Annals of Diagnostic Pathology 41 (2019) 24–37

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Original Contribution

Expression of programmed death ligand (PD-L1) in different tumors. Comparison of several current available antibody clones and antibody profiling

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ARTICLE INFO

Keywords:
PD-L1 expression in tumor cells
PD-L1 antibody clones
Biomarker
Antibody profiling
Immunohistochemistry
Non-small cell lung cancer

ABSTRACT

PD-L1 is a surface molecule which is expressed on different types of cells, including antigen presenting cells, vascular endothelial cells and other cells of human tissues. Expression of PD-L1 is also found on human tumor cells. PD-L1 as the ligand to PD1 receptor molecule of CD8+ T cells inhibits its cytotoxic effect on the tumor cell. The modern target therapy uses this interaction to inhibit the PD-1 molecule of T cells to stimulate tumor necrosis. To compare expression differences, twelve frequent types of malignant tumors with ten patients per group were selected. Immunohistochemical stains with different antibodies for PD-L1 (DAKO, Spring Bioscience, Ventana, Cell Signaling, Biocare Medical, Abcam, Zeta Corporation) were performed, analyzed and compared. To summarize, we detected variable expression pattern of PD-L1 with general higher mean value of expression of tumor cells with clone SP263 in most tumor groups. In the comparison of selected cases of lung cancer, therapy relevant differences of PD-L1 expression on tumor cells with different antibodies were observed. Additionally, the profiling study of several PD-L1-antibody clones (28-8 Abcam and 28-8 DAKO, SP142, SP263) with Signal-to-Amino Acid Residue Plots was performed with interesting findings of cross-activity of SP142 with two peptides from PD-1, which can explain why clone SP142 stains immune cells more intensively, as previously published.

1. Introduction

PD-L1 is one of the membranous bound ligands to the programmed cell death 1 receptor (PD-1) and could be found on professional and nonprofessional antigen-presenting cells (APC's), endothelial cells of vessels, and also in cells of several organs: placenta, testes, eye and pancreas [1]. PD-1 and its ligands are playing an important role in regulation of the activity of different T cell types and are involved in such processes as T-cell tolerance and autoimmunity, especially in the following autoimmune diseases: type 1 diabetes, multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis [2].

At the same time, an expression of PD-L1 in different tumor types was explored and the correlation of PD-L1 expression and prognosis with controversial results for some tumor types (lung cancer, colorectal carcinoma and melanoma) was observed [3].

Previously, a possible role of PD-L1 in evasion from host immunity on immunogenic tumor cells was described. Researchers demonstrated that tumor growth of PD-L1-expressing "myeloma cells in normal syngeneic mice was inhibited significantly albeit transiently by the administration of anti-PD-L1 Ab in vivo and was suppressed completely in the syngeneic PD-1-deficient mice" [4].

The evasion of tumor cells from host immunity can also be characterized as an immune resistance, which could be divided in 1) innate immune resistance, in which PD-L1 expression on tumor cells results from activation of some oncogenic pathways and 2) adaptive immune resistance, in which upregulation of PD-L1 on tumor cells results from interaction with T cells (induced by interferon or inflammatory cytokine [5]) with following inhibition of $PD1^+$-T cells [6].

These facts suggest that "blockade of PD-1-PD-L1 interaction may provide an effective approach for specific tumor immunotherapy" [4] with subsequent tumor cell death.

So called "immune checkpoint inhibitors" are antibodies which inhibit different (macromolecular) targets of cells in tumor microenvironment and spleen/lymph node microenvironment [7] with the aim of activation of anti-tumor-immunity. Some examples are anti-PD-1- or anti-PD-L1-antibodies which are in clinical development or

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https://doi.org/10.1016/j.anndiagpath.2019.05.005

already approved as a therapeutic agent for patients with different malignancies. The immunohistochemical detection of PD-L1 on tumor cells was explored as a promising biomarker for possible selection of patients who can most respond to this therapy [8].

Actually different mono- and polyclonal PD-L1 antibodies for immunohistochemical analysis are commercially available from different providers. Some antibody clones are also integrated in clinical studies of different therapeutic drugs [9]: nivolumab (clone 28-8), atezolizumab (SP142), durvalumab (SP263) and pembrolizumab (22C3). In the investigation of other new drugs such as avelumab (an anti-PD-L1 antibody for therapy of metastatic Merkel cell carcinoma) initially no PD-L1 expression was evaluated [10].

Currently two **companion diagnostic** tests are approved by the U.S. Food and Drug Administration (FDA) [11] –

1) "PD-L1 immunohistochemistry (IHC) 22C3 pharmDx is a qualitative immunohistochemical assay using Monoclonal Mouse Anti-PD-L1, Clone 22C3, intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC), gastric or gastroesophageal junction (GEJ) adenocarcinoma, cervical cancer, and urothelial carcinoma tissues using EnVision FLEX visualization system on Autostainer Link 48", for treatment with KEYTRUDA (pembrolizumab). In the case of NSCLC, the so called Tumor Proportion Score (TPS) is predetermined, "which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity". In this type of carcinoma "the specimen should be considered to have PD-L1 expression if TPS ≥ 1% and high PD-L1 expression if TPS ≥ 50%". In other carcinomas (gastric or GEJ adenocarcinoma, cervical cancer and urothelial carcinoma) so called Combined Positive Score (CPS) is required, "which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100". Especially for urothelial carcinoma, "the specimen should be considered to have PD-L1 expression if CPS ≥ 10", in other last-mentioned carcinoma types "the specimen should be considered to have PD-L1 expression if CPS ≥ 1".

In Europe, pembrolizumab has also different therapy indications for treatment of various cancer entities with or without cutoffs of PD-L1 expression of tumor specimen with no reference of an explicit PD-L1 test [12]. For example, "pembrolizumab as monotherapy is indicated for the first-line treatment of metastatic non-small cell lung carcinoma (NSCLC) in adults whose tumors express PD-L1 with a ≥ 50% tumor proportion score (TPS) with no EGFR or ALK positive tumor mutations". At the same time, "pembrolizumab as monotherapy is indicated for the treatment of locally advanced or metastatic NSCLC in adults whose tumors express PD-L1 with a ≥ 1% TPS and who have received at least one prior chemotherapy regimen. Patients with EGFR or ALK positive tumor mutations should also have received targeted therapy before receiving pembrolizumab". Another indication for pembrolizumab with a cutoff of PD-L1 expression is "the treatment of recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) in adults whose tumors express PD-L1 with a ≥ 50% TPS and progressing on or after platinum-containing chemotherapy". No CPS at the time of access of EMA webpage was mentioned (December 1, 2018).

2) "VENTANA PD-L1 (SP142) Assay is a qualitative immunohistochemical assay using the rabbit monoclonal anti-PD-L1 clone SP142 intended for use in the assessment of the PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) urothelial carcinoma and non-small cell lung cancer (NSCLC) tissue stained with OptiView DAB IHC Detection Kit and OptiView Amplification Kit on a VENTANA BenchMark ULTRA instrument". In this assay beside tumor cells (TC) also tumor-infiltrating immune cells (IC) should be determined separately. According to the FDA, for urothelial carcinoma only evaluation of IC is necessary (≥5%) "as an aid in identifying urothelial carcinoma patients for treatment with TECENTRIQ (atezolizumab)." For NSCLC both TC and IC are required: "PD-L1 expression in ≥50% TC or ≥10% IC …may be associated with enhanced overall survival from TECENTRIQ (atezolizumab)".

Due to the European Medicines Agency (EMA), Tecentriq in Europe "as monotherapy is indicated for the treatment of adult patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) after prior chemotherapy. Patients with EGFR activating mutations or ALK-positive tumor mutations should also have received targeted therapy before receiving Tecentriq", here no cut offs for PD-L1 expression are mentioned [13].

In Europe, Tecentiq has similar requirements for treatment of urothelial carcinoma, although no specific test is mentioned: "Tecentriq as monotherapy is indicated for the treatment of adult patients with locally advanced or metastatic urothelial carcinoma (UC) after prior platinum-containing chemotherapy, or who are considered cisplatin ineligible, and whose tumors have a PD-L1 expression ≥5%…Patients with previously untreated UC should be selected for treatment based on the tumor expression of PD-L1 confirmed by a validated test [14]". Recently it was published that "EMA restricts use of Keytruda and Tecentriq in bladder cancer", because "data show lower survival in some patients with low levels of cancer protein PD-L1 [15]".

As **complementary diagnostic** assay (in US) is known: VENTANA PD-L1 (SP263) Assay "for the assessment of the PD-L1 protein in formalin-fixed, paraffin-embedded urothelial carcinoma tissue" for treatment with durvalumab (IMFINZI) [16]. There is also FDA approval expansion from February 16, 2018 of "Imfinzi (durvalumab) for the treatment of patients with stage III non-small cell lung cancer (NSCLC) whose tumors are not able to be surgically removed (unresectable) and whose cancer has not progressed after treatment with chemotherapy and radiation (chemoradiation)" [17].

In Europe, "the full indication is: imfinzi as monotherapy is indicated for the treatment of locally advanced, unresectable non-small cell lung cancer (NSCLC) in adults whose tumors express PD-L1 on ≥1% of tumor cells and whose disease has not progressed following platinum-based chemoradiation therapy". No indication for urothelial carcinoma is mentioned at the time of access of EMA webpage [18].

Another assay, previously published as complementary diagnostic assay PD-L1 IHC 28-8 for treatment of NSCLC and melanoma with OPDIVO (nivolumab) [19,20] is not findable on the FDA Homepage with the keywords "complementary diagnostic assay" (accessed at September 29, 2018), but becomes apparent after an intensive search with the following approval order statement: "approval for the device which will be marketed under the trade name PD-L1 IHC 28 - 8 pharmDx and is indicated for a qualitative immunohistochemical assay using Monoclonal Rabbit Anti-PD-L1, Clone 28-8 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-squamous non-small cell lung cancer (NSCLC) and melanoma tissues using EnVision FLEX visualization system on Autostainer Link 48. PD-L1 protein expression is defined as the percentage of evaluable tumor cells exhibiting partial or complete membrane staining at any intensity. PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in non-squamous NSCLC may be associated with enhanced survival from OPDIVO® (nivolumab). Positive PD-L1 status as determined by PD-L1 IHC 28-8 pharmDx in melanoma is correlated with the magnitude of the treatment effect on progression-free survival from OPDIVO®" [21].

In Europe, OPDIVO has various therapy indications for treatment of melanoma, NSCLC, renal cell carcinoma, classical Hodgkin lymphoma, squamous cell cancer of the head and neck and urothelial carcinoma, where just in the case of melanoma some information about PD-L1 expression is mentioned: "relative to nivolumab monotherapy, an increase in progression-free survival (PFS) and overall survival (OS) for the combination of nivolumab with ipilimumab is established only in patients with low tumor PD-L1 expression" [22].

In view of these approvals and data, the routine diagnostic of PD-L1-

expression in advanced tumors, requested by attending physician, actually plays an essential role for patients who may profit of a therapy with check-point-inhibitors. The usage of all previously described assays on the same patient's sample, usually a tiny biopsy in the case of NSCLC, appears not meaningful because of possible insufficient cancer tissue for other important diagnostic investigations (other target-therapy relevant molecular aberrations) [23]. For this reason, each pathology laboratory needs to determine which PD-L1 clone of the actually available, or PD-L1 assay shows the most reliable staining and which is also the most cost-effective in daily use. Another difficulty of choice of certain antibody clones is the variable staining of tumor and immune cells. And the evaluation of PD-L1 staining without paying attention to these details can lead to pitfalls with false positive and false negative results due to misinterpretation of different types of cells, located nearby [24].

The aim of this study was to compare the expression of different current available anti-PD-L1-antibodies on tumor cells to evaluate the differences in intensity and reactivity and to determine characteristics of binding sides of the most common antibodies.

2. Materials and methods

2.1. Immunohistochemistry

Twelve frequent types of malignant tumors (malignant melanoma; colorectal cancer with/without microsatellite instability; two subtypes of non-small-cell lung cancer; two subtypes of invasive carcinoma of breast; head and neck squamous cell carcinoma; carcinomas of pancreas, stomach, prostate, urinary bladder; clear cell renal carcinoma and mesothelioma) and ten patient's samples each were selected (Table 1). After histological examination of haematoxilin and eosin stained tumor sections from formalin fixed paraffin embedded tissue from the archive of institute of pathology of the University Hospital RWTH Aachen, Aachen, Germany, selected from 2011 till 2016 and anonymized (covered by the vote of ethics committee of the university clinic of RWTH Aachen, ek 173/06), six tissue microarrays (TMA) with triplets of 1 mm tumor cores of each case were constructed with using of self-built TMA metal form and using of punch needles (from Beecher Instruments, Inc., Sun Prairie, Wisconsin, United States). Initially, immunohistochemical stains using different antibody clones for PD-L1 from DAKO, Spring Bioscience, Ventana, Cell Signaling, Biocare Medical, Abcam, Zeta Corporation (Table 2) were established by testing on autostainer DAKO AS 48 Link (Agilent, Santa Clara, United States) of different pre-treatments and dilutions on placenta, tonsil and vermiform appendix tissue as a positive control, with respect of data sheet recommendations of each antibody. All stainings were performed using three different protocols for polyclonal, monoclonal mouse and monoclonal rabbit antibodies with FLEX system (Agilent). First, paraffin slides were pretreated with PT Link system. Then, as the same step in all protocols, previous to primary antibody incubation, an endogenous enzyme block with Flex Peroxidase Block was performed. After incubation of the primary antibody, in the case of monoclonal antibodies, a secondary reagent (Flex+ Mouse or Rabbit LINKER) for intensification of signal was used. Following this in all protocols, an application of labelled polymer (Flex/HRP (horseradish peroxidase)), of substrate chromogen (Flex DAB (diaminobenzidine) + Substrate Chromogen) and finally a counterstain with Flex Hematoxylin was applied. After every using of new reagent, sections were rinsed with buffer. The staining procedure of TMAs was performed by an autostainer DAKO AS 48 Link (Agilent). After cover slipping, slides were scanned by whole slide scanner Hamamatsu NanoZoomer 2.0HT (Hamamatsu, Geldern, Germany). Scans were analyzed and microphotographs were taken by using the software ndpview (Hamamatsu).

A cut-off for PD-L1 positive stained tumor in each case was 1% of cells, the expression < 1% was evaluated as negative. Complete or incomplete, only membranous staining was evaluated regardless

Table 1
Characteristics of examined tumors.

Tumor type	Subtype	Grade	Stage	Localization	Gender distribution male:female
Non-small cell lung cancer	Squamous cell carcinoma	Mostly G2, two cases G3	pT1 - pT4	6 cases in left lung, 4 cases in right lung	10:0
Non-small cell lung cancer	Adenocarcinoma	Mostly G2, three cases G3	pT1 - pT4	3 cases in left lung, 5 cases in right lung and 2 cases unknown	6:4
Breast cancer	Invasive lobular	Mostly G2, one case G3	pT1-pT3	7 cases in left and 3 cases in right breast	0:10
Breast cancer	Mostly NST, one case NST with medullary features	G2-G3	pT1-pT4	6 cases in left and 4 cases in right breast	0:10
Colorectal cancer with microsatellite instability	Some cases mucinous or partially mucinous, one case mucinous with partially signet ring cell differentiation, one case partially solid and partially mucinous	G2 or low molecular grade	pT2-pT4, pM1	Cecum, colon ascendens, right flexure, colon transversum, left flexure, spleen and liver metastasis	7:3
Colorectal cancer without microsatellite instability	Some cases mucinous	G1-G3	pT2-pT4	Satellite in small intestine, ileocecal valve, cecum, colon ascendens, colon descendens, left flexure, colon sigmoideum, rectum	6:4
Pancreatic adenocarcinoma	Mostly ductal, one case mucinous	G1-G3	pT3	Mostly pancreas head, one case pancreas cauda	7:3
Gastric cancer	Diffuse, intestinal or mixed, focal neuroendocrine differentiation	G2-G3	pT1-pT4	–	4:6
Prostate adenocarcinoma	Acinar	Gleason-Score: 6–7	pT2-pT3	–	–
Clear cell renal carcinoma	–	G1-G3	pT1-pT3	–	5:5
Infiltrating urothelial carcinoma	–	G2-G3	pT2-pT4	–	5:5
Head and neck squamous cell carcinoma	–	G2-G3	pT1-pT4	Vestubulum nasi, oral cavity, mandibula, maxilla, oropharynx, larynx	4:6

(continued on next page)

Table 1 (*continued*)

Tumor type	Subtype	Grade	Stage	Localization	Gender distribution male:female
Malignant melanoma	Nodular, superficial spreading	–	pT3-pT4, pN1, pM1, subcutaneous tumor satellite	Cutis, subcutis, pN1, pM1	6:4
Malignant mesothelioma	Mostly epitheloid, one case of sarcomatoid variant	–	pTx	Peritoneum, pleura, omentum, colon ascendens, chest wall	5:4

intensity. Cytoplasmic and nuclear staining was excluded from scoring. Additionally, a proportion Score (modified 'Cologne Score' [30]) for tumor cells was applied with following scoring-criteria: score 0 with expression < 1%; score 1 with expression of 1–4%; score 2 with expression of 5–9%; score 3 with expression of 10–24%; score 4 with expression of 25–49% and score 5 with expression of > 50%. Supplementary mean values of expression of different PD-L1 clones in different tumor types between the cases were calculated.

The immune cells were not evaluated.

2.2. Peptide library generation and microarray assay

The peptide library for PD-L1 was generated based on all isoforms available in Uniprot [25] (Q9NZQ7, Q9NZQ7-2, Q9NZQ7-3) and the missense variants collected in the phase 31,000 genomes browser for gene product ENSG00000120217 [26]. An optimal scan resulted in 645 peptides including all single amino acid variants. In addition, peptides from PD-1 (Q15116), PD-L2 (Q9BQ51) and CD276 antigen/B7-H3 (Q5ZPR3), including annotated isoforms and sequence variants, were included in the library.

The peptides were synthesized and printed on peptide microarray slides as described previously [27]. In brief, the peptides were synthesized using SPOT synthesis [28], cleaved from the solid support and chemoselectively immobilized on functionalized glass slides as described in [26]. Each peptide was deposited on the microarray in triplicates. The microarrays were incubated with 1.0 µg/mL of each PD-L1-antibody clone in an HS 4800 microarray processing station (Tecan Group Ltd., Männedorf, Switzerland) for two hours at 30 °C, followed by incubation with 1.0 µg/mL fluorescently labelled secondary antibody (Cy5-anti-rabbit IgG antibody (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK, 111-175-144) or DyLight 650-anti-mouse-IgG antibody (ThermoFisher Scientific, Waltham (MA), USA, 84545)). Washing steps were performed prior to every incubation step with 0.1% Tween-20 in 1× TBS. After the final incubation step the microarrays were washed (0.05% Tween-20 in 0.1× SSC) and dried in a stream of nitrogen. Each microarray was scanned using a GenePix Autoloader 4300 SL50 (Molecular Devices, San Jose (CA), USA, Pixel size: 10 µm). Signal intensities were evaluated using GenePix Pro 7.0 analysis software (Molecular Devices). For each peptide, the mean of the three triplicates was calculated. Further evaluation of results was performed using the statistical computing and graphics software R, Version 3.0.0 [29].

3. Results

3.1. Immunohistochemical analysis of tumor cells

The greatest number of positive stained cases was detected in both groups of NSCLC (Fig. 1). In the group of squamous cell carcinoma (***SCC*** ***of lung*** no positivity in the staining with the polyclonal anti-PD-L1 antibody (Zeta corporation) was shown. The staining with CAL10 and E1J2J antibody clones showed similar results with just one positive case per group (not same case), but the expression was completely different (3% in the staining with E1J2J and 90% in CAL10). The immuno-stainings with other anti-PD-L1 antibody clones showed expression of ≥ 5 cases in the group of SCC. A similar high expression of PD-L1 of two cases (≥ 50%) in the stainings with clones 28-8 (DAKO and Abcam), 22C3, SP142, E1L3N and 405.9A11 was detected. The highest number of positive stained cases (6) with clone SP263 with an expression of ≥ 50% of tumor cells was detected.

In the group of ***adenocarcinoma of lung*** the highest number of positive stained cases was detected with clones 28-8 from DAKO and SP142 with 9 positive stained cases with similar variability of 1 to 100% positive stained tumor cells per case (Fig. 2). The stainings with eight of the ten used anti-PD-L1 antibody clones with the exception of clone E1J2J and polyclonal antibody showed in ≥ 3 cases per group a

Table 2
Characteristics of used PD-L1 antibody clones for immunohistochemistry.

Antibody clone	Clonality rabbit/mouse	Cellular localization	Dilution	Concentration	Price per slide (Euro)
DAKO, clone 28-8 (Agilent, Santa Clara, US)	mAb-r	Membrane	RTU	No information on data sheet	~100
DAKO, clone 22C3 (Agilent, Santa Clara, US)	mAb-m	Membrane	RTU	3 µg/mL	~50
Spring bioscience, clone SP142 (Roche Diagnostics Deutschland GmbH, Mannheim, Germany)	mAb-r	Membrane	1:1000	~7 µg/mL	4,335
Ventana, clone SP263 (Roche Diagnostics Deutschland GmbH, Mannheim, Germany)	mAb-r	Membranous/cytoplasmic	RTU	~1,61 µg/mL	122,28
Cell Signaling, E1L3N (Cell Signaling Technology Europe, B.V., Frankfurt am Main, Deutschland)	mAb-r	No information on data sheet	1:6400	874 µg/mL	0,62
Cell Signaling, E1J2J (Cell Signaling Technology Europe, B.V., Frankfurt am Main, Deutschland)	mAb-r		1:50	9 µg/mL	79,9
Cell Signaling, 405.9A11 (Cell Signaling Technology Europe, B.V., Frankfurt am Main, Deutschland)	mAb-m		1:400	100 µg/mL	9,99
Biocare Medical, CAL10 (Zytomed Systems GmbH, Berlin, Germany)	mAb-r	Membranous/cytoplasmic	1:400	~10 mg/mL	0,46
Abcam, 28-8 (Abcam, Cambridge, UK)	mAb-r	Cell membrane and endomembrane system	1:500	1096 mg/mL	1,796
Zeta corporation (Zeta corporation, Sierra Madre, USA)	pAb-r	Membrane	1:50	No information on data sheet	84

high PD-L1 expression of tumor cells (50–100%). In the staining with E1J2J antibody clone only two cases with just 1% positivity were detected. Similarly, in the staining with the polyclonal antibody clone just one case showed a low positivity for PD-L1 (2%).

The breast cancer group of ***non-special type*** (***NST***) was characterized by a low expression of PD-L1. Seven of the ten used clones did not show any positivity for PD-L1. The stainings with clones 28-8 (DAK0), SP263 and zeta corporation showed positivity of one (with 1%), two (with 1 and 6%) and three cases (2 cases with 30% and one case with 40%), respectively. As just mentioned, the highest percentage of positive stained tumor cells was detected by the polyclonal antibody with 40%.

In the second group of ***invasive lobular carcinoma of breast*** no positivity of tumor cells for PD-L1 with all used clones was shown.

A variable expression of PD-L1 in groups of ***colorectal cancer*** (***CRC***) ***with and without microsatellite instability*** (***MSI***) was detected. An absent expression of PD-L1 with clones E1J2J, 28-8 (Abcam), SP142 and CAL10 was observed. In both groups of CRC the staining with clone

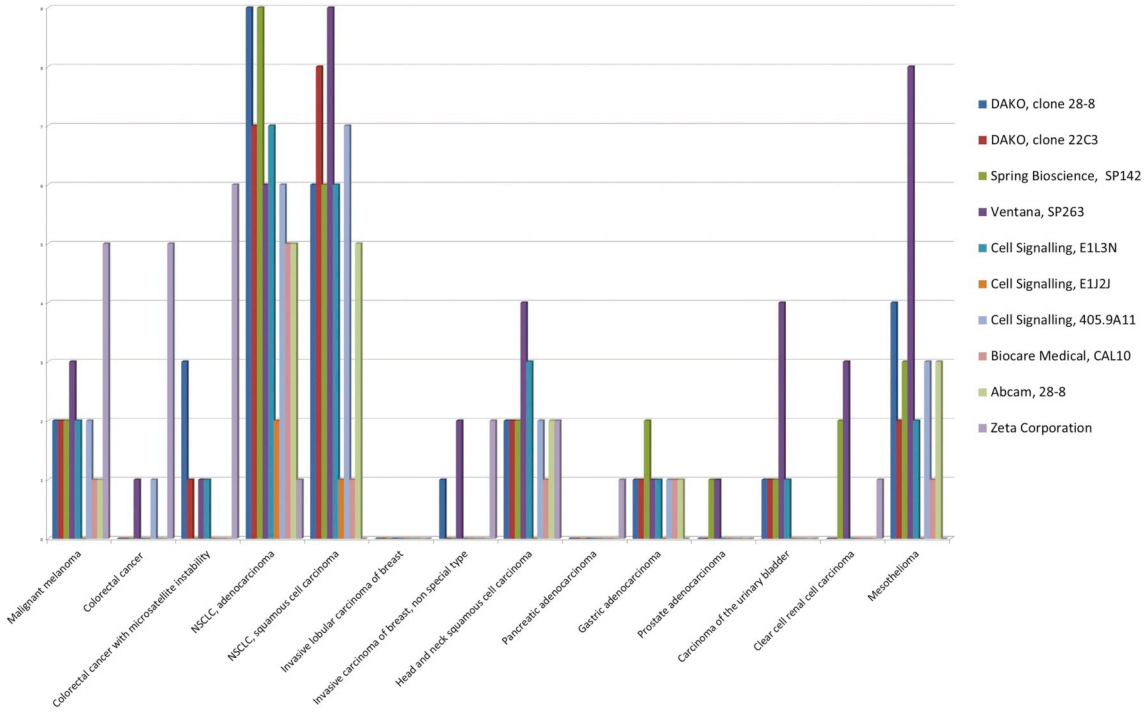

Fig. 1. Number of positive stained cases with different anti-PDL1-antibodies (x-axis: number of cases, y-axis: tumor group, right side: differently colored PD-L1 antibody clones).

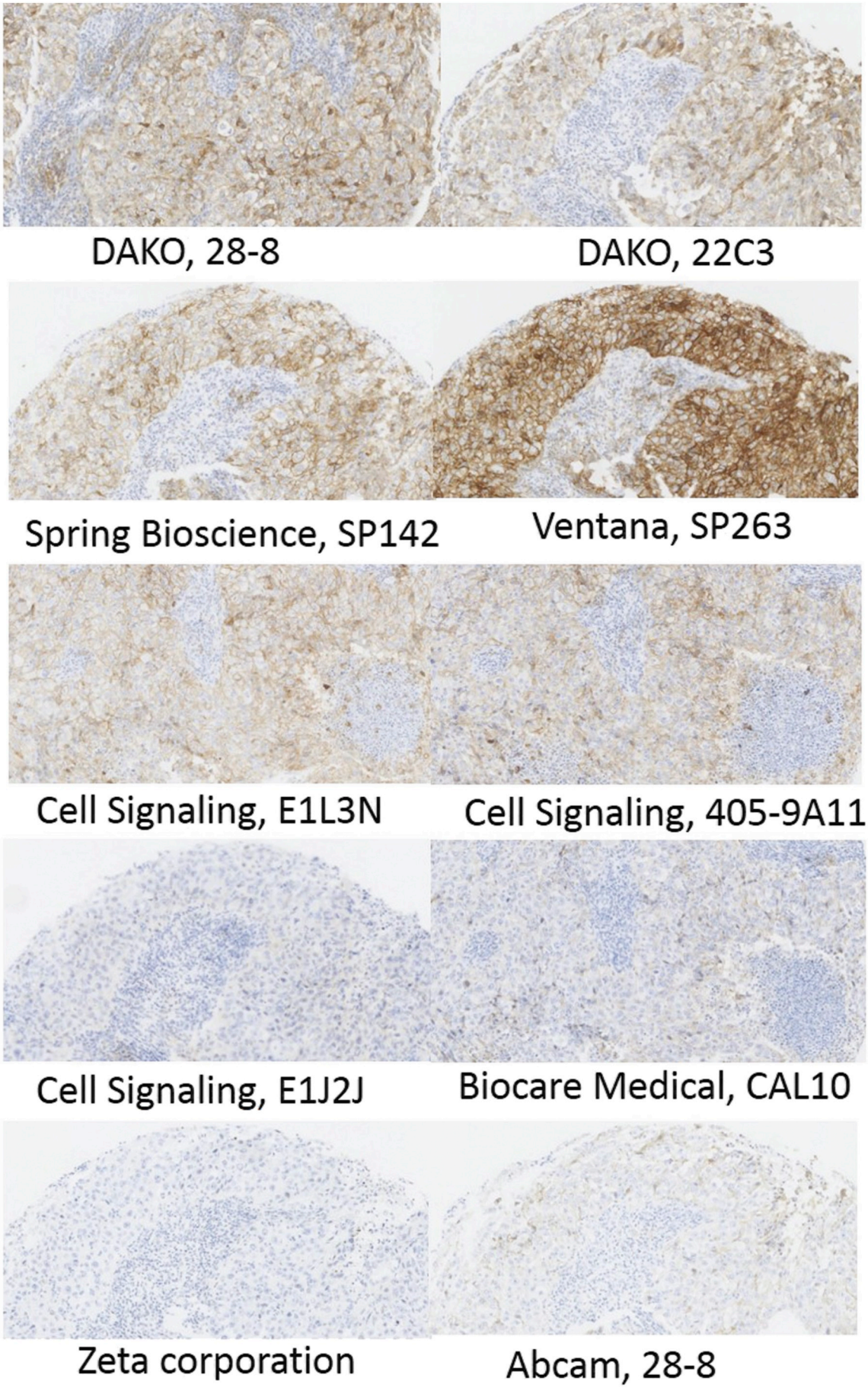

Fig. 2. Expression of different PD-L1 clones in a solid adenocarcinoma of lung.

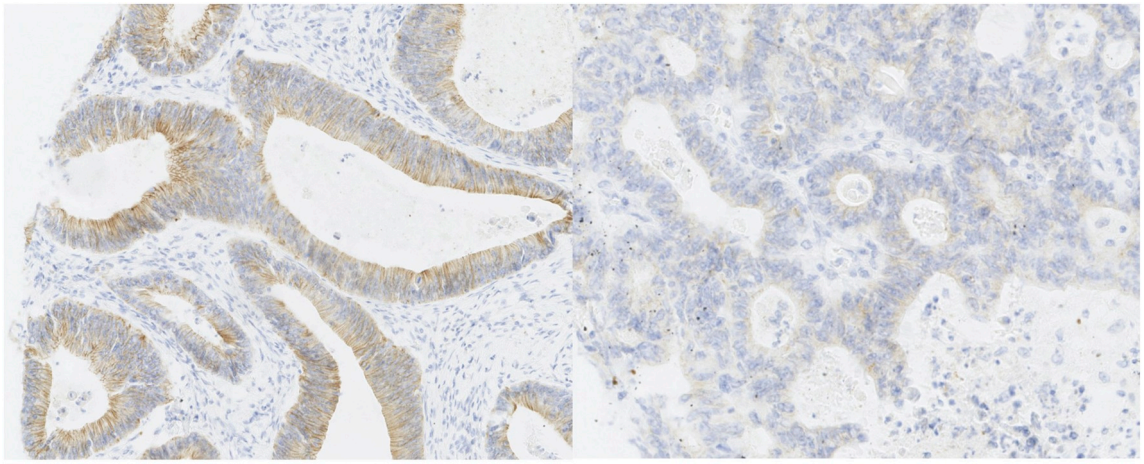

Fig. 3. Expression of polyclonal PD-L1 (Zeta Corporation) antibody in colorectal cancer.

SP263, just one case from ten examined was positive each, although the intensity of the staining was variable (1% without MSI and 40% with MSI). The highest number of positive stained cases in both groups of CRC in the staining with the polyclonal antibody with five cases in the group without MSI and six cases in the group with MSI was revealed. The percentage of positive stained tumor cells was visible in the staining with the polyclonal antibody, ≥60% in the group without MSI and up to 50% in the group with MSI (Fig. 3).

The staining with antibodies 28-8 (DAKO), 22C3 and E1L3N revealed a higher number of positive stained cases in the group of CRC with MSI in comparison to CRC without MSI (3 vs 0, 1 vs 0 and 1 vs 0, respectively). Reversely, a higher number of positive stained cases in the group of CRC without MSI in comparison to CRC with MSI was shown in the staining with clone 405.9A11 (1 vs 0, respectively).

Nine of the ten examined antibody clones did not show any positivity in the **pancreatic cancer** group. Only in the staining with the polyclonal PD-L1 antibody (zeta corporation) four cases with expression from 7% to 60% were identified.

A similar low number of positive stained cases (1/10) in the **gastric cancer group** with eight of the ten examined clones with the exception of E1J2J and SP142 clones were detected. The percentage of positive stained cells of the same positive stained case was variable – from 5% with clone CAL10 to 100% with clone SP263. No positivity in the staining with clone E1J2J was observed and two cases were positive (with 2% and 95% positivity) in the staining with clone SP142.

Just two of ten examined antibodies (SP142 and SP263) showed a low positivity of one same case in the group of **prostate adenocarcinoma** with 1% and 4% positivity correspondingly. The remaining cases stained with these antibodies were negative. The other eight used anti-PD-L1-clones did not shown any positivity.

In the group of **clear cell renal carcinoma** just three of the ten examined antibodies (polyclonal, SP263 and SP142) showed positive expression of PD-L1 in 2–3 positive stained cases. The percentage of positive stained cells was variable – from 2% to 43%. Other clones did not show an expression.

A similar low number of positive stained cases (1/9 or 1/10) in the **group of invasive carcinoma of urinary bladder** with clones 28-8 (DAKO), 22C3, SP142 and E1L3N was observed, at which identical staining of 10% of tumor cells of the same case in all mentioned clones with the exception of 28-8 DAKO were observed. The last one stained another case with 1% positivity of tumor cells. In the staining with clone SP263 four cases with variable expression from 1% to 99% were positive. Other antibodies did not show any positivity.

In the group of **head and neck SCC** all examined clones with exception of E1J2J showed expression of PD-L1 with one (CAL10) to four positive stained cases (SP263). The lowest expression (1% of tumor cells) with the polyclonal PD-L1 antibody and highest expression with clones 28-8 (DAKO) and SP263 with 100% of positive stained tumor cells was observed.

Up to three positive cases in the group of **malignant melanoma** in the stainings with nine of the ten examined clones, with the exception of E1J2J (no positivity), were detected. In a comparison of one same positive stained case, a similar high expression of PD-L1 (90–99%) in tumor cells with clones 28-8 (DAKO, Abcam), SP263, SP142, C22C3, 405-9A11 and E1L3N was detected. Analyzing the same case, a low positivity of PD-L1 in the staining with CAL10 (1%) and no positivity in the staining with polyclonal antibody was demonstrated.

In the group of **malignant mesothelioma**, all clones with exception of polyclonal antibody showed an expression of PD-L1 in tumor cells. A variable positivity of one (E1J2J with 3%, CAL10 with 30%) to eight cases (SP263 with 5 to 100%) in the group was detected. A similar high expression of PD-L1 (95–100%) in one same positive stained case with exception of clone CAL10 (30%) was observed.

In the comparison of mean values of expression of different PD-L1 clones in different tumor types, three of ten examined PD-L1 clones showed different staining pattern (Table 3):

1) A clone E1J2J (Cell Signaling) stained < 1% of tumor cells in all examined tumor groups.
2) In contrast to other clones, a clone CAL10 (Biocare medical) showed a weak positivity of cells (< 1%) in such tumor types as malignant melanoma and gastric carcinoma. The expression of CAL10 in other tumor groups showed with exception of NSCLC (adenocarcinoma) a generally lower amount of stained cells compared to other clones.
3) A polyclonal antibody (Zeta corporation) showed a lower expression level of stained tumors compared to other clones, and interestingly stained tumor cells, which was not stained with other clones (colorectal cancer with/without MSI, invasive breast carcinoma of NST, pancreatic adenocarcinoma and clear cell renal carcinoma).

Remarkably, the staining with the same clone 28-8 from different vendors (DAKO and Abcam) did not show exact same results (different mean values of expression) with dissimilarities in such tumor groups as lung cancer (both subgroups) and head and neck SCC.

3.2. Profiling study of several PD-L1 antibody clones

Additionally, profiling of PD-L1-antibody clones 28-8 (Abcam and DAKO), 22C3, SP263 and SP142, used in the immunohistochemical staining, was performed by our co-operation partners from JPT Peptide

Table 3
Results of immunohistochemistry. Mean values of expression of different PD-L1 clones in different tumor types.

Tumor type/PD-L1 antibody clone	DAKO, clone 28-8	DAKO, clone 22C3	SB, SP142	Ventana, SP263	CS, E1L3N	CS, E1J2J	CS, 405.9A11	BM, CAL10	Abcam, clone 28-8	ZC
Malignant melanoma	10%	10,4%	11,11%	21,4%	10%	0%	9,6%	0,1%	9,5%	3,2%
Colorectal cancer	0%	0%	0%	0,1%	0%	0%	0,2%	0%	0%	40,8%
Colorectal cancer with MSI	1,2%	0,3%	0%	4%	0,2%	0%	0%	0%	0%	10,6%
NSCLC, adenocarcinoma	62,2%	46%	49,7%	69,3%	45,6%	0,2%	43,3%	28,5%	22,9%	0,2%
NSCLC, SCC	30,2%	26,3%	20,9%	44,9%	21,5%	0,3%	20,2%	9%	20,1%	0%
Invasive lobular carcinoma of breast	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Invasive carcinoma of breast, NST	0,1%	0%	0%	0,7%	0%	0%	0%	0%	0%	7%
Head and neck SCC	19,3%	9,3%	11%	25,2%	17,5%	0%	13,7%	1,1%	9,8%	0,2%
Pancreatic adenocarcinoma	0%	0%	0%	0%	0%	0%	0%	0%	0%	8,7%
Gastric adenocarcinoma	9,9%	9,9%	9,7%	10,0%	9,8%	0%	9,3%	0,5%	6%	1,5%
Prostate adenocarcinoma	0%	0%	0,1%	0,4%	0%	0%	0%	0%	0%	0%
Carcinoma of the urinary bladder	0,1%	1%	1%	10,9%	1,11%	0%	0%	0%	0%	0%
Clear cell renal carcinoma	0%	0%	0,5%	8,5%	0%	0%	0%	0%	0%	4%
Mesothelioma	14,5%	18,4%	14,7%	27,5%	15,5%	0,3%	12,6%	3%	13,6%	0%

There is a six step score for % of stained tumor cells from white background to dark blue ranging from 0 to 5: 0 ≤ 1%; 1 = 1–4%, 2 = 5–9%, 3 = 10–24%, 4 = 25–49%, 5 ≥ 50%.

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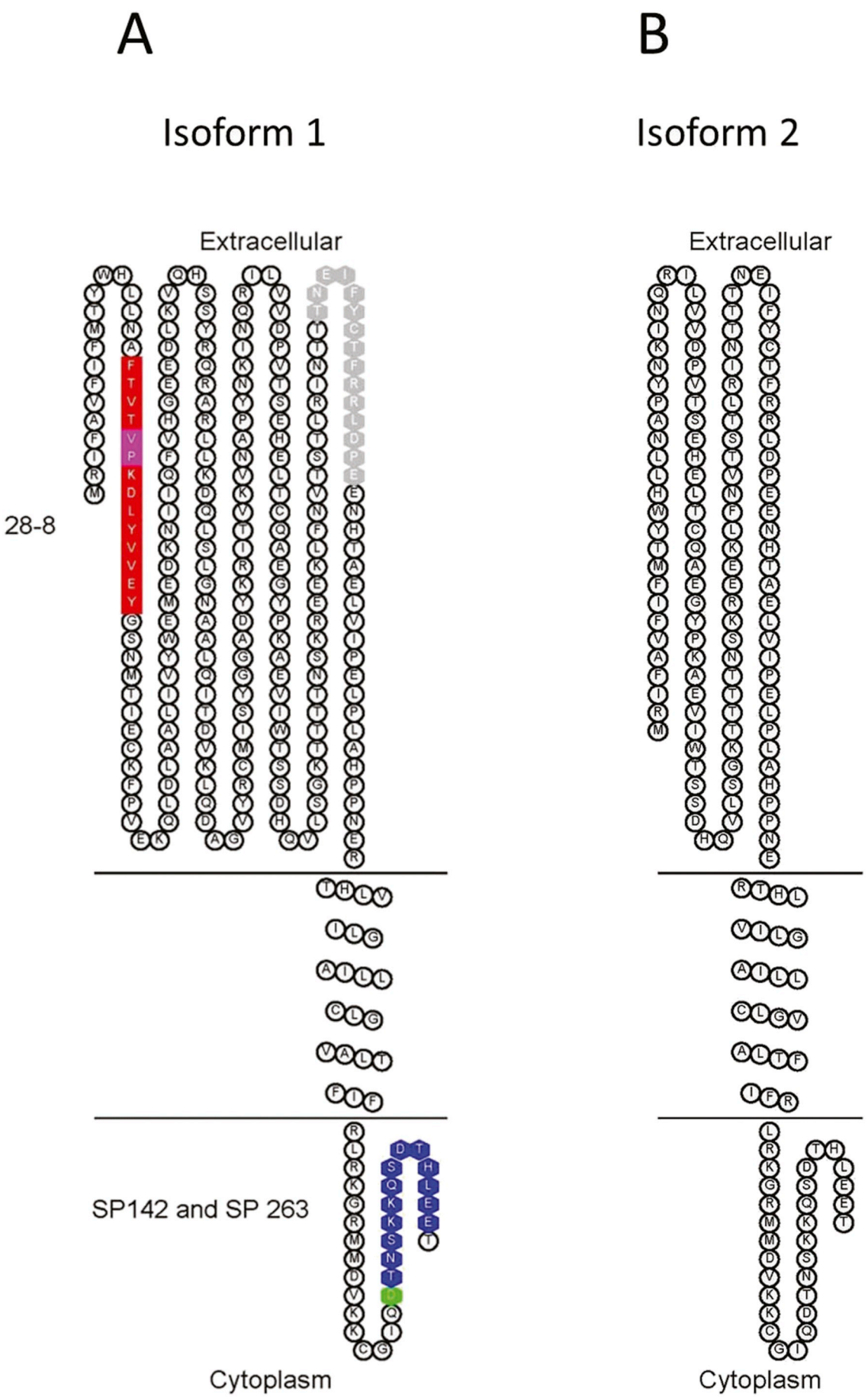
Based on antibody binding profiles and overlapping peptide sequences, linear epitopes of PD-L1-antibody clones 28-8 (Abcam), SP263 (Ventana) and SP142 (SB) could be identified (Table 4). For SP142 and SP263 highest fluorescent signals have been observed with peptides spanning the amino acids 275–289 for SP142 and 275–290 for SP263, respectively, which resemble the C-terminal intracellular region of PD-L1 isoform 1 and 2 (Table 5, Fig. 4) indicating a high likeliness of mapping an epitope within this part of the protein. However, binding of SP263 has also been observed with peptides covering the extracellular domain at positions AA 203–217. Nevertheless, SP263 did not react with any other peptides derived from PD-1, PD-L1 or CD276 antigen/B7-H3. SP142 showed reactivity with two peptides from PD-1 Q15116. Since signal occurred in non-adjacent peptides and consensus sequence spanned only three amino acids at positions 225–227, it could be considered that the signal though being strong might not be based on epitope-specific interaction. Besides this observation no other cross-reactivity has been observed with the other clones.

Table 4
Binding epitopes of PD-L1-antibody clones 28-8 (Abcam), SP263 (Ventana) and SP142 (Ventana) identified with peptide microarray. Peptides showing significant antibody binding are marked bold. Epitopes can be derived from the overlapping peptide sequences (marked red). Differences of the mutated (Mut) sequences in comparison to the wild type (Wt) are highlighted with blue color.

Origin	Sequence	Signal
PDL-1-Ventana-SP263		
Wt	KKCGIQDTNSKKQSD	201.67
Wt	**QDTNSKKQSDTHLEE**	**55078.67**
Wt	**DTNSKKQSDTHLEET**	**45299**
Mut	**YTNSKKQSDTHLEET**	**39918.33**
Mut	**HTNSKKQSDTHLEET**	**30453.33**
Wt	INTTTNEIFYCTFRR	1634.33
Wt	**TNEIFYCTFRRLDPE**	**46621**
Wt	FYCTFRRLDPEENHT	188.67
PDL-1-abcam-28-8		
Wt	HLLNAFTVTVPKDLY	806
Mut	**AFTVMVPKDLYVVEY**	**53950.67**
Mut	**AFTITVPKDLYVVEY**	**50410.33**
Wt	**FTVTVPKDLYVVEY**G	**54717**
Mut	VPKDLYVVEYGTNMT	210.67
PDL-1-Ventana_SP142		
Mut	GIQHTNSKKQSDTHL	50
Wt	**QDTNSKKQSDTHLEE**	**42231**
Wt	DTNSKKQSDTHLEET	56.33
Wt	LKEDPSAVPVFSVDY	58.33
Wt	**PSAVPVFSVDYGELD**	**47943.67**
Wt	**ELDFQWREKTPEPPV**	**51091.33**
Wt	QWREKTPEPPVPCVP	58.67

Table 5
Peptides demonstrating highly significant binding of PD-L1 antibody clones. The mean signal values are shown as color coded ranging from white (zero or low binding) over yellow (middle binding) to red (strong binding). Overlapping sequences are marked red.

Peptide	Origen	Sequence position (referred to Isoform 1)	Microarray readout signal		
			SP142 (Ventana)	SP263 (Ventana)	28-8 (Abcam)
QDTNSKKQSDTHLEE **DTNSKKQSDTHLEET**	**PD1L1_HUMAN Isoform 1/2 (Uniprot: Q9NZQ7-1/Q9NZQ7-2)**	**275-289**	**42231**	**55079**	**162**
		276-290	**56**	**45299**	**149**
FTVTVPKDLYVVEYG	**PD1L1_HUMAN Isoform 1/3 (Uniprot: Q9NZQ7-1/Q9NZQ7-3)**	**19-33**	**58**	**922**	**54717**



(caption on next page)

Fig. 4. Schematic structure of PD-L1 isoform 1 and 2 and position of mapped linear epitope regions. A. Full length PD-L1 isoform 1 with likely epitopes identified by microarray assay. Red peptides recognized by clone 28-8, positions with annotated missense mutations are marked in magenta. Blue peptides recognized by SP142 and SP263, positions with annotated missense mutations in green. Light grey peptide also bound by SP263 located at the extracellular domain. B. Schematic view of PD-L1 Isoform 2 showing a truncation of AA 19-132. Figure has been created using TOPO2 Transmembrane protein display software by Johns SJ [46].

The clone 28-8 from Abcam showed the strongest signals with an epitope from the extracellular domain between AA 19–32 (Table 5). Additional reactivity with peptides from other parts of the protein have not been observed. To our surprise, clone 28-8 from a different vendor (DAKO) did not show any binding at all even at different dilutions. Clone 22C3 which was derived from immunization with an extracellular fusion protein also did not exhibit any significant binding indicating the absence of a linear epitope.

To identify PD-L1 sequence variants which could be recognized by the antibody clones mentioned above, Signal-to-Amino Acid Residue Plots were computed representing aligned sequences of all PD-L1 isoforms (Q9NZQ7, Q9NZQ7-2, Q9NZQ7-3) as well as of missense variants. According to these data, PDL1-antibody clone SP263 (Ventana) with different affinity recognized additional sequence variants (Q9NZQ7-2_D162H; Q9NZQ7_D276H; Q9NZQ7 – 2_T163S; Q9NZQ7 – 2_D162Y; Q9NZQ7_D276Y; Q9NZQ7_T277S) which could not be detected by SP142 (SB).

The remaining PD-L1-antibody clones 28-8 (DAKO) and 22C3 (DAKO) did not show any significant binding to peptides.

4. Discussion

In the discussion part we would like to focus on the comparison of PD-L1 staining on tumor tissue of NSCLC due to importance of potential involvement of patient into a treatment with available target therapy depend of expression of PD-L1 in tumor cells. Other reasons in favor are various published data about PD-L1 harmonization studies, performed on NSCLC. In addition, by data of the profiling study, different binding characteristics are discussed. Other tumor entities will be also briefly addressed.

Hirsch et al. analyzed four trial-validated PD-L1 IHC assays in NSCLC in the Blueprint project and revealed that “the percentage of PD-L1-stained tumor cells was comparable when the 22C3, 28-8, und SP263 assays were used, whereas the SP142 assay exhibited fewer stained tumor cells overall” [30]. In the study of Scheel AH et al. [31], a harmonized PD-L1 immunohistochemistry for pulmonary squamous-cell and adenocarcinomas was performed. Similarly to the previous study, clones 28-8, 22C3, SP142 und SP263 were examined. “The assays 28-8 and 22C3 stained similar proportions of carcinoma cells in 12 of 15 cases. SP142 stained fewer carcinoma cells compared to 28-8, 22C3, and SP263 in four cases, whereas SP263 stained more carcinoma cells in nine cases”. In the study of Ratcliffe MJ et al., after examination of 493 samples of NSCLC, similar results of tumor cell staining with assays SP263, DAKO 22C3 and 28-8 was observed [32]. In another multicenter study of Adam J et al. [33], 41 NSCLC tumor samples were analyzed after resection with five antibody clones (28-8, 22C3, SP263, SP142, and E1L3N) with high concordant result for assays 28-8, 22C3 and SP263 in the staining of tumor cells. Also the laboratory-developed tests (LDT), such as SP142 and E1L3N, were compared with reference PD-L1 assay (SP263) and two of seven centers in the case of SP142, and four of seven in the case of E1L3N, were classified as concordant. Rimm DL et al. analyzed in another multi-institutional study four PD-L1 antibodies by staining of 90 NSCLC specimen on Dako Link 48 for 28-8 and 22c3, on Ventana Benchmark for SP142 and Leica Bond for E1L3N [34]. Interestingly, the expression of PD-L1 in tumor and immune cells using SP142 assay was significant lower than in other assays. Similar to SP142, clone 22c3 also stained less tumor cells, but slightly. No significant difference between 28 and 8 and E1L3N assays was found. Another study of Scheel AH et al. investigated the PD-L1 immunohistochemistry of four assays and eleven LDTs (with 5 primary antibodies such 22C3 Dako, 28-8 Abcam, SP263 Roche Diagnostics, E1L3N Cell Signaling and QR1 Quartett Immunodiagnostic, using different protocols) on NSCLC tissue microarrays at ten German testing sites [35]. The staining of examined assays demonstrated similar results of 22C3, 28-8 and SP263. The SP142 showed less staining of tumor cells due to additional granular pattern (in seven of 15 cases of carcinoma cells and in six of 11 cell lines). In the case of LTDs, similar results were observed with 22C3 and 28-8 assays in six of eleven analyzed protocols, where just one protocol for E1L3N and QR1 was examined.

In our study in both groups of NSCLC (adenocarcinoma and SCC), different mean values of PD-L1 expression between ten examined clones, in contrast to mentioned studies, were detected. Similar low mean value (< 1%) in the staining with clones E1J2J (Cell signaling) and polyclonal antibody (Zeta corporation) were observed.

4.1. Differences in NSCLC, adenocarcinoma

Comparable, but relative low PD-L1 expression levels were detected with clones CAL10 (BM) with 28,5% and 28-8 (Abcam) with 22,9%. Comparable, intermediate PD-L1 expression was shown with clones 405.9A11 (CS), E1L3N (CS), 22C3 and SP142 (SB) with mean values of expression from 43,3 to 49,7%. Highest mean values of PD-L1 expression were shown with clones SP263 and 28-8 (DAKO) with 69,3% and 62,2%, respectively.

If we look at our results of different PD-L1 stainings among came cases in the group of NSCLC (adenocarcinoma) and apply different cutoffs of positivity (≥1% and ≥50% of stained tumor cells/case) there are noticeable differences in seven of ten examined cases, where just three cases showed similar positivity or negativity (after exclusion from the comparison of E1J2J and ZC with general low expression of PD-L1)

Table 6
Percentage of positive stained tumor cells in the group of NSCLC (adenocarcinoma). Italicized are cases with no differences by using cutoffs of ≥1% and ≥50% of stained tumor cells/case.

PD-L1 antibody	DAKO, clone 28-8	DAKO, clone 22C3	SB, SP142	Ventana, SP263	CS, E1L3N	CS, E1J2J	CS, 405.9A11	BM, CAL10	Abcam, clone 28-8	ZC
Case 1	***100%***	***100%***	***100%***	***100%***	***98%***	0%	***98%***	***95%***	***80%***	0%
Case 2	80%	10%	15%	100%	0%	0%	0%	0%	0%	0%
Case 3	50%	0%	10%	0%	2%	0%	2%	0%	0%	0%
Case 4	100%	93%	100%	100%	100%	1%	98%	10%	50%	0%
Case 5	100%	95%	99%	100%	97%	0%	98%	90%	3%	0%
Case 6	11%	2%	2%	95%	0%	0%	0%	0%	1%	2%
Case 7	***100%***	***100%***	***100%***	***100%***	***99%***	1%	***97%***	***90%***	***95%***	0%
Case 8	80%	60%	70%	98%	60%	0%	40%	0%	0%	0%
Case 9	***0%***	***0%***	***0%***	***0%***	***0%***	0%	***0%***	***0%***	0%	0%
Case 10	1%	0%	1%	0%	0%	0%	0%	0%	0%	0%

Table 7
Percentage of positive stained tumor cells in the group of NSCLC (SCC). Italicized are case with no differences by using cutoffs of ≥1% and ≥50% of stained tumor cells/case.

PD-L1 antibody	DAKO, clone 28-8	DAKO, clone 22C3	SB, SP142	Ventana, SP263	CS, E1L3N	CS, E1J2J	CS, 405.9A11	BM, CAL10	Abcam, clone 28-8	ZC
Case 1	100%	100%	98%	100%	97%	0%	95%	0%	99%	0%
Case 2	40%	4%	3%	30%	1%	0%	1%	0%	0%	0%
Case 3	***100%***	***99%***	***100%***	***100%***	***99%***	0%	***99%***	***90%***	***97%***	0%
Case 4	0%	1%	0%	0%	0%	0%	2%	0%	0%	0%
Case 5	20%	16%	3%	50%	13%	0%	2%	0%	1%	0%
Case 6	2%	0%	0%	3%	0%	0%	0%	0%	0%	0%
Case 7	40%	20%	4%	50%	3%	3%	2%	0%	3%	0%
Case 8	0%	20%	0%	53%	0%	0%	0%	0%	0%	0%
Case 9	0%	0%	0%	3%	0%	0%	0%	0%	0%	0%
Case 10	0%	3%	1%	60%	2%	0%	1%	0%	1%	0%

(Table 6).

4.2. Differences in NSCLC, SCC

Interestingly, that clone CAL10 demonstrated the PD-L1 expression in the lower range (9%). Also a relative low PD-L1 expression levels were detected with clones SP142, E1L3N, 405.9A11, 28-8 (Abcam) with range from 20,1 to 21,5%. Other DAKO clones and SP263 showed higher mean values of expression with the highest PD-L1 expression by SP263 with 44,9%.

By same application of PD-L1 positivity cutoffs by ≥1% and ≥50% of stained tumor cells/case in the group of NSCLC (SCC), and also after exclusion of E1J2J and ZC from comparison, just one case showed comparable results, while other nine demonstrated therapy-relevant differences (Table 7).

According to other tumor entities, for examples, melanoma, urothelial carcinoma of the bladder, hypopharyngeal squamous cell carcinoma or gastric/gastroesophageal junction adenocarcinoma, some studies to PD-L1 are actually published.

In the case of melanoma Sunshine JC et al. [36] examined clones 22C3, 28-8 (Abcam), SP263, SP142 (SB), 5H1 and demonstrated, that "strong correlations were observed between the percentage of PD-L1(+) cells across all clones studied" while examined "the percentage of total cells (including melanocytes and immune cells)". Four of mentioned antibodies were also examined in our study (we analyzed just tumor cells): stained cases ranged from 1/10 (28-8 Abcam) to 3/10 (SP263). In the comparison of the same tumor core between different clones no wide variation of percentage of positive stained tumor cells was detected: from 90% tumor cells (SP142, SB), over 95% (28-8 Abcam) to 99% (22C3 and SP263).

Recently published study from Hodgson A et al. [37] examined a PD-L1 expression on TCs and ICs on TMAs of urothelial carcinoma of the bladder and hypopharyngeal (HP) squamous cell carcinoma to compare three kits (Dako 22C3, Ventana SP263 and Ventana SP142) and a platform-independent test E1L3N (from Cell Signaling Technologies). Using different cutoffs depends on tumor entity and assay they demonstrated, that "12% of bladder and 15% HP cases showed discrepant PD-L1 classification results. Regardless of the scoring algorithm used, E1L3N provided comparable PD-L1 staining results". In our study we analyzed just tumor cells, and in the group of head and neck SCC, a very variable mean value of PD-L1 expression was detected – from 0% (J1J2J) to 25,2% (SP263). In the group of urinary bladder carcinoma, the most of examined clones showed 0 to 1,11% mean value of PD-L1 expression, in contrast to them, SP263 showed the highest mean value of PD-L1 expression of 10,9%.

In the case of advanced gastric carcinoma, according to approval of FDA, a therapy with pembrolizumab can be performed, when the tumor has CPS ≥ 1, measured after immunohistochemistry with 22C3 pharmDx assay [11]. Some authors believe, that PD-L1 testing may not be enough, because "tumor mutational load, microsatellite instability (MSI), and alternative PD-L1 testing thresholds may serve as predictive biomarkers for response to immune checkpoint inhibition, and standard PD-L1 testing will not identify all patients who may benefit from this therapy" [38].

Koppel et al. published recently that "a systemic and comprehensive comparison of PD-L1 immunohistochemistry protocols on the four most commonly used immunohistochemistry platforms using the Abcam anti-PD-L1 28-8 antibody in lung cancer, melanoma, and head and neck cancer, using tissue microarray samples, showed adequate concordance and good reproducibility when compared with reference PD-L1 IHC 28-8 pharmDx kit" [39]. Contrary to this, in our study in the comparison of staining with PD-L1 clones 28-8 from different vendors (Abcam vs. DAKO), a generally slightly lower mean value of expression with clone 28-8 from Abcam was detected. To concentrate on NSCL, the marked lower percentage of expression in the group of adenocarcinoma (NSCLC) with a difference of almost 40% was observed. Both clones are monoclonal rabbit antibodies and have membranous staining. These differences of staining could not be explained considering an epitope mapping or due to inaccurate establishment in the test phase. Interestingly, that Schats et al. [40] published that "differences in IC and/or TC sensitivity could not be observed on cell controls or placenta tissue (often suggested as staining control). Thus, validation of PD-L1 IHC staining requires additional inclusion of tonsil and tumor samples". We validated our staining with usage of placenta, tonsil and vermiform appendix tissue without using of additional tumor samples.

The comparison of the PD-L1 expression between other clones (28-8 DAKO, 22C3 DAKO, SP263 and SP142) showed in our study a higher mean value of expression in NSCLC, using clone SP263 (concordant to earlier published study of Sheel et al. [31]), and also in other positive stained tumor groups. Considering the adenocarcinoma group of NSCLC, both clones 28-8 DAKO and SP263 showed interestingly a higher percentage of mean value of PD-L1 expression compared to other two clones, with clinically important differences (cutoff of 50% is overstepped). Similar to previously published studies, which analyzed SP142 from Ventana [29,30,33,34], in the SCC group we observed a lower percentage of mean value of PD-L1 expression in the staining with SP142 (Spring Bioscience).

In our study we performed all staining of different antibody clones on one same platform (autostainer DAKO AS 48 Link, Agilent). Schats et al. announced recently in the investigation of tumor and immune cells of lung carcinoma and melanoma that "the detection method used in the PD-L1 IHC assay determines the staining pattern of a specific cell type or cellular compartment. Changing the detection method has important consequences on TC and/or IC sensitivity, affecting the clinical cutoff values and subsequently, patients' PD-L1 positivity status" [40]. In that study researchers examined Dako assays (22C3 and 28-8) on Autostainer Link 48; Ventana assays (SP142 and SP263) on Ventana Benchmark Ultra platform; 22C3 from Dako and 28-8 from Abcam on Ventana Benchmark XT platform, and SP142 from Spring Bioscience and SP263 from Ventana on Lab Vision Autostainer 480S. According to tumor cells, authors described that "the SP142 L + F protocol showed more TC staining than the kit insert and was more concordant with the

22C3 and 28-8 kits". Possibly, some our discrepant results could be caused by using just one IHC platform. At the same time, Munari et al. postulated after examination of non-small cell lung cancer cases that "assays 22C3 and SP263 show important discrepancies in identifying programed death-ligand 1-positive cases at clinically relevant cutoffs, with possible underestimation of patients suitable for pembrolizumab therapy" [41]. In that study examined assays were performed on different platforms (Dako Link-48 and Ventana Benchmark Ultra). We also observed in our study in the comparison of 22C3 and SP263 stainigs on lung cancer that in adenocarcinoma group two cases of ten showed therapy relevant differences with staining of more tumor cells by SP263. In SCC group of NSCLC, comparing same two clones, interestingly one case (case 4, Table 7) showed more stained cells with 22C3 as with SP263, one other case showed the same positivity of 100% cells (case 1, Table 7), and other cases demonstrated a higher PD-L1 positivity in the staining with SP263 as with 22C3 with therapy relevant differences in six cases.

Another possible reason of variable mean value expressions of different PD-L1 antibodies could be the nature of antibodies. In our study we performed microarray antibody profiling with selected PD-L1 antibodies, which we used for immunohistochemistry and which play an essential role as part of diagnostic assays. The clones 28-8 (Abcam), SP263 and SP142 showed highly significant interactions with some overlapping peptides representing different PD-L1 sequence variants. Linear epitopes identified for SP263 and SP142 could be located to the cytoplasmic regions which is in line with previous findings [42,43]. In addition clone SP142 also shows some minor binding do a peptide derived from the extracellular domain.

Surprisingly, with antibody clone 28-8 obtained from DAKO no binding was observed in the microarray whereas with the antibody of the same clone designation provided from Abcam an epitope could be mapped to the amino acids 19–33 of full length PD-L1 (Isoform 1). The location of the epitope within in this section of the protein is also supported to by the fact that Phillipps et al. [44] created clone 28-8 upon rabbit immunization with an extracellular domain spanning the amino acid 19–293. Different performance in microarray could be explained due to matrix or concentration effects but also in line with a dissimilar staining performance observed in this study.

In 2004 a N-terminally shortened isoform of PD-L1 (isoform 2) lacking residues 19–132 was published by He et al. [45]. Although its physiological function is still unresolved it should be kept in mind that 28-8 might not detect this isoform due the lack of the epitope whereas SP124 and SP263 might also detect this variant since epitopes are located downstream of the lacking sequence. Once the detailed expression and function of PD-L1 isoform 2 is discovered, the latter antibodies might provide a broader specificity.

Despite PD-L1 antibody clones SP263 and SP142 showing strong binding within the same region ((Q)DTNSKKQSDTHLEE), SP263 turned out to be less susceptible for mutation-derived amino acid exchange at p276 and it also recognizes missense variants of the peptide (p.D276H, pD276Y) while SP142 did not bind to the mutated peptides. As reported by Hirsch et al. 2017 [30] (and seen in our study for NSCLC, Table 3), SP142 would stain less tumor cells in contrast to SP263, it is tempting to speculate that this could be addressed to an overall "broader specificity" or affinity of SP263 as it is less sensitive to amino acid exchange.

According to SP142, after detected cross-activity of SP142 with two peptides from PD-1 Q15116 we can suppose that this interesting finding can be a good explanation that clone SP142 stains immune cells more intensely [30] in immunohistochemistry, compared with other PD-L1 antibody clones. Lymphocytes or so called tumor-infiltration lymphocytes (TILs) also belong to immune cells, which express PD-1 [46]. However this cross-activity was not detected for SP263, which also stains immune cells more intensely [30].

To verify our described supposed correlations between the microarray data and staining behavior, future biophysical comparison of the antibodies such as affinity measurements, identification of the complementary-determining region (CDRs) or PD-L1 sequencing of specimens are necessary.

In summary, actually different anti-PD-L1 antibody clones are available for immunohistochemistry for variable costs, individually or within the assay. We performed staining of multiple antibody clones on different tumor types and compared our findings with other published data with evaluation of partial similar data. Our results are limited due to several factors (examination of tissue microarrays, low number of examined cases within the group, one stain platform). A more nuanced understanding could be achieved by studying a greater number of cases with hole slide tumor sections with additional performance on several platforms, because of essential diagnostic information for patient with advanced tumor, which can/can't profit of target therapy, depending of PD-L1 stain result.

Declaration of Competing Interest

None.

Acknowledgments

We would like to thank Zytomed and Ventana for providing us with PD-L1 antibodies.

Formatting of funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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