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Expression of programmed death ligand 1 and 2 in adrenocortical cancer tissues: An exploratory study[☆]

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ABSTRACT

Background: Inhibition of the interaction of programmed death 1 with programmed death ligand 1 and 2 has been used successfully for treatment of multiple advanced cancers, but expression has not been studied in adrenocortical carcinoma. In this study, we investigated programmed death ligand 1 and 2 expression in adrenocortical carcinoma to determine the potential usefulness of checkpoint inhibitors in these malignant neoplasms.
Methods: A total of 56 tissue samples from patients with adrenocortical carcinoma (34) and benign adrenal tissues (22) were identified. Immunohistochemistry was performed for programmed death ligand 1, programmed death ligand 2, and CD8 and scored for membranous staining on adrenal and stromal tissue according to the immunoreactive score and absolute percentage, respectively. Descriptive statistics, a Mann-Whitney U test, and Fisher exact tests were calculated.
Results: In total, 15 adrenocortical carcinoma (44%) stained positive for programmed death ligand 2 and 1 adrenocortical carcinoma for programmed death ligand 1 ($P = .03$). Adrenocortical carcinoma samples were more likely to express programmed death ligand 2 on tumor cells or in stromal tissues than benign samples (OR = 2.3, $P = .03$). There was no relationship between programmed death ligand 2 and CD8 expression ($P = .08$). There were also no relationships between programmed death ligand 2 or CD8 expression and tumor characteristics.
Conclusion: Programmed death ligand 2, but not programmed death ligand 1, is expressed commonly in adrenocortical carcinoma samples. The utility of certain checkpoint inhibitors should, therefore, be evaluated in further studies.

Introduction

Adrenocortical carcinoma (ACC) is a rare but lethal malignancy with an estimated worldwide incidence of 2 per million.[1] It is the second most aggressive endocrine malignancy behind anaplastic thyroid cancer, and its overall median survival of 32 months has not improved in the past 3 decades.[1] Complete resection is considered the most important component of treatment, but patients frequently recur locally or distally, even after a curative resection. Systemic therapy with mitotane, etoposide, doxorubicin, and cisplatin has a low objective response rate and does not improve overall survival, even in an adjuvant setting.[2] Phase II investigations of newer therapies, such as sunitinib and vascular endothelial growth factor (VEGF)-inhibitors, also have not shown encouraging response rates.[3,4] Therefore, novel, effective therapies are needed for this disease.

Programmed death 1 (PD-1) is a tyrosine-kinase receptor protein expressed by B- and T-lymphocytes that is involved in regulation of the immune response.[5,6] Specifically, PD-1 inhibits T-cell proliferation when activated by either of its ligands, programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2).[5] Blockade of the interaction between PD-1 and PD-L1 has been well

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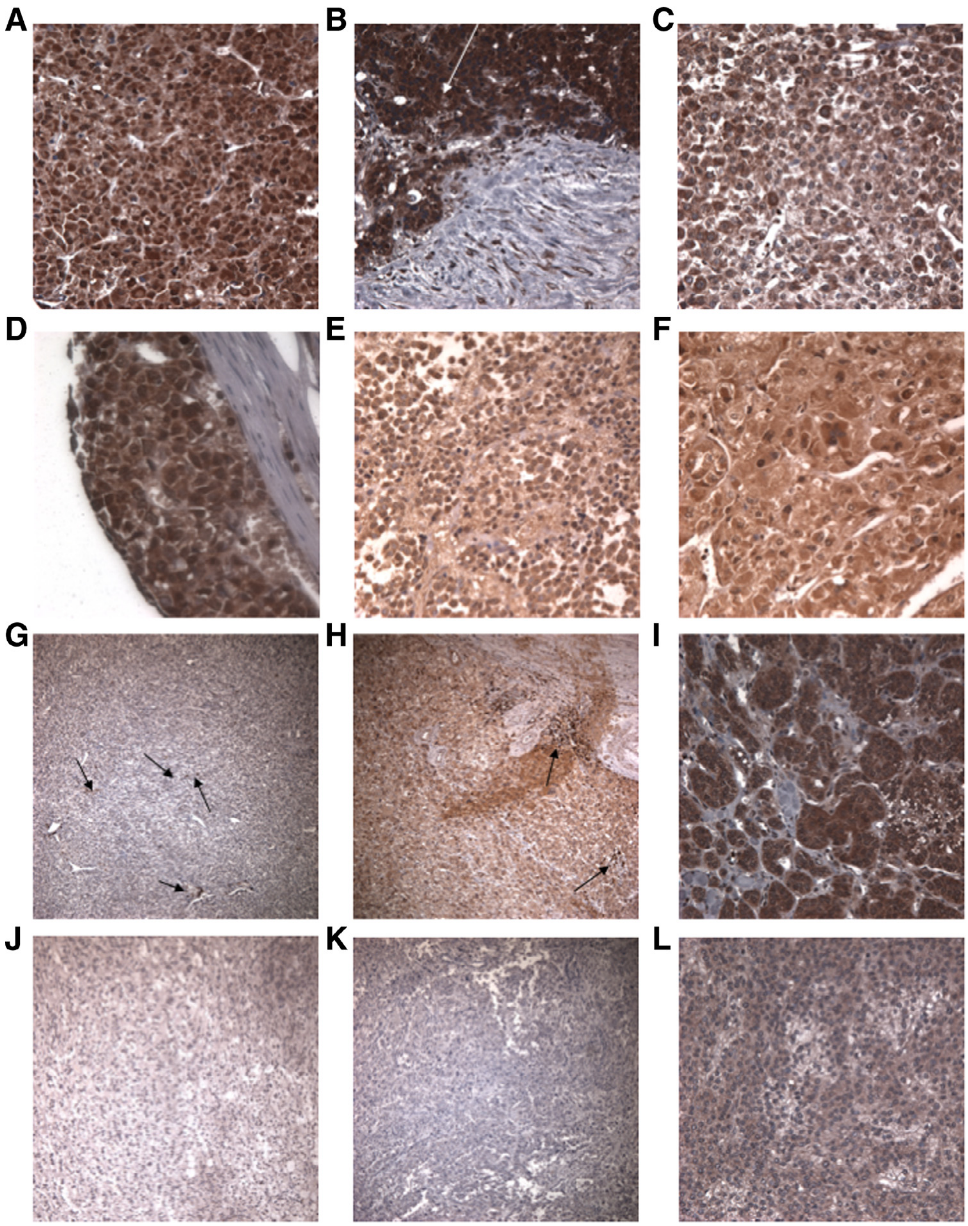

Figure. PD-L2 expression in adrenal tissue. (*A*) High PD-L2 expression in an ACC sample mounted on a TMA, with 95% of cells staining positive at 3+ intensity (IRS = 12). (*B*) High PD-L2 expression in ACC tissue on a TMA (*arrow*), with a lesser expression in stroma (IRS = 12); (*C*) PD-L2 expressed in ACC tissue on a TMA (IRS = 6), with 60% of cell staining positive at 2+ intensity; (*D*) High-power image of positive membranous and cytoplasmic PD-L2 staining in ACC on TMA (IRS = 8). (*E*) Low-power image of positive PD-L2 staining of ACC tissue on an individual glass slide, with 60% of cells staining at 2+ intensity; (*F*) High-power image of positive PD-L2 staining of ACC tissue on an individual glass slide, with 60% of cells staining at 2+ intensity. (*G* and *H*) Diffuse CD8+ lymphocyte infiltration in ACC tissue (*arrows*). (*I*) PD-L2 positive adrenal adenoma on TMA (IRS = 12). (*J*) PD-L2 negative adrenal adenoma on an individual slide (IRS = 0). (*K*) PD-L2 negative ACC sample on an individual slide (IRS = 0). (*L*) PD-L2 negative ACC sample on TMA (IRS = 0). PD-L2, programmed death ligand 2; ACC, adrenocortical carcinoma; IRS, immunoreactive score; TMA, tissue microarray.

described in many cancers, including non–small cell lung cancer, esophageal cancer, triple-negative breast cancer, and melanoma.[7,8] The only previous study to examine the role of PD-L1 in ACC, however, found that its expression on tumor cells was limited to a low percentage of samples without correlation with clinical outcomes or pathologic features.[9]

Despite the more intense focus on PD-L1 in tumor immunotherapy, there is some evidence that PD-L2 is associated with decreased survival and decreased presence of CD8+ tumor-infiltrating lymphocytes in patients with esophageal cancer and could be a viable treatment target for many cancers because it has a much greater affinity for the PD-1 receptor than PD-L1.[6,10]

We, therefore, sought to examine the expression levels of PD-L1 and PD-L2 in human ACC samples to determine whether checkpoint inhibitors should be considered as a therapeutic option in these patients. Secondary objectives included correlating PD-L1 and PD-L2 expression with presence of tumor-infiltrating CD8+ lymphocytes and with pathologic and clinical features.

Methods

A total of 56 tissue samples from patients with ACC, indeterminate adrenocortical tumors (ACT), adrenal adenomas (AA), and normal adrenal tissue (NA) were obtained from separate institutional biorepositories at Rush University Medical Center (RUMC) and NewYork-Presbyterian/Weill Cornell Medical Center (NYP-WCMC). The RUMC Institutional Review Board approved this study, the research offices of each institution approved material transfer, and all

Table 1
Definition of the IRS[11]

Percentage of positive cells (A)	Intensity of staining (B)	IRS (A x B)
0 = no positive cells 1 = <10% positive cells 2 = 10–50% positive cells 3 = 51–80% positive cells 4 = 81–100% positive cells	0 = no reaction 1 = mild reaction 2 = moderate reaction 3 = intense reaction	0–1 = negative 2–5 = weak expression 6–12 = strong expression

data obtained from NYP-WCMC were de-identified before transfer. Consent and Health Insurance Portability and Accountability Act authorization were waived owing to the retrospective nature of the study.

Clinical and pathologic data, including patient sex, age at diagnosis, presence of metastases at diagnosis, whether the tumor secreted hormones, and survival were collected from the medical records when available. Samples were classified according to their diagnosis on final clinical pathology performed at the time of tumor removal. Samples that did not meet Weiss criteria for ACC but had more than 5 mitoses per 50 high-power fields were considered diagnosed as ACTs by the clinical pathologist who reviewed the initial pathology. These tumors were treated as benign lesions in this study because the clinical course of these patients was not consistent with ACC.

Immunohistochemical analysis

Five-micron sections were made from formalin-fixed, paraffin-embedded tissue from 34 primary ACCs, 16 AAs, 3 NAs, and 3 ACTs. Samples from RUMC were mounted on individual glass slides, and samples from NYP-WCMC were obtained on a tissue microarray (TMA) because these samples had been cored and mounted previously.

PD-L1, PD-L2, and CD8 staining was performed using commercially available antibodies. Slides were first deparaffinized with xylene and rehydrated. A heat-mediated retrieval was performed using citrate buffer (SignalStain® Citrate Unmasking Solution, Cell Signaling Technology, Danvers, MA). Endogenous peroxidases were blocked using hydrogen peroxide. Slides were incubated for 2 h with animal-free blocking solution (Cell Signaling Technology, Danvers, MA) for protein blocking. Slides were then incubated overnight with rabbit monoclonal anti-PD-L2 (Abcam, ab214221, 1:250, Cambridge, MA), mouse monoclonal anti-PD-L1 (Dako, 22c3 pharmDx, Santa Clara, CA), and rabbit polyclonal anti-CD8 (Abcam, ab4055, 1:200, Cambridge, MA) antibodies. Rabbit SignalStain® Boost Detection reagent secondary (Cell Signaling Technology, Danvers, MA) was then applied. Slides were developed using SignalStain® DAB Substrate Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions and counterstained with hematoxylin (Harris Hematoxylin, BBC Biochemical, Mount Vernon, WA). Finally, they were washed, dehydrated in alcohol and xylene, mounted, and coverslipped.

Immunohistochemical scoring

All samples were reviewed and scored blindly by 2, board-certified pathologists and scored for PD-L1 and PD-L2 expression on tumor cell membranes according to the percent of positive tumor cells and intensity. Intensity was scored as absent (0), low (1), moderate (2), or high (3). Each tumor sample on the TMA was represented by 1 to 3 TMA cores; the percentages and intensities from each core were averaged.

For evaluation of tumor tissue, percentage scores were then converted to the following integer values on a 0–4 scale: a value of 0 was assigned to slides that stained 0% of cells; 1 to <10%; 2 to 10%–50%; 3 to 51%–80%; and 4 to 81%–100%. An immunoreactive score (IRS) was calculated by multiplying the intensity score and the integer value for the percentage score, as described previously (Table 1).[11] Positive staining of adrenal samples in this study was defined as an IRS ≥6 (>50% of positive cells and ≥2+ staining intensity).

Positive staining of stromal tissue was determined by the percent of stromal cells that stained positive, with a cutoff of ≥10%, as described previously.[10] CD8+ tumor infiltrating lymphocyte staining was classified as absent, focal, or diffuse. When differences in IRS, percent of positive stromal cells, or CD8+ lymphocyte infiltration existed between pathologists, the average score was used.

Statistical analysis

Descriptive statistics were calculated for the rate of PD-L1, PD-L2, and CD8+ lymphocyte positivity in tumor and stromal tissues according each histologic diagnosis. The proportion of ACC samples expressing PD-L2 and the proportion expressing PD-L1, and the rates of PD-L2 positivity between ACC and benign samples in adrenal and stromal tissues, were both compared using Fisher exact tests. For ACC samples, relationships between PD-L2 positivity and CD8+ lymphocyte infiltration, metastases at diagnosis, patient sex, and whether the tumor secreted hormones were also compared with Fisher exact tests. The relationship between PD-L2 positivity and patient age was compared using a Mann-Whitney *U* test. All analyses were conducted in R 3.3.2.[12]

Results

Samples from 34 ACC, 3 ACT, 16 AA, and 3 NA were analyzed. A total of 19 samples mounted on individual glass slides were obtained from the RUMC institutional biorepository and 37 tissue cores mounted on a TMA were obtained from NYP-WCMC. Sufficient stromal tissue was present to analyze stromal protein expression in 34 samples.

PD-L1 tumor staining was observed in only 1 ACC sample. No benign samples stained positive for PD-L1. Of note, the ACC sample that expressed PD-L1 did not also express PD-L2.

Strong PD-L2 tumor was found in 15 ACC and stromal staining in 8 ACC samples. Although tumor or stromal PD-L2 expression individually was not greater when comparing malignant to benign lesions ($P = .11$ and $P = .16$), ACC samples were more likely to express PD-L2 than benign samples when all samples with positive tumor and stromal tissue were included (odds ratio [OR] 2.3, $P = .03$; Table 2). ACC samples were also more likely to express PD-L2 than to express PD-L1 ($P < .001$).

ACC samples on the TMA were more likely to be PD-L2 positive in the tumor and stroma than samples on individual glass slides ($P = .013$ and $< .001$).

A total of 65% (22 of 34) of ACC samples were either focally or diffusely positive for CD8+ tumor infiltrating lymphocytes. There was no relationship between PD-L2 expression in tumor or stroma and CD8+ lymphocyte infiltration ($P = .46$, $P = .14$). There was also no difference in the presence of CD8+ tumor infiltrating lympho-

Table 2
PD-L1 and PD-L2 expression in ACC and benign adrenal tissue

	ACC (%)	AA (%)	NA (%)	ACT (%)	All benign	*P* value*
Number of samples	34	16	3	3	22	
RUMC	13 (38)	3 (19)	0 (0)	3	6 (27)	
NYP-WCMC	21 (62)	13 (81)	3 (100)	0	16 (73)	
PD-L1 positive (adrenal tissue)	1	0	0	0	0	1
PD-L2 positive (adrenal tissue)	15 (44)	4 (25)	0	1	5 (23)	.16
PD-L2 positive (stroma)	8 (37)	1	0	0	1	.11
PD-L2 positive (adrenal + stroma)	18 (53)	4 (25)	0	1	5 (23)	**.03**
CD8 absent	12 (35)					
CD8 focal	18 (53)					
CD8 diffuse	4 (12)					

* *P* value refers to comparison between ACC and total benign groups

Table 3
Association of clinical characteristics with PD-L2 expression in tumor and stroma

Clinical characteristic	Tumor			Stroma		
	PD-L2 positive (%)	Patient number	*P* value	PD-L2 positive (%)	Patient number	*P* value
Metastases at diagnosis	5 (42)	20	.64	3 (33)	16	.21
Male sex	5 (50)	33	.7	3 (50)	22	.62
Hormone-secreting	3 (38)	14	.62	3 (43)	11	.99
Age > median (51.5 y)	8 (62)	29	.14	3 (30)	22	.67

cyte or PD-L1 expression between the TMA and individual slide groups ($P = .13$ and $P = .99$).

Of the patients with ACC, 10 of 33 were male (30%), and the median age was 52 years. Of the 14 ACCs, 8 (57%) secreted hormones, most commonly cortisol. In total, 60% of patients (12 of 20) had metastases at diagnosis. There was no relationship between sex, age, metastases at diagnosis, or functional status, and PD-L2 positivity in the tumor or stroma (all *P* values >.1). There was no difference in sex, age, metastases at diagnosis, or functional status between the TMA and individual slide groups (all *P* values >.2–.7; Table 3).

Discussion

In this study, we sought to investigate the expression of PD-L1 and PD-L2 in ACC tumor samples. We demonstrated that although PD-L1 is rarely expressed in ACC, PD-L2 is strongly expressed in nearly half of the ACCs, and that PD-L2 is expressed more frequently in the tumor or stromal tissues of ACCs than in benign samples, suggesting that it may be an interesting target for therapy.

Blockade of the interaction of PD-1 with its ligands, PD-L1 and PD-L2, has been shown to improve survival in a variety of cancers.[5,7,8,13–15] PD-L1 is commonly tested in clinical pathology to determine if patients with non–small cell lung cancer and esophageal adenocarcinoma will respond to treatment with blockade of the PD-1 pathway, but some patients who are PD-L1 negative will also respond to this therapy.[16] It has been hypothesized that PD-L2 expression could promote response to PD-1 inhibition in PD-L1 negative patients.[13] Although PD-L1 is tested and expressed more commonly in tumor tissue of all cancer types, PD-L2 has greater affinity for PD-1.[6] It was initially posited that PD-L1 is constitutively expressed on all tissues, whereas PD-L2 is only expressed by antigen-presenting cells, but this is now known to be untrue.[6,10] PD-L2 expression has been shown to be upregulated in the presence of certain stimuli in the microenvironment, including Th2 cytokines such as IL-4, IFN-gamma, and toll-like receptor ligands, and has been demonstrated in cancer fibroblasts from multiple tumor types, although these mechanisms have not been studied in ACC.[6] PD-L2 expression in tumor tissue, therefore, is an attractive area for further investigation.

When a PD-1 receptor on the surface of a lymphocyte is activated by its ligand, downstream effects eventually lead to impaired cytokine production and cytolysis, and eventually, to apoptosis of the lymphocyte.[6] Physiologically, PD-1 activation dampens the immune response to protect against autoimmune and ineffective immune responses, but its mechanism also leads to immune dysfunction in chronic viral infections and cancer.[5] Tumor cell expression of a PD-1 ligand such as PD-L2 allows tumor cells to target host T-cells, and thereby evade the immune response.[9] T-lymphocytes themselves upregulate PD-L1 expression on tumor cells by secreting IFN-gamma.[14] Although this pathway has been better described for PD-L1, PD-L2 expression is also IFN-gamma-dependent.

A subset of drugs targeting the PD-1 pathway, including atezolizumab, bind to PD-L1 and, therefore only block the interaction of PD-1 with PD-L1. Other drugs, however, including nivolumab and pembrolizumab, bind PD-1 directly and block its interaction with both PD-L1 and PD-L2.[13] The use of this second class of drugs is more likely to be beneficial in ACC because PD-L1 expression appears to be rare in this cancer.

Only 1 previous study by Fay et al reported PD-L1 expression in ACC by examining 28 samples. The authors found that PD-L1 was expressed in only 10% of ACCs but by tumor infiltrating mononuclear cells in 68% of cases.[9] The rate of PD-L1 expression on tumor tissue reported in our study (3%) was less than the rate reported previously, and no PD-L1 expression was seen in stromal tissue. Although the study by Fay et al determined that a sample was positive for PD-L1 if 10% of tumor cells were positive, the ACC samples in our study would still have been considered negative even at this lesser threshold. After reviewing both studies, we can conclude that PD-L1 expression in ACC is uncommon. The previously reported high expression of PD-L1 on tumor-infiltrating mononuclear cells combined with the expression of PD-L2 on tumor tissue and in stroma reported in this study suggests that the PD-1 pathway is still perhaps a viable target for immunotherapy for a select number of patients with ACCs.

This study has several limitations. First, the number of ACC samples is relatively small, which limits the power of any comparisons with clinical and pathologic features. These tumors are rare and thus acquiring a large *n* value is difficult. Second, immunohistochemistry has an inherent observational bias because it uses subjective scoring from a pathologist. We tried to correct for this by having 2 pathologists assess these samples independently and blindly. Third, we were unable to analyze the effect of PD-L2 expression on patient survival because long-term follow-up and treatment information were not available and the number of patients was small. The available clinical information, such as the presence of metastases at diagnosis, does not necessarily correspond with tumor aggressiveness and was not available for all patients. Inhibition of thePD-1 pathway has been an effective therapy even in cancers for which there is no apparent relationship between increased PD-L1 expression and prognosis.[17,18] Fourth, some samples were assessed using a TMA whereas others were mounted on individual glass slides. We initially hypothesized that the use of a TMA might lead to underestimation of PD-L1 and PD-L2 expression if these proteins were not expressed in the area of the tumor used for the TMA core, but we found that TMA samples were actually more likely to express PD-L2 than the samples on individual slides. The majority of samples on individual slides that did not express PD-L2 had no staining but expression was relatively uniform in samples that did express the marker, so it is likely that the greater expression on the TMA accurately represents the tumor at large.

Despite these limitations, we have shown that PD-L2 is expressed in nearly half of ACCs and could represent a viable target for further investigation. Future research should focus on testing of PD-1 inhibitors such as pembrolizumab in preclinical cell line and animal models as well as correlation of PD-L2 expression with patient survival.

Conflicts of interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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Discussion

Dr Brian Untch (New York, NY): I think this has a lot of interest for us because we are in the midst of finishing a phase II study with pembrolizumab for advanced patients with ACC.

We have been picking patients mostly based on microsatellite instability and neoantigenic burden, and I am wondering if we should be using PD-L2 IHC instead. I would like your comments on that.

Secondly, I imagine some of these patients have undergone some genomic sequencing because it looked like it was a large group. So I am wondering if you have done any of that, and if you have been able to correlate genomic lesions with PD-L2 expression.

Dr John F. Tierney: I think working with PD-L2 IHC could potentially be usable to help select patients for that study. And I am curious if you have been looking at PD-L1 IHC for those patients, and if you found that PD-L1 was expressed on their tumors.

Then to your second question, many of these patients dated back a couple of decades, so I don't know how many of them underwent genomic sequencing.

Dr John Phay (Columbus, OH): For specific checkpoint inhibitors, there's certain levels of what is considered positivity for the tumor cells. And for some, it's as low as 25%. Did you see many more positive cells, if you lowered your criteria?

Dr John F. Tierney: We would not have seen that many more positive cells–especially for PD-L1. We would have seen a few more for PD-L2. But if we had lowered the threshold for PD-L1, we would not have seen any more positive cells.

Dr Janet Li (New York, NY): I am interested to know if you had any patients that only stained positive in the stroma and not in the tumor and if you anticipate that would affect tumor infiltration of the T-cells.

Dr John F. Tierney: We did have, I believe, only 3 patients who stained positive in the stroma and not in the tumor.

If the stroma is expressing the ligand, then that could still serve to bind PD-1 and therefore inhibit the T-cell efficacy, so that's been seen in other cancers that stroma expression of PD-L1 can inhibit T-cell.

Dr Mark Cohen (Ann Arbor, MI): Did you happen to correlate PD-L2 expression levels with mitotic rate? It might be interesting to look at that.

Dr John F. Tierney: We did not.

Dr Edwin Kaplan (Chicago, IL): Very nice talk. Could you tell us the ones who are not that familiar with this, a drug like KEYTRUDA is a PD-L1 inhibitor I believe. What are the PD-L2 inhibitors?

Dr John F. Tierney: Actually, KEYTRUDA is a PD-1 inhibitor. PD-1 is the receptor that's on the T-cells. And then PD-L1 and PD-L2 bind PD-1.

But KEYTRUDA is a PD-1 inhibitor, and that will block both PD-L1 and PD-L2 from binding PD-1, so the PD-L1 inhibitors will only block PD-L1, but PD-1 inhibitors will block both.

There aren't any specific PD-L2 inhibitors, but the PD-1 inhibitors could potentially work for PD-L2 positive tumors.

Dr Oliver Gimm (Linkoping, Sweden): Very nice presentation. Thank you. As we know, these tumor cells are very heterogenous. I was wondering whether you were able to see a correlation between those patients that were doing well and those that were doing less well.

Dr John F. Tierney: We didn't have very long-term follow-up data for most of our patients, so we were not able to correlate that, unfortunately.