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Tumoral and Stromal Pdl1 and Pdl2 Checkpoints Immunohistochemical Expression in Pancreatic Ductal Adenocarcinoma, a Promising Field Of Study

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Abstract

BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) is world-widely considered as one of the most malignant tumors. Programmed cell death protein 1 (PD-1) through its ligands PDL1 and PDL2 plays a critical role in cancer immunoediting. The ligands are expressed in many solid tumors and there is an emerging hope of using anti-PDL in cancer immunotherapy.

MATERIALS AND METHODS: This study included that 40 patients with PDAC who underwent pancreaticoduodenectomy. PDL1 and PDL2 pancreatic expression were evaluated in these patients using immunohistochemical staining and correlated their expression levels with each patient's reported clinicopathological features.

RESULTS: There were a significant correlations between high tumoral PDL1 expression and the PDAC tumor histologic grade ($p = 0.021$) and the tumor status (T) ($p = 0.022$), while the stromal expression of PDL1 showed non-significant correlation with any of the studied features. There were a significant correlations between high tumoral PDL2 expression and tumor stage ($p = 0.012$), while the stromal expression of PDL2 showed a significant correlation with tumor status, lymph node status, tumor stage, and the presence lymphovascular invasion with P value equal 0.001, 0.009, 0.009, and 0.045, respectively.

CONCLUSION: This study showed that in PDAC patients, high tumoral PDL1 and PDL2 expression was associated with some important prognostic factors, while only stromal PDL2 expression was significantly associated with most of the studied prognostic features emphasizing a role of both markers in the prognosis of this neoplasm.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal cancer worldwide with an overall 5-year relative survival rate of 8% [1]. This was related to lack of efficient therapeutic modalities as it is unresponsive or mildly responsive to chemotherapy, radiotherapy, and immunotherapy [2]. There is still a lack of knowledge about molecular mechanisms underlying such unresponsiveness of pancreatic cancer [3].

Many factors contribute to the decreased responsivity of PDAC to therapy including the densely desmoplastic stroma which represents about 80% of the tumor mass [4] that is highly infiltrated by immunosuppressive cells that contribute to the downregulation of antitumor immune response and poor tumor immunogenicity [5].

In addition, PDAC is characterized by low mutational load compared with other types of cancer [6]. High mutational load cancers can be easily recognized by host immune cells when compared to cancers with low mutational load [7] which acts as an additional factor for poor response to therapy.

Many emerging evidences have shown that the coinhibitory receptors, such as programmed cell death protein 1 (PD-1), play a critical role in cancer immunoediting [8]. Anti-PD-1/PDL1 immunotherapy was found to strengthen antitumor immunity and has exhibited inspiring efficacy in various cancer types, resulting in FDA approval and wide clinical application [9], [10], [11].

PD-1, a member of the CD28 family, is an immune-checkpoint receptor expressed on a variety of immune cells, such as T-cells, monocytes, B-cells, dendritic cells, and tumor-infiltrating lymphocytes [12]. Its major role is to limit the activity of T-cells in peripheral tissues at the time of an inflammatory response to constrain autoimmunity and tissue damage [12], [13].

PD-1 possess two ligands, namely, PDL1 and PDL2 [14]. It binds to these ligands on solid tumors [15], on tumor-infiltrating dendritic cells [16], and on tumor associated-macrophages [17], to prevent chronic activation of T-cells [18]. Hence, PD-1 attenuates tumor immunity and infectious immunity and facilitates tumor progression [19], [20].

The expression of PDL1 and PDL2 has been found in many solid tumors and hematologic malignancies [21], [22], [23]. Their expression on tumor cells was strongly correlated to an unfavorable prognosis, which had been manifested in a variety of cancers containing pancreatic, bladder, gastric, renal and ovarian cancers, and melanoma [24], [25], [26], [27], [28], [29].

Nowadays, different clinical trials using immune checkpoint inhibitors alone or in combination with other therapeutic agents in the treatment of PDAC patients are under clinical evaluation. Results of early clinical trial of anti-PD1/anti-PD1 axis blockade as single-agent therapy (anti- PDL1 monoclonal antibody) in pancreatic cancer patients showed no apparent therapeutic effect [30]. However, some advances have already been achieved in combination with anti-PD-1 treatment for PDAC including immunotherapy, chemotherapy or radiotherapy [31].

Recently, a rising number of studies have investigated the prognostic implication of expression of both PDL1 and PDL2 proteins in various types of solid tumors, while the results remain controversial. Their expression in PDAC has lately paid great attention with emerging few numbers of studies with no single published Egyptian study yet.

Material and Methods

Study group and histologic examination

Samples were collected retrospectively from paraffin blocks of 40 PDAC cases. All were obtained from radical pancreaticoduodenectomy specimens, from the Pathology Department, Faculty of Medicine, in time period from January 2017 to December 2020. The study attained approval by the Ethical Committee for the release of the archival medical records and the utilization of the patient samples for scientific research. Patients' data such as age, sex, and tumor histopathological findings including tumor histological type, extent, grade, lymphovascular and perineural invasion, and nodal status were recorded. The histologic features were re-assessed according to the fifth edition of the WHO Classification of Tumors [32] and the tumor was staged according to the American Joint Committee on Cancer (AJCC) eighth edition [33]. The hematoxylin and eosin (H&E)-stained slides of the collected cases were also reviewed to select the best block to perform immunohistochemical staining on.

Immunohistochemical examination

From each chosen paraffin block, two unstained slides were sliced at 4-micron thickness for further immunohistochemical staining for PDL1 and PDL2. Sections were deparaffinized by xylene and rehydrated by graded alcohol.

Inhibition of endogenous peroxidase activity was done by 3% hydrogen peroxide in methanol for 40 min at room temperature. Primary antibodies (PDL1 and PDL2) were diluted. Reactions with primary antibodies rabbit monoclonal anti-PDL1 antibody (clone 22C3, 1:50, Agilent/Dako, Santa Clara, USA) and mouse monoclonal anti-PDL2 (MAB1224–100, 1:1000, R&D, USA) were undergone for 2 h at room temperature, then reaction with a secondary antibody EnVision HRP-Labeled Polymer (Dako, Carpinteria, CA) followed. Visualization of the reactions was performed by 3,3'-diaminobenzidine chromogen (Dako), and slides were counterstained with hematoxylin (Muto Pure Chemical Ltd., Tokyo, Japan). Tonsillar tissue was used as a positive internal control [34]. Each slide was independently evaluated by the three investigators without knowledge of any clinical data.

PDL1 staining was defined as complete or partial circumferential linear cellular membrane staining at any intensity that could be differentiated from the background as well as diffuse cytoplasmic staining [34].

The ratio of PDL1 and PDL2 expression was calculated by counting the positive tumor cells and tumor proportion score (TPS) was applied (TPS; Null < 1%, low expression; 1 ≤ TPS ≤ 49%, and high expression; ≥50%) [35]. Null and low expression cases were combined for statistical reasons.

The peritumoral stroma was defined as the stroma directly adjacent to tumor cell areas and the evaluation of stromal PDL1 and PDL2 expression was classified as negative/positive [36].

Statistical analysis

Microsoft Excel 2013 was used for data entry and the Statistical Package for the Social Science (SPSS) version 21 (SPSS, Armonk, New York: International Business Machines Corporation) was used for data analysis. Simple descriptive statistics (arithmetic mean and standard deviation) were used for summary of quantitative data and frequencies used for qualitative data. Bivariate relationship was displayed in cross tabulations and comparison of proportions was performed using the Chi-square test or Fisher exact whenever appropriate. The level of significance was set at probability $p < 0.05$.

Results

Clinicopathologic features of studied cases

Thirty patients (75%) of total number of the studied PDAC cases (total = 40) were males and only 10 cases (25%) were females. The tumor expression of PDL1 was high in 21 (52.5%) of patients and null/low in 19 (47.5%) cases, while its stromal expression was positive in 26 (65%) and negative in 14 (35%) (Figure 1). The tumor expression of PDL2 was high in 17 (42%) and null/low in 23 (58%) cases, while its stromal expression was positive in 13 (32.5%) and negative in 27 (67.5%) (Figure 2). The rest of the features are listed in Table 1.

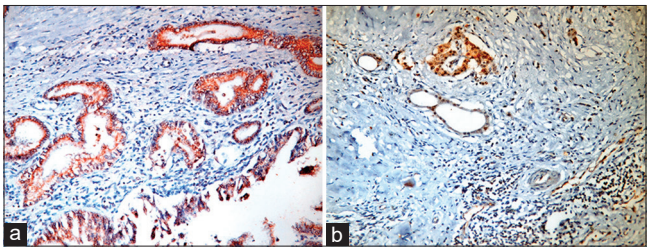

Figure 1: Immunohistochemical staining for PDL1 in pancreatic ductal adenocarcinoma patients (×400): Positive staining (high expression) in tumor cells (a), positive staining (low expression) in tumor cells, and positive staining of stromal cells (b)

Association of PDL1 and PDL2 expression and clinicopathologic features

The results showed that there were a significant correlations between high tumoral PDL1 expression and the histologic grade (p = 0.021) and the T status (p = 0.022), while the stromal expression of PDL1 showed non-significant correlation with any of the studied features (Table 2).

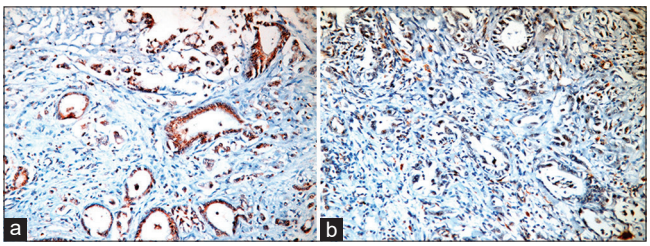

Figure 2: Immunohistochemical staining for PDL2 in pancreatic ductal adenocarcinoma patients (×400): Positive staining (high expression) in tumor cells and positive staining of stromal cells (a), negative staining in tumor cells and positive staining of stromal cells (b)

There were a significant correlations between high tumoral PDL2 expression and cancer stage only (p = 0.012), while the stromal expression of PDL2 showed significant correlation with T status, lymph node status, staging according to the AJCC, and the presence of lymphovascular invasion with P value equal 0.001, 0.009, 0.009, and 0.045, respectively (Table 3). There was no significant correlation between tumoral PDL1 and PDL2 expression (p = 0.385).

Table 1: Clinicopathologic features of studied PDAC cases (n = 40)

Clinicopathologic feature	Number	Percentage
Age		
<60 years	22	55
≥60 years	18	45
Histologic grade		
II	31	77.5
III	9	22.5
T status*		
T1& T2	29	72.5
T3	11	27.5
Lymph node status		
Positive	16	40
Negative	24	60
AJCC stage		
I	18	45
II and III	22	55
Perineural invasion		
Positive	35	87.5
Negative	5	12.5
Lymphovascular invasion		
Positive	13	32.5
Negative	27	67.5
Tumor PDL1		
High expression	21	52.5
Null/low expression	19	47.5
Stromal PDL1 expression		
Positive	26	65
Negative	14	35
Tumor PDL2		
High expression	17	42
Null/low expression	23	58
Stromal PDL2 expression		
Positive	13	32.5
Negative	27	67.5

*T status was defined according to the American Joint Committee on Cancer (AJCC) 8th ed..ition [33].
PDAC: Pancreatic ductal adenocarcinoma.

Discussion

PDAC remains a challenge for oncologist and immunotherapy as T-cell exhaustion remains one of the main resistance factors against PDAC immunotherapy. Since many factors can lead to T-cell dysfunction, it is not easy to find a single responsible factor [3].

Table 2: Correlations between the clinicopathologic features and PDL1 immunostaining in studied PDAC cases (n = 40)

Clinicopathologic feature	Tumoral PDL1 expression			Stromal PDL1 expression		
	High N (%)	Null/Low N (%)	p value	Positive N (%)	Negative N (%)	p value
Age						
<60 years	12 (57.1)	10 (52.6)	0.775	13 (50)	9 (64.3)	0.386
≥60 years	9 (42.9)	9 (47.4)		13 (50)	5 (35.7)	
Histologic grade						
II	13 (61.9)	18 (94.7)	0.021*	18 (69.2)	13 (92.9)	0.088
III	8 (38.1)	1 (5.3)		8 (30.8)	1 (7.1)	
Tumor status						
T1& T2	12 (57.1)	17 (89.5)	0.022*	17 (65.4)	12 (85.7)	0.17
T3	9 (42.9)	2 (10.5)		9 (34.6)	2 (14.3)	
Lymph node status						
Positive	8 (38.1)	8 (42.1)	0.796	9 (34.6)	7 (50)	0.343
Negative	13 (61.9)	11 (57.9)		17 (65.4)	7 (50)	
AJCC stage						
I	9 (42.9)	9 (47.4)	0.775	11 (42.3)	7 (50)	0.641
II and III	12 (57.1)	10 (52.6)		15 (57.7)	7 (50)	
Perineural invasion						
Positive	20 (95.2)	15 (78.9)	0.172	24 (92.3)	11 (78.6)	0.322
Negative	1 (4.8)	4 (21.1)		2 (7.7)	3 (21.4)	
Lymphovascular invasion						
Positive	9 (42.9)	4 (21.1)	0.141	6 (23.1)	7 (50)	0.083
Negative	12 (57.1)	15 (78.9)		20 (76.9)	7 (50)	

*p < 0.05. PDAC: Pancreatic ductal adenocarcinoma.

The human immune system is the main biological system that defends the body against the surrounding adverse environment. It has a necessary role in the tumorigenesis and tumor progression [37]. Costimulatory molecules on T lymphocytes are essential immune checkpoints including positive and negative

Table 3: Relations between the clinicopathologic features and PDL2 immunostaining in studied PDAC cases (n = 40)

Clinicopathologic feature	Tumoral PDL2 expression			Stromal PDL2 expression		
	High N (%)	Null/Low N (%)	p value	Positive N (%)	Negative N (%)	p value
Age						
<60 years	9 (52.9)	13 (56.5)	0.822	7 (53.8)	15 (55.6)	0.919
≥60 years	8 (47.1)	10 (43.5)		6 (46.2)	12 (44.4)	
Histologic grade						
II	14 (82.4)	17 (73.9)	0.707	8 (61.5)	23 (85.2)	0.93
III	3 (17.6)	6 (26.1)		5 (38.5)	4 (14.8)	
Tumor status						
T1& T2	11 (64.7)	17 (73.9)	0.53	5 (38.5)	24 (88.9)	0.001*
T3	6 (35.3)	6 (26.1)		8 (61.5)	3 (11.1)	
Lymph node status						
Positive	5 (29.4)	19 (82.6)	0.069	9 (69.2)	7 (25.9)	0.009*
Negative	12 (70.6)	4 (17.4)		4 (30.8)	20 (74.1)	
AJCC stage						
I	5 (29.4)	16 (69.6)	0.012*	2 (15.4)	16 (59.3)	0.009*
II and III	12 (70.6)	7 (30.4)		11 (84.6)	11 (40.7)	
Perineural invasion						
Positive	16 (94.1)	20 (87)	0.624	11 (84.6)	24 (88.9)	0.702
Negative	1 (5.9)	3 (13)		2 (15.4)	3 (11.1)	
Lymphovascular invasion						
Positive	8 (47.1)	5 (21.7)	0.091	7 (53.8)	6 (22.2)	0.045*
Negative	9 (52.9)	18 (78.3)		6 (46.2)	21 (77.8)	

*p < 0.05. PDAC: Pancreatic ductal adenocarcinoma.

acting molecules. Positive immune checkpoint can produce a positive signal and promote lymphocytes proliferation, differentiation, and functions, while negative immune checkpoint, such as PD-1, can generate a negative signal and suppress lymphocytes functions [38].

PD-1/PDL1 axis represents one of the ways used by tumor cells to avoid immune surveillance [39]. In antigenic overexposure, PD-1/PDL1 signaling makes a positive feedback loop where this signaling generates an exhausted T-cell population within the tumor and its periphery by inhibiting T-cell activation [40]. In addition, PDL1 positive cells are able to induce T-cell apoptosis protecting tumor cells from being killed and interfering with PD-1/PDL1 axis is described to reactivate the immune response against cancer [41].

The majority of PDAC excluding mismatch repair deficiencies are considered as resistant or immune-quiescent tumors and are non-responsive to single checkpoint treatment, like anti-PD-1 [42]. However, some improvements have already been reached in combination with anti-PD-1 treatment for PDAC [43].

To date, the association between PDL1 and PDAC patients remains inconclusive [44]. In a meta-analysis examined, data from a total of 1058 PDAC patients from 10 independent studies. PDL1 expression was examined by immunohistochemistry in eight of these studies, while the other two studies used quantitative reverse transcription polymerase chain reaction, the pooled results showed that positive PDL1 expression was highly correlated with a poorer overall survival in PADC patients. Moreover, the high-level PDL1 expression was correlated with poor differentiation and neural invasion. However, the analysis found no significant correlations between PDL1 expression and other clinicopathologic characteristics, including tumor status, pathologic (TNM) stage, metastatic status, lymph node metastasis, and vascular invasion [45].

In this study, a significant correlation was detected between high tumoral PDL1 expression and histologic grade (p = 0.021) and the tumor size (p = 0.022) while there was no significant correlation with other clinicopathologic features. These data were similar to that of Wang *et al.* who reported a correlation between PDL1 expression and pathological grade and TNM stage [46] and unlike that of Yamaki *et al.* that found no significant correlation between PDL1 expression and tumor size and lymph node metastasis [24].

The significant correlation between high-level PDL1 expression and poor differentiation may provide an additional indication for the application of anti-PDL1 treatment modalities for PDAC patients with poorly differentiated tumor [45].

The molecular mechanism of PDL1 overexpression in PDAC remains obscure. This upregulation could be stimulated by cytokines produced by infiltrating immune cells, such as interferon-γ, interleukin (IL)-4, IL-10, vascular endothelial growth factor, and growth stem cell factor, in some solid tumors [47].

In this study, stromal PDL1 expression was not related to any of the studied clinicopathologic features. This might be related to small sample size. To the best of our knowledge, after searching the literature, no previous study examined stromal PDL1 expression in PDAC; however, some recent articles discussed its stromal expression in other types of cancer as non-small-cell lung cancer, T-cell leukemia/lymphoma, cancer breast, colon cancer, and cholangiocarcinoma [48], [49], [50], [51], [52].

In this study, tumoral PDL2 was expressed in 17 (42%) of cases and significant relation was detected between high tumoral PDL2 expression and only tumor stage. While stromal PDL2 was expressed in 13 (32.5%) of the studied cases and its expression was significantly associated with tumor size, lymph node status, stage, and the presence lymphovascular invasion with P value equal 0.001, 0.009, 0.009, and 0.045, respectively.

In most studies, PDL2 was expressed in only small percentage of the studied patients and perhaps not only its expression by tumor cells, but rather by stromal cells which could be more significant [53].

PDL2 is basically an inhibitory molecule, expressed in antigen presenting cells and other immune cells including T-cells and non-immune cells in an inducible manner, mainly in the modulation of Th2 responses [53]. Its expression is present in a variety of tumor types, and while generally associated with PDL1, PDL2 expression can also occur in the absence of PDL1 [54].

In general, the tumoral PDL2 expression was almost linked to a worse prognosis in the majority of tumor types, with conflicting results observed in

esophageal carcinoma [55]. In addition, a recent study found that tumoral PDL2 expression was associated with worse overall survival in PDAC patients [36].

However, whether PDL2 expressed by tumor cells or other cells in the tumor-microenvironment plays, the commanding role is unclear. PDL2 has also been found to be expressed by stromal cells and appears to be functional [56], [57]. Perhaps not only PDL2 expression in tumor cells but also its expression in stromal cells plays an effective role in immune suppression and affects prognosis [58] and this could be matching the large number of significant relations between stromal PDL2 expression and the studied clinicopathologic features in this study.

Data about targeting PDL2 in cancer are scarce. It is a less studied PD-1 ligand compared to PDL1 and has yet to be fully explored, especially in PDAC. Although PDL2 has not been as fully explored in immunological research as PDL1, it is still strongly related to immunoregulation and tumor progression and provides valuable prospects for the future tumor management [36].

Conclusion

This study highlights the significance of the relation of PDL1 and PDL2 expression with important clinicopathologic features in PDAC with clear distinction between tumoral and tumor stromal expression of both PDL1 and PDL2. The significant association found between stromal PDL2 expression and most of the studied clinicopathologic features of PDAC patients showed pay the attention for further studies to rule out the benefits of these findings. However, it has several limitations including the sample size and the lack of specification of the nature of the stained stromal cells.

Declarations

Ethics approval

The study was approved by the Institutional Medical Ethical Committee (Faculty of Medicine, Cairo University) (Mentioned inside the text in method section) and consent to participate: Not applicable.

Authors' contributions

Manuscript has been read and approved by all the authors.

Category 1

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(b) Analysis of data: All authors
(c) Interpretation of data: All authors.

Category 2

(a) Drafting the article: All authors
(b) Revising it critically for important intellectual content: All authors.

Category 3

Final approval of the version to be published: All authors.

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