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Characterization of Two New Recombinant Rabbit anti-PDL1 Antibodies In Bladder Cancer, NSCLC, and Melanoma With Immune Cell Markers CD3, CD8A, CD20, CD68 and FOXP3

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Abstract

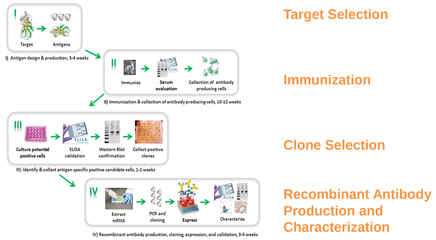
Immunohistochemistry (IHC) is an important diagnostic tool to determine the expression level of PD-L1 in tumor cells. However, the PD-L1 antibodies also stain tumor infiltrating lymphocytes/immune cells. Studies have shown that separating positive immune cell staining from positive tumor cell staining is a better diagnostic indicator for PD-L1/PD-1 immunotherapy. Two FDA approved PD-L1 antibodies clones (SP142 and 28-8) present different staining patterns when evaluated on the same tumor tissue. In this study, we assessed multiple PD-L1 antibodies measuring PD-L1 expression of immune cells and tumor cells. To do this, we evaluated multiple immune cell markers with the PD-L1 antibodies. IHC screens were done with 5 PD-L1 antibodies, 2 recombinant rabbit monoclonal antibodies (clone OR-5E3 and OR-5H8), one mouse anti PD-L1 clone UMAB229, and the FDA approved antibodies clone (SP142 and 28-8). The immune cell markers used in the IHC screen were CD3, CD8A, CD20, CD68, and FOXP3. The screen was done on sequential sections of bladder cancer, melanoma, and NSCLC tumors. On a sequential section, the PD-L1 clone OR-5E3 and OR-5H8 stained the same positive immune cells as clone SP142 but not as strongly; conversely, PD-L1 clones OR-5E3 and OR-5H8 stained tumor cells stronger and picked up low expression of PD-L1 in that tumor. The mouse monoclonal anti-PDL1 clone UMAB229 stained high and low PD-L1 expression similar to clones OR-5E3 and OR-5H8 in tumor cells, and stained immune cells similar to SP-142. The five PD-L1 antibodies may be from different antigens which may contribute to their sensitivity and specificity to detect PD-L1 in tumor and immune cells. PD-L1 antibody clones SP142 and 28-8 were the first generation of antibodies at the time PD-L1/PD1 immune therapy started; this study suggests a new generation of PD-L1 antibodies may be a better diagnostic tool to screen PD-L1 expression in tumor cells by IHC.

Introduction

Targeted PD-L1 and PD-1 therapy successes to fight the progression of melanoma has expanded to include treatment of non-small-cell lung cancer, bladder cancer, head and neck cancer, renal cell cancer, with clinical trials of other solid tumor ongoing. Clinical studies have shown that positive PD-L1 protein expression in these tumors are associated with higher response rates from targeted PD-L1 /PD-1 immunotherapy. However tumor responses are not mediated by the antibody per se, but by tumor PD-L1 antigen interaction with specific T cells that had been previously blocked by the PD-1–PD-L1 interaction (1). Here we look at the we looked at the expression profile of immune cell markers CD3, CD8A, CD20, CD68 and FOXP3 with the new OriGene new rabbit mono PD-L1 antibodies clone OR-5E3 and OR-5H8, mouse monoclonal clone UMAB229 and the FDA approved rabbit mono clonal anti-PDL1 clone SP142 and clone 28-8 for targeted immunotherapy drugs atezolizumab and OPDIVO® (nivolumab) respectively. NSCLC, Bladder Cancer, and Melanoma immune cells, as indicated by the CD3, CD8A, CD20, CD68 and FOXP3 staining, generated different distribution patterns in the three tumor types. The five PD-L1 antibodies showed variation in detection of both immune and tumor cells.

Design & Methods

Figure 1: Rabbit Mono Antibody Development:



Target Selection

Immunization

Clone Selection

Recombinant Antibody Production and Characterization

Fig.1 Rabbit recombinant monoclonal antibody platform was developed using B cells from peripheral blood. Briefly, B cells were isolated from the whole blood of rabbits immunized with PD-L1 peptides. Immune response positive cells were selected after they were cultured for 7-10 days. Rabbit IgG variable light and heavy chain were PCR amplified and cloned into vectors. Positive clones were sequenced. Both light and heavy chain were co- transfected 293 cells for antibody expression. More than 10 positive PD-L1 clones, which were first screened by immunocytochemistry and then immunohistochemistry. The study is on the two best two clones (OR-5E3 and OR-5H3) for immunohistochemistry however we have multiple other clones for ELISA and Westerns that are available.

Immunohistochemistry:

Manual IHC staining of paraffin-embedded human and mouse tissues using anti PD-L1 rabbit mono antibodies clone SP142 [Spring Biosciences -Pleasanton, CA] , clone 28-8 [Abcam - Cambridge, MA], clone OR-5E3 and OR-5H8, [C/N TA591003 &TA591004 OriGene Technologies-Rockville MD], and other OriGene antibodies are listed on the table here.

Table 1 Antibody Information and Dilution

Target	Clone	OriGene Cat #	Dilution
CD3e	UMAB54	UM500048	1:200dil
CD8A	UMAB241	UM800133	1:200dil
CD20	UMAB37	UM800001	1:200dil
CD68	UMAB150	UM800047	1:200dil
FOXP3	UMAB248	UM800140	1:200dil
PD-L1	UMAB229	UM800121	1:200dil
PD-L1	OR-5H8	TA591003	1:100dil
PD-L1	OR-5E3	TA591004	1:100dil

All PD-L1 antibodies required heat induced epitope retrieval HIER using OriGene-ACCEL Tris-EDTA buffer pH8.7 for clone OR-5H8 or OR-5E3; OriGene TEE pH9.0 for clone SP142; BioCare DIVA DeCloaker for clone 28-8 at 120C for 3 minutes in BioCare Decloaker chamber. PD-L1 clones SP142 and 28-8 were diluted 1:50; PD-L1 clone OR-5H8 & OR-5E3 were diluted 1:100 and incubated for 1hr at room temperature. OriGene Polink-1 a one step anti- rabbit polymer HRP detection (Cat# D13-100) was used except for clone 28-8 which used 2 step Rb Polymer Polink2Plus (D39) and DAB chromogen according to manufactures protocol.

Immunohistochemistry Scoring:

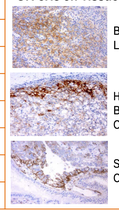
For this study PD-L1 intensity of stain was not incorporated into the overall score 20% 1+ and a 20% 3+ was considered 20% positive tumor. Tissues were score twice. First pass they were given an overall score of positive tumor cell and positive immune cells. Intensity of the stain was not evaluated. Second the same cases were compared to each other to see if the scores were correct and adjusted against each clone for that tissue. This was to insure that similar staining patterns received a similar score.

Results

Table 2: New PD-L1 clones OR-5H8 and OR-5E3 Staining on Normal and Cancer Tissue Array

Normal Tissue	Clone OR-5H8	Clone OR-5E3	Cancer Tissue	Clone OR-5E3	Clone OR-5H8
Adrenal	0 of 1	0 of 1	B-cell lymphoma	3 of 3	2 of 3
Bone Marrow	0 of 1	1 of 1	Breast Cancer (Her2-)	1 of 3	0 of 3
Breast	0 of 2	0 of 2	Breast Cancer (Her2+)	1 of 3	1 of 3
Cerebellum	0 of 3	0 of 3	Carcinoid	0 of 3	0 of 3
Cerebrum	0 of 3	0 of 3	Colon Cancer	0 of 3	0 of 3
Cervix	0 of 4	0 of 4	Uterine Carcinoma	0 of 3	2 of 3
Colon	0 of 5	0 of 5	Glioma	0 of 2	0 of 2
Esophogus	0 of 3	0 of 3	Liver Cancer	2 of 3	2 of 3
Heart	0 of 3	0 of 3	Lung cancer	3 of 3	3 of 3
Kidney	0 of 2	0 of 2	Melanoma	4 of 4	4 of 4
Liver	0 of 3	0 of 3	Ovarian Cancer	1 of 3	1 of 3
Lung	0 of 3	0 of 3	Pancreatic Cancer	1 of 3	1 of 3
Mesothelium/ Omentum	0 of 3	0 of 3	Prostate Cancer (>1%)	2 of 3	2 of 3
Ovary	0 of 3	0 of 3	Renal Cell Carcinoma	0 of 3	0 of 3
Pancreas (?)	1 of 3	1 of 3	Stomach Cancer	3 of 3	2 of 3
Pituitary	0 of 2	0 of 2			
Placenta	3 of 3	3 of 3			
Prostate	0 of 3	0 of 2			
Spleen (1+)	3 of 3	3 of 3			
Testies	0 of 3	0 of 3			
Thymus	2 of 3	2 of 3			
Thyroid	0 of 3	0 of 3			
Tonsil	2 of 2	2 of 2			
Uterus	0 of 3	1 of 4			

Figure 2: Positive PD-L1 OR-5H8 on Tissue Array



Bcell Lymphoma

HER2+ Breast Cancer

Stomach Cancer

Figure 3: PD-L1 Mouse Tissue

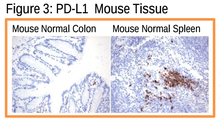


Fig-3 PD-L1 clone OR-5E3 on mouse colon and spleen were screen using the same protocol as human tissue. The images show positive staining on the mouse immune cells.

Figure 4: IHC Screen of Multiple PD-L1 Rabbit Mono Antibodies on NSCLC, Bladder Cancer, & Melanoma

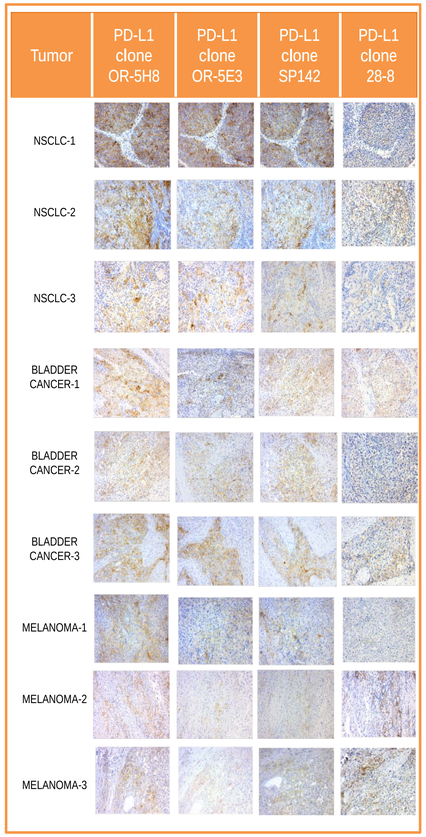


Table 3: PD-L1 Rb Mono Antibodies IHC Results Summarize of Fig-4

PD-L1 Clone Results	TC <1%	TC 1-50%	TC >50%
OR-5H8 NSCLC	4/13	4/13	5/13
OR-5E3 NSCLC	4/13	6/13	3/13
UMAB229 NSCLC	4/13	4/13	5/13
SP142 NSCLC	4/13	7/13	2/13
28-8 NSCLC	5/12	5/12	2/12
OR-5H8 Bladder Cancer	6/13	4/13	3/13
OR-5E3 Bladder Cancer	7/13	3/13	3/13
UMAB229 Bladder Cancer	7/14	4/14	3/14
SP142 Bladder Cancer	8/13	3/13	2/13
28-8 Bladder Cancer	9/14	2/14	3/14
OR-5H8 Melanoma	0/11	10/11	1/10
OR-5E3 Melanoma	3/9	5/9	1/9
UMAB229 Melanoma	2/11	8/11	1/11
SP142 Melanoma	2/11	9/11	0/11
28-8 Melanoma	4/10	6/10	0/10

Fig-4 Show examples of tumors stained with new and FDA approved rabbit PD-L1 clones on NSCLC (lung), bladder cancer, and melanoma. Results show new rabbit clones stain stronger. Comparison was done using manual staining. PD-L1 clone 28-8 required 2-step polymer amplification one step resulted in weak or no staining.

Fig-5 Shows various staining pattern of immune cell markers with PD-L1 expression in NSCLC (lung), bladder cancer, and melanoma.

Fig- 6 Summarizes the results of the immune cell expression levels in the tumors. Note CD20 tended to be on the periphery or large foci adjacent to the tumor where CD3E tended to be more diffuse throughout tumor. FOXP3 was rare 1 to3 per 20x field.

Figure 5: PD-L1, CD3E, CD8A, CD20, CD68, FOXP3 Antibodies IHC Stain on NSCLC, Bladder Cancer, & Melanoma

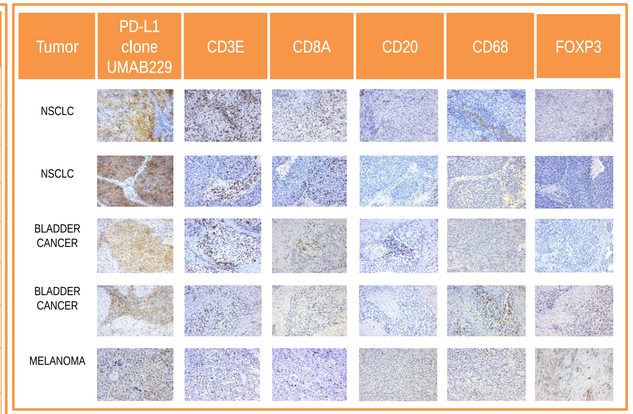
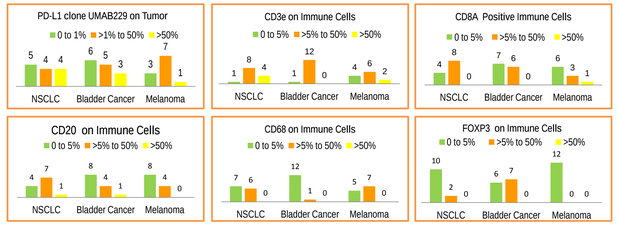


Figure 6: PD-L1, CD3E, CD8A, CD20, CD68, FOXP3 Antibodies IHC Summary on NSCLC, Bladder Cancer, & Melanoma (Score on based on total immune cells not total tissue.)



Conclusion

1. Clones OR-5H8 and OR-5E3 perform as well or better than the FDA approved clones. In bladder cancer case 3 you see that OR-5H8 stains 90% of the tumor but both the FDA approved drugs show less than 25% positive tumors. New Rabbit clones OR-5H8 and OR-5E3 were easier to see PD-L1 positive tumor cells in the presence of PD-L1 positive immune cells.
2. Immune cell markers were use to evaluate the presence of immune cells in the tumor contributing to PD-L1. Immune CD3, CD8A, CD20, CD68, and FOXP3 cell markers show different staining pattern between the three cancers evaluate. They also show a different expression levels and distribution pattern throughout the tumor. Tumors that had strong staining at the edge of tumor often seem to have a lot of immune cells present that were PD-L1 positive. Future studies will look at double staining with PD-L1 and the immune cell marker to how much influence PD-L1 contributed to the distribution pattern.
3. PD-L1 clones OR-5H8 and OR-5E3 work very well on mouse tissue for screening PD-L1 positive immune cells.