clone SP263 was generated using a short peptide based on amino acid residues 272-290 as an immunogen; it was epitope mapped to both intracellular and extracellular domains (Schats et al., 2017). These findings indicate that the expression patterns for PD-L1 in IHC studies
are influenced by features of the antibody used, such as the binding affinity and target site. Notably, about 10% of patients with low PD-L1 expression still respond to ICIs (Shukuya and Carbone, 2016), indicating that the prognostic and/or predictive value of different PD-L1 antibodies remains sub-optimal (Ilie et al., 2016; Shukuya and Carbone, 2016).

Membrane-localized PD-L1 is considered to be the active form and most useful for clinical evaluation of malignant tumors (Escors et al., 2018). Here, we generated a novel mAb targeting the extracellular domain of PD-L1. The antibody was validated using IHC and immunoblotting of cancer cells and tumor tissues from NSCLC patients. Furthermore, PD-L1 expression patterns were determined by IHC in various malignant tissues.

Materials and methods
PD-L1 epitope identification and monoclonal antibody generation
To identify possible antigenic regions of human PD-L1 (accession number: EAW58763), in-built algorithms of the MacVector™ software (v.16.0) (Waterbeach, Cambridge, UK) were used to predict epitope antigenicity (Parkers and Protrusion antigenicity; Kyte/Doolittle and Hopp/Woods hydrophilicity). Selected peptide sequences were analyzed by an online program for predicting B-cell epitopes (BepiPred-2.0, Lyngby, DK) (Jespersen et al., 2017) and by the Basic Local Alignment Search Tool (BLAST) (Rockville Pike, MD, USA) to identify any sequence overlap with other proteins. The mAb was generated by Cyrsbioscience (New Taipei City, Taiwan). The HC16 peptide (HQVLSGKT1TTNSKRE, PD-L1 residues 172-187) and N-terminus biotinylated full-length and truncated versions (HQVLSGKT, LSGKTTTT, KTTTNNSK, TTNNSKRE) were synthesized and purified (GL Biochem Ltd., Shanghai, China). For antibody generation, mice were immunized with HC16 peptide by standard protocols. Briefly, mice were subcutaneously injected with synthesized HC16 plus complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) at day 1 followed by three immunizations done at day 21, 35, 51 with synthesized HC16 plus incomplete adjuvant (Sigma-Aldrich). Blood samples were obtained from mice for measurement of serum antibodies by enzyme-linked immunosorbent assay (ELISA). The fifth immunization was done at day 65 by intravenous injection of synthesized HC16 without adjuvant. At day 68, mice were euthanized and spleens were removed for in vitro hybridoma cell production through the 38% polyethylene glycol (PEG)-mediated cell fusion approach (Greenfield, 2018). The murine myeloma Sp2/0-Ag14 cell was used as a fusion partner to produce hybridoma. After selection, hybridoma clones were maintained and processed for the production of monoclonal antibody by the mouse ascites method (Jackson et al., 1999). For the epitope mapping experiment, 100 ng biotinylated peptide, full length HC16 (FL) or truncated peptides, were incubated with streptavidin coated plate (Pierce™, ThermoFisher Scientific) for 2 h at room temperature (RT). After three washes by PBS with 0.05% Tween-20 (PBST), each well was incubated with the HC16 antibody (1:1000) for 1 h at RT followed by donkey anti-mouse secondary antibody (1:5000) (GE Healthcare Life Science, Buckinghamshire, UK) for 1 h at RT. After three washes, developing solution and stop solution were added, and the optical density (OD) at 450 nm was measured using a spectrophotometer (SpectraMax→ i3, Molecular Devices, Lagerhausstrasse, Wals, Austria) within 5 min, with a reference wavelength of 655 nm.

Cell culture
Cells were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) – A549 (BCRC60074), MDA-MB231 (BCRC 60425), MCF7 (BCRC 60429) – or the American Type Culture Collection (ATCC, VA, USA) – HCC827 (ATCC CRL-2868). Cells were cultured as recommended. Hybridoma cells were grown in Iscove’s Modified Dulbecco’s Medium (Thermo Fisher Scientific Inc., Grand Island, NY, USA) supplemented with 15% fetal-bovine serum (FBS), 2 mM L-glutamine, and penicillin/streptomycin at 37°C with 5% CO2. Half medium replacements were made until the cell density reached 1.6 · 10^6 cells/mL.

**Western blotting and co-immunoprecipitation**

Total protein extracts were prepared and Western blots were performed by standard protocols. Primary antibodies were as follows: PD-L1 (1:1,000), α-tubulin (1:2,000, clone DM1A, Cell Signaling, MA, USA), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10,000, clone 6C5, EMD Millipore, Burlington, MA). Membranes were visualized with enhanced chemiluminescence (Merck Millipore, Billerica, MA, USA) and detected by an imaging system (UVP, BioSpectrum™ 500). For the competition assay, the membrane was incubated with the HC16 blocking peptide prior to the addition of the primary antibody. For the co-immunoprecipitation (co-IP), 500 µg equal protein lysate from the HCC827 cells were harvested for IP using Dynabeads™ magnetic beads (Thermo Fisher Scientific Inc., Dynal, Oslo, Norway), in accordance with the recommended procedures.

**Immunocytochemical and immunohistochemical studies**

For immunocytochemical studies, cells stained with antibodies (PD-L1/1:500; Integrinα5/1:500, Cell Signaling Inc., MA, USA) were mounted in ProLong Gold Antifade Reagent (ThermoFisher Scientific). Samples were observed with a FV3000 laser-scanning confocal microscope (Olympus), and images were acquired with FV31S software (Olympus). Tissue arrays were purchased from SUPER BIO CHIPS (CC5, CCA4, CBA4, and MB4). Paraffin sections were processed by standard protocols with antigen retrieval for 32 min. Slides were stained using the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ). The slides were incubated with the PD-L1 antibody (1:200) for 16 min at 37°C and then overnight at 4°C. After three rinses in PBS, the slides were incubated with the secondary antibodies (EnVisionTM System, HRP, anti-mouse, DakoCytomation). Slides were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate chromogen solution (DakoCytomation) and counterstained with hematoxylin, dehydrated, and mounted. Images were obtained with an inverted microscope (Olympus, IX71) coupled to a digital camera (Olympus), using a 4- (UPlanFL 10×/0.13 PhL) and 20- (LUCPlanFL 20×/0.40 Ph1) objective lens. CellSens standard software (Olympus) was used for image acquisition. Quantitation of the PD-L1 signal intensity was performed with ImageJ software (National Institutes of Health) using the IHC tool box plugins.

**PD-L1 blockade assay**

PD-L1 blockade assay was performed using PD-1[Biotinylated]:PD-L1 Inhibitor Screening Assay Kit (BPS Bioscience) following the recommended protocol. Briefly, 50 µL diluted PD-L1 (100 ng) was coated on a 96-well plate. Uncoated wells served as the Ligand control. After three washes with 1× Immuno buffer and blocking for 1 h at RT, 25-µL reaction mixtures (10 µL 3× Immuno buffer + 15 µL H2O) were added into each
Five microliters of the same solution was added to the Ligand control and Positive control groups, while 5µL PD-L1 antibody solution (125, 250 or 500 ng) was added into the Sample groups. Diluted biotinylated PD-1 (10 ng) was then added into each well and incubated for 2 h at RT. After three washes with 1· Immuno buffer and blocking for 10 min at RT, diluted streptavidin-HRP was added and incubated for 10 min at RT. After washing, freshly prepared chemiluminescent reagent was added to each well. Chemiluminescence was measured with a luminescence reader (Molecular Devices).

Statistical analysis. Data from repeated assays were analyzed by Prism 7 software (GraphPad Inc). Values represent the mean ± SD, and statistical significance was determined by applying a two-tailed unpaired Mann-Whitney t-test or one-way ANOVA test. Differences were considered statistically significant at $P < 0.05$.

Results

Generation of a mAb targeting human PD-L1

A short peptide sequence covering 16 amino acids (residues 172-187) within the Ig-like C2 type domain of human PD-L1 (Fig. 1A) was used for mAb production. Selection of the target peptide was based on antigenicity prediction algorithms (Fig. 1B, boxed by a red line) and validated by a web server that predicts B-cell epitopes (Jespersen et al., 2017). The selected antigen partly overlaps with that of clone 22C3 but not with the other commercially available clones (Fig. 1A). Synthesized peptide was then used to immunize mice. Antibody-producing cells were collected for fusion with myeloma cells, and one hybridoma clone with the strong anti-PD-L1 activity was selected for cell expansion and mAb production in mice.

Expression pattern of PD-L1 in cancer cell lines

Immunocytochemical staining of PD-L1 with purified clone HC16 was performed in epidermal growth factor receptor (EGFR)-mutated HCC827 cells, EGFR-wild-type A549 lung cancer cells, triple-negative MDA-MB-231 breast cancer cells, and estrogen receptor/progesterone receptor (ER/PR)-positive MCF7 cells. PD-L1 was expressed on the membrane and intracellular regions of all tested cells (Fig. 2A, pink arrows indicate membrane-localized PD-L1). Interestingly, different levels of PD-L1 glycosylation were observed in different cancers under normal culture conditions (Fig. 2B), suggesting that distinctive mechanisms regulate PD-L1 in the cell lines (Fig. 2B and C). The antibody binding specificity was confirmed by competition assays (Fig. 2B, middle panel). Clone HC16 only recognized full length peptide (FL) and not truncated versions in ELISA (Fig. 2D) and competition assays (Fig. 2E). PD-L1 residues Ala-121 to Arg-125 are conserved across species and are required for the interaction with PD-1 (Lin et al., 2008). This site is far from the binding site for clone HC16, so we tested whether the antibody might influence the PD-L1-PD-1 interaction by an ELISA-based approach. The result showed that clone HC16 only has minor effects on the interaction between PD-1 and PD-L1 (Fig. 2F).

PD-L1 expression may be associated with clinical stage and subtype in lung cancer

We subsequently determined the profiles of PD-L1 expression in cancer tissue arrays by IHC with clone HC16. The intensity of PD-L1 staining was quantified in each tissue sample and normalized to the average of nine matched normal adjacent tissue (NAT) samples (Fig. 3Aa). A normalized value less than 1.5 was considered weak expression (Fig. 3Ab); 1.5 to 3.0 was defined as high (Fig. 3Ac), and over 3.0 (Fig. 3Ad)
indicated strong expression. Staining was observed within tumor cells both intracellularly and extracellularly (Fig. 3Ad). A significant elevation of PD-L1 intensity was observed in lung cancer tissues over NAT (Fig. 3B, n = 93). Interestingly, the PD-L1 level was significantly higher in stage I samples compared to those from stage II/III patients (Fig. 3C). Correlation analysis further showed that PD-L1 expression might be weakly correlated (not statistically significant) with follow-up survival data in stage I/II NSCLC patients (Fig. 3D and E, Spearman $r = 0.1472$ in stage I and 0.1984 in stage II patients); while a negative correlation was observed in stage III patients (Fig. 3F, Spearman $r = -0.06717$). Among the 31 stage I patients, 21 showed high or strong expression of PD-L1 (Fig. 3D). More surviving patients were observed in this group (61.1% [11/18] surviving stage I patients) than the stage II/III groups (43.8% [7/16] stage II patients and 33.3% [3/9] stage III patients were surviving) (Fig. 3E and F). Among different cancer types, bronchioloalveolar carcinoma (BAC), squamous carcinoma (SCC), and adenocarcinoma (ADC) groups had more patients with strong PD-L1 expression at stage I, but the numbers were reduced in stage II/III groups (Fig. 3G–I). Strikingly, all stage I patients (n = 6) with BAC showed strong PD-L1 immunoactivity (Fig. 3G), while PD-L1 staining in stage II/III patients was relatively low (Fig. 3H and I). Lung cancer tissues were obtained from 12 female and 47 male individuals. The median follow-up time was 70.78 months (range 2-149 months). To validate the results of our IHC studies, we also measured PD-L1 levels in NSCLC clinical samples by immunoblotting. The results show that three out of five stage I patients (diagnosed as BAC, SCC and ADC) had strong PD-L1 immunoactivity; meanwhile, the level of PD-L1 was lower in three other patients staged II–IV (Fig. 4).

A similar analysis was then used to evaluate PD-L1 expression in breast cancer tissue arrays (Fig. 5A). PD-L1 staining was observed within the tumor cells intracellularly and extracellularly (Fig. 5Ad). Significantly higher PD-L1 immunoactivity was observed in breast cancer tissues compared to nine NATs (Fig. 5B, n = 48). No significant differences in PD-L1 levels were observed between the stage II and III patients (Fig. 5C). Including all breast cancer types, 34.6% (9/26) of stage II patients and 23.8% (5/21) of stage III patients had strong PD-L1 expression (Fig. 5D–F). All breast cancer tissues were obtained from female patients. The median follow-up time was 70.75 months (range 9-84 months). Finally, we analyzed the association of PD-L1 expression with three known predictive markers in breast cancers (38/48 patients; 22 stage II cases and 16 stage III cases). The results showed that PD-L1 expression was detectable in various breast cancer subtypes (Fig. 5F).

Discussion

In this study, we introduce a novel mAb, clone HC16, with unique target specificity to human PD-L1. Its utility was validated using cancer cell lines and arrays of malignant tissues. Significant elevation of PD-L1 level was observed in both lung cancer and breast cancer tissues, and strong PD-L1 expression was particularly notable in stage I NSCLC patients.

Clone HC16 targets the Ig-like C2 type domain of PD-L1 (Fig. 1A), which is also targeted by clone 22C3 as one of two different binding sites (Schat et al., 2017). The partially overlapping binding site includes residues 172-178 (Fig. 1A). An earlier report utilized clone 22C3 to show that PD-L1 expression is a favorable prognostic factor in early stage NSCLC (Cooper et al., 2015). We also observed that PD-L1 level is significantly higher in stage I patients than stage II/III patients (Fig. 3C) in a limited number of cases. A larger sample size is needed to confirm our findings. The study with clone 22C3 further showed that PD-L1 tended to be more highly expressed in SCC and LCC than ADC, but the differences were not statistically significant;
also, high PD-L1 expression in NSCLC was significantly correlated with longer OS (Cooper et al., 2015). The survival analysis of stage I NSCLC patients in our study revealed that individuals with high or strong PD-L1 staining activity might have improved survival status (4/6 surviving BAC patients; 2/3 surviving ADC patients; 4/7 surviving SCC patients; 2/2 surviving LCC patients; 1/1 surviving MEC patients) (Fig. 3E–G). In addition, we found that all patients diagnosed with BAC showed strong PD-L1 staining (Fig. 3E). BAC is a subtype of ADC characterized by the growth of malignant cells over the alveoli and bronchioles but without invasion (Brambilla et al., 2001). Patients with small non-invasive BACs (tumor size < 2 cm) have excellent survival after surgical resection (Noguchi et al., 1995). Extensive BAC with an aggressive malignant component may occur with invasion and destruction of the pulmonary architecture, and is categorized as a mixed subtype of ADC (Brambilla et al., 2001). Therefore, the presence or absence of invasion seems to be a critical prognostic factor. Follow-up survival data revealed that only 2/3 ADC patients with strong PD-L1 expression (among 8 total stage I patients) were alive (Fig. 3G). Moreover, PD-L1-positive cases tended to decrease with stage: 21/33 stage I cases (63.6%), 16/38 stage II cases (42.1%), 9/22 stage III cases (40.9%) (Fig. 3D). These results echo the previous finding that PD-L1 staining with clone 22C3 may be of prognostic value for early-stage NSCLC (Cooper et al., 2015). Furthermore, the finding that PD-L1 expression is more relevant in BAC than ADC subtypes suggests that PD-L1 regulatory activities may differ among lung cancer types. miR-197 is a reported prognostic indicator for NSCLC that regulates PD-L1 expression through the cyclin-dependent kinase CDC28 protein kinase regulatory subunit 1B (CKS1B) and signal transducer and activator of transcription 3 (STAT3) signaling cascade in chemo-resistant NSCLC cells (Fujita et al., 2015; Mavridis et al., 2015). Expression of miR-197 is associated with larger tumor size and SCC histotype in early or advanced NSCLC (Mavridis et al., 2015). In addition, a variant form of the cancer stem cell (CSC) marker, CD44v, may serve as a negative prognostic marker in SCC (Nguyen et al., 2000; Leung et al., 2010). However, in ADC, improved OS was observed for patients with CD44-expressing tumors (Leung et al., 2010). Intriguingly, ADC tumors may contain a population of CD44v-expressing cells that are highly proliferative and resistant to chemotherapy (Nishino et al., 2017). Tumor cells with a stemness phenotype (i.e. CD44v positive) exhibit WNT-mediated constitutive activation of PD-L1 in triple negative breast cancer (TNBC), which facilitates immune evasion (Castagnoli et al., 2019). In addition, PD-L1 promotes OCT4 and Nanog expression in breast CSCs through activation of PI3K/AKT (Almozyan et al., 2017). Constitutive and inducible expression of PD-L1 in CD44-positive head and neck SCC suppresses T-cell-mediated immunity (Lee et al., 2016). Furthermore, PD-L1 binds to H-Ras, subsequently triggering Erk-mediated epithelial mesenchymal transition (EMT) signaling to promote Glioblastoma multiforme formation and invasion in a rodent model (Qiu et al., 2018). A transcriptomic study conducted in human glioblastoma cells revealed that PD-L1 significantly alters expression of genes involved in cell growth/migration/invasion (Qiu et al., 2018), suggesting that PD-L1 is associated with EMT in malignant cells. Alternatively, anti-tumor activity of PD-L1 has also been reported. Cells with low PD-L1 expression isolated from two human cholangiocarcinoma cell lines harbor pluripotent stem cell-like characteristics and are highly tumorigenic in immunodeficient NOD/SCID/γcnull mice, but knockdown of PD-L1 in the cells suppresses tumorigenicity (Tamai et al., 2014). Survival analysis revealed that low PD-L1 expression correlates with poor prognosis in cholangiocarcinoma patients (Tamai et al., 2014). Taking our finding that PD-L1 is strongly expressed in the BAC subtype in
NSCLC together with other studies showing high PD-L1 expression at early stages of lung cancer may have improved survival status, one may hypothesize that a distinct population of NSCLC cells with very different oncogenic properties could exist, and their correlation with the overall PD-L1 profile should be carefully evaluated.

Strong PD-L1 expression was detected in different breast cancer subtypes by clone HC16 (Fig. 5D–F). No significant difference in PD-L1 staining was observed between stage II and III breast cancer patients, but more patients with strong PD-L1 activity were found in the stage II group (Fig. 5F). Among patients with known predictive factors, 4/10 TNBC cases showed strong PD-L1 activity (Fig. 5Ad). Significant upregulation of PD-L1 in TNBC has been reported and validated with IHC (Gatalica et al., 2014; Mittendorf et al., 2014; Ali et al., 2015; Polonia et al., 2017). However, the expression of PD-L1 in TNBC tissues can vary when using different mAbs (Sun et al., 2016). Clones 28-8 and E1L3N produced more positive tumor cell staining than clone SP142; moreover, clone SP142 was almost devoid of immune cell staining compared to the two other clones (Sun et al., 2016). The staining profile with clone HC16 is much more similar to clones 28-8 and E1L3N, with positive staining of both immune and tumor cells (Fig. 5Ad). Clones 28-8 and E1L3N both recognize intracellular domains of PD-L1, while clone SP142 targets both intracellular and extracellular epitopes (Schats et al., 2017). In this work, we did not experimentally verify the HC16 binding sites in PD-L1. Thus it is still unclear whether HC16 can also recognize intracellular portions of PD-L1.

Studies of the correlations between PD-L1 expression and breast cancer subtypes or prognosis remain controversial (Stovgaard et al., 2019). For example, PD-L1 expression is frequently detected in basal-like breast cancers and is correlated with improved disease-free survival (DFS) in ER− breast cancers (Ali et al., 2015). A high level of PD-L1 in TNBC patients was correlated with the prevalence of TILs and had significantly longer DFS and OS (AiErken et al., 2017). Increased PD-L1 expression in tumor cells was strongly associated with better DFS but not OS in TNBC (Botti et al., 2017). By contrast, other studies showed PD-L1 is associated with the TNBC subtype and decreased OS (Polonia et al., 2017; Adams et al., 2018; Choi et al., 2018). Indeed, different PD-L1 antibodies show different binding characteristics that may lead to distinctive IHC patterns. Splicing variants of PD-L1 have been identified and their cellular functions have been characterized (He et al., 2005; Nielsen et al., 2005; Hassounah et al., 2019; Mahoney et al., 2019). A secreted form of PD-L1, which lacks the C-terminal transmembrane domain and cytoplasmic region, has been found to function as an inhibitory immune regulator within the tumor without the need for cell-cell interactions (Mahoney et al., 2019). Conversely, the PD-L1 splicing isoform 2, which lacks the Ig-like V-type domain (residues 19-132), shows an intracellular localization pattern (He et al., 2005). Therefore, failure of an antibody to bind specific domains of PD-L1 may not fully reveal the specific expression patterns of all isoforms.

Overall, a single anti-PD-L1 antibody clone may not fully reveal the status of every patient, since post-transcriptional modulation of PD-L1 may result in differential immune regulation. Considering the requirement for high diagnostic accuracy to identify patients who should receive immune therapy, two or more positive tests may be required to determine the most appropriate treatment options. The novel PD-L1 mAb, clone HC16, has excellent PD-L1 target specificity. However, further investigations to fully characterize the targeted epitope and PD-L1 conformation are still required before further development of diagnostic tools.
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Availability of data and materials
The materials and data generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

Authors' contributions
BC Su and CH Ting performed the experiments. KY Lee, SM Wu, PH Feng, and YF Chan designed the study. BC-Su, CH-Ting, and JY Chen wrote the manuscript.

Ethics approval and consent to participate
The use of human tissue specimens was approved by the Institutional Review Board at Shuang Ho Hospital, Taipei Medical University (IRB No: N201702026). This study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication
Not applicable.

Competing interests
The authors have no competing financial interests to declare.
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Figure legends

Fig. 1. Antigenic analysis of human PD-L1. (A) Schematic shows the domain characteristics of PD-L1. The epitope selected for antibody production is shown in yellow. Several epitopes recognized by commercially available PD-L1 antibodies are indicated, including clone 28-8 (brown), clone 22C3 (shown in blue; boxed by blue dashed line), clone SP263 (green), clone SP142 (grey line), and clone E1L3N (red line). (B) Parker, Protrusion antigenic index and Kyte/Doolittle, Hopp/Woods hydrophilicity index algorithms were used to predict the potential antigenic sequences. Red boxed region indicates amino acids 172-187.

Fig. 2. Expression patterns of PD-L1 in cancer cell-lines. (A) Localization of PD-L1 in cancer cells. Cells were stained for PD-L1 (green), Integrin5α (red), and nuclei (blue; Hoechst33258). Boxed regions are magnified to the right of the merged image. Scale bar: 20 µm (B) Total lysates from cancer cells were analyzed by Western blot using antibodies against PD-L1 and GAPDH. For the competition assay, blocking peptide was incubated with the primary antibody before blotting. (C) HCC827 cell lysates were harvested for co-IP using the PD-L1 antibody. IP eluates were analyzed by Western blot for PD-L1. (D) Biotinylated HC16 peptide (FL) and its truncated variants (named a to d) were incubated with streptavidin-coated wells and recognized by PD-L1 antibody. BSA served as a negative control. (E) Total cell lysates were analyzed by Western blot using antibodies against PD-L1 with or without the incubation of HC16 peptide or its truncated variants. (F) Biotinylated PD-1 was incubated with PD-L1-coated wells without or with HC16 antibody. PD-L1 and PD-1 binding was determined with a fluorescence reader and relative luminescence was calculated by dividing the signal by its ligand control (Lig.). The ELISA assays shown in (D) and (F) represent at least duplicate wells. Data were collected from three independent assays. Results represent the mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
**Fig. 3.** Immunohistochemical analysis of PD-L1 profile in lung cancer. (A) Matched NAT (n = 9) and lung cancer samples (n = 93) were stained for PD-L1 with DAB signal enhancement. Scale bars: 200 µm, left; 50 µm, right. Red boxed regions are magnified to the right. (B) Quantification of PD-L1 staining intensity in lung cancer and matched NAT samples. (C) PD-L1 staining intensity was normalized to matched NAT and stratified by disease stage. (D–F), Correlation analysis of relative PD-L1 level and survival status in stage I (D), II (E), and III NSCLC patients (F). On the right, survival data are shown according to relative PD-L1 expression level. S: Strong; H: high; L: low PD-L1 expression. Blue dashed line divides low and high/strong PD-L1 expression. Orange and black dots respectively denote dead and surviving patients. (G–I) PD-L1 expression in stage I (G), stage II (H), and stage III (I) NSCLC patients with various cancer types. Red and green dashed line denote the value 1.5 and 3.0; a value less than 1.5 was considered weak PD-L1 expression, between 1.5 and 3.0 was defined as high, and over 3.0 was defined as strong. The red and black dots respectively denote dead and alive patients. ADC: Adenocarcinoma; BAC: Bronchioloalveolar carcinoma; SCC: Squamous carcinoma; LCC: Large cell carcinoma; MEC: Mucoepidermoid carcinoma; CS: Carcinosarcoma; LCNC: Large cell neuroendocrine carcinoma; MM: Malignant mesothelioma; Ad-SCC: Adenosquamous carcinoma; ACC: Adenoid cystic carcinoma. Quantitative results in (B) and (C) show mean ± SD. Mann-Whitney t-test: ns, not significant; *P < 0.05; **P < 0.01.

**Fig. 4.** Immunoblotting of PD-L1 with lung cancer tissues. Tissue extracts from eight lung cancer patients staged I to IV were analyzed by Western blot using antibodies against PD-L1 and αTubulin. For the competition assay, blocking peptide was incubated with the primary antibody before blotting. General information for each patient is shown below the blot.

**Fig. 5.** Immunohistochemistry for PD-L1 in breast cancer. (A) Matched NAT (n = 9) and cancer samples (n = 48) were stained for PD-L1 with DAB signal enhancement. Scale bar: 200 µm, left; 50 µm, right. Red boxed regions are magnified to the right. (B) Quantification of PD-L1 staining intensity in cancer and matched NAT samples. (C) PD-L1 staining intensity was normalized to matched NAT and stratified by disease stage. (D–F) PD-L1 expression in stage II (D) and stage III (F) breast cancer patients with various cancer types. Red and green dashed lines denote the values of 1.5 and 3.0; less than 1.5 was considered weak PD-L1 expression, between 1.5 and 3.0 was defined as high, and over 3.0 was defined as strong. IDC: Infiltrating duct carcinoma; AMC: Atypical medullary carcinoma; MC: Metaplastic carcinoma; IPC: Intraductal papillary carcinoma; SC: Sarcomatoid carcinoma; LC: Lobular carcinoma. Quantitative results shown in (B) and (C) represent the mean ± SD (two-tailed unpaired Mann-Whitney t-test: ns, not significant; *P < 0.05). (F), Expression of PD-L1 is shown for various subtypes of breast cancer at different stages. ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; TNBC: ER/PR/HER2.
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Legend:
- WT: Wild Type
- L858R: EGFR L858R mutation
- ADC: Adenocarcinoma
- BAC: Bronchioloalveolar carcinoma
- SCC: Squamous cell carcinoma
- LCNC: Large cell neuroendocrine carcinoma
- MEC: Medullary carcinoma
Infiltrating duct carcinoma/IIIc

Atypical medullary carcinoma/I1b

Infiltrating duct carcinoma/I1b

Predictive markers | Case (n) | Strong PD-L1 expression (n)
--- | --- | ---
Stage II
ER+ | 1 | 1
PR+ | 2 | 1
ER+/PR+ | 5 | 2
ER+/PR+/HER2+ | 1 | 1
HER2+ | 3 | 4
TNBC | 10 | 4
Stage III
ER+ | 3 | 2
PR+ | 1 | 1
ER+/PR+ | 1 | 1
ER+/HER2+ | 1 | 1
HER2+ | 5 | 4
TNBC | 5 | 4

Relative PD-L1 level (Normalized to NAT)

Stage II:
- SC
- IPC
- MC
- AMC
- IDC

Stage III:
- IDC+LC
- IDC+LC