The expression of Programmed death ligand 2 in patients with thymoma and thymomatous Myasthenia Gravis

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Abstract

Abstract Background There is a growing relationship between PD-1/PD-Ls and autoimmune disease We attempted to explore the relationship of the PD-L2 expression in patients with thymoma, especially with Myasthenia Gravis (thymomatous MG). Methods Seventy patients with thymoma who underwent surgical resection between January 2017 to December 2018 were retrospectively reviewed. The PD-L2 expression was evaluated by immunohistochemistry. The association between the PD-L2 expression and the clinicopathologic features was investigated, especially thymomatous MG. Results The PD-L2 expression was positive in 41 patients (58.6%) and negative in 29 patients (41.4%). The PD-L2 expression was significantly associated with WHO histology of type B2 and B3 thymoma (p=0.008) and the status of MG (p=0.002). In addition, PD-L2 positive tumors showed a significantly smaller tumor size (P=0.017). Ectopic thymus was significantly more often seen in the PD-L2 positive group (p=0.035). The patients with MG (p=0.001) and WHO type B2 and B3 (p=0.007) have significantly higher PD-L2 scores. Multivariate logistic regression model showed the status of MG (OR 12.601, 95%CI 2.406-65.995, p=0.02) and age (OR 0.199, 95%CI 0.046-0.863, p=0.031) were significantly associated with the expression of PD-L2. The analysis of 33 patients with MG shows age was not associated with the expression of PD-L2 (OR 0.04, 95%CI 0.001-3.2, p=0.15). Conclusions A strong expression of PD-L2 in thymoma was significantly associated with thymomatous MG and WHO histologic type B2 and B3. In addition, PD-L2 may play a potential role in the pathogenesis of thymomatous MG.

Introduction

Thymoma is the most common anterior mediastinal mass in adult[1]. About 30% of patients with thymoma have MG (thymomatous MG) [2]. Almost all patients with thymomatous MG have antibodies to ACHR [3]. However, the pathogenesis of thymomatous MG and the signal path of antibodies generation are not entirely clear. Studies reveal that TFH cells play a fundamental role in humoral immunity deriving from their ability to provide help for germinal center (GC) formation, B cell differentiation into plasma cells and memory cells, and antibody production in secondary lymphoid tissues [4]. Furthermore, Lieping Chen and his colleagues found that PD-1 expression on T cells and PD-L2 expression on B cells controlled TFH and PC numbers. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cell[5].

What's more, thymic TFH cells might involve in the pathogenesis of MG with thymoma [6,7].

PD-1 is highly expressed in TFH. The roles of PD-1/PD-L1,2 signaling in the pathogenesis of thymomatous MG has been virtually unstudied. However, numerous studies showed that PD-L1 protein expression was not associates with the status of MG [8,9]. In that case, PD-L2 may be an alternative and valuable marker. Previous studies shown that PD-L2 was expressed on solid tumor, APC (Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses) and medullary thymic epithelial cells[10] with high expression in some organs (like lung, liver and heart) and low expression in some others (like spleen, lymph

node and thymus. And there is a growing relationship between PD-1/PD-Ls and autoimmune disease[11]. So, we have a guess that PD-L2 may play an important role in the status of MG in patients with thymoma.

The purposes of this retrospective study were to characterize the association between the expression of PD-L2 in thymoma and the clinicopathologic features of the patients and to evaluate the relationship of PD-L2 expression and the status of MG.

Patients and methods

This study was a retrospective review of patients with thymoma and was approved by the Institutional Review Board in the first affiliated hospital of SAN-YAT-SUN university. Seventy patients who had undergone surgical resection for thymomas at the first affiliated hospital of SAN-YAT-SUN university between January 2017 to December 2018 were enrolled, and their paraffin-embedded specimens were used to assess the expression of PD-L2 by immunohistochemistry (IHC). Clinical data were obtained from patients' medical charts, including patient age, sex, tumor size, World Health Organization histologic type, status of myasthenia gravis, Masaoka-Koga stage, ectopic thymus. For the patients with MG, Myasthenia Gravis Foundation of America (MGFA) Clinical Classification at diagnosis were obtained.

Immunohistochemistry Studies Immunohistochemistry studies were performed on formalin-fixed, paraffin-embedded tissue sections from each tumor. Each section was cut to a 4-mm thickness. Anti-PD-L2 mouse immunoglobulin G monoclonal antibody (clone UMAB223; ZSGB Bioscience) were diluted to 1:100 and used as a primary antibody. The IHC staining was performed automatically with the Ventana BenchMark XT Stainer (Roche Diagnostics, Basel, Switzerland).

Scoring of PD-L2 Positivity Two pathologists evaluated the expression of PD-L2 on tumor cells. They reviewed each sample independently then discussed the findings of each sample and determined the proportion of PD-L2 positive tumor cells together. According to a previous consensus, a tumor cell was defined as "PD-L2 positive" when the cell membranes were partially or completely stained [12] (Fig 1). In contrast, PD-L1 staining in the cytoplasm of a tumor cell was defined as "negative." The PD-L2 positive immune cells, such as lymphocytes and macrophages, were excluded from the cell counts. Tumor cells were quantified by evaluating the ratio of stained to unstained tumor cells. The PD-L2 positivity was evaluated based on based on the proportion of PD-L2 positive tumor cells. A PD-L2 expression rate of 1% or greater was defined as PD-L2 positive, and all other cases were PD-L2 negative in the present study. The intensity of PD-L2 (from 0 to 3: 0, negative; 1, very weak; 2, moderate; and 3, strong) in tumor cells were evaluated for each core. And mean PD-L2 expression scores were calculated by multiplying the percentage of tumor area stained by the staining intensity.

Descriptive statistics were used to describe the patients' baseline. Univariate analysis was conducted using non-parametric test, the Chi square ($\chi 2$) test and the Student t-test. If necessary, fisher's exact test was conducted for the categorical variables. The logistic regression model was used for the analysis of the correlation between patients' characteristics and PD-L2 expression. The p values of< 0.05 were defined as significant.

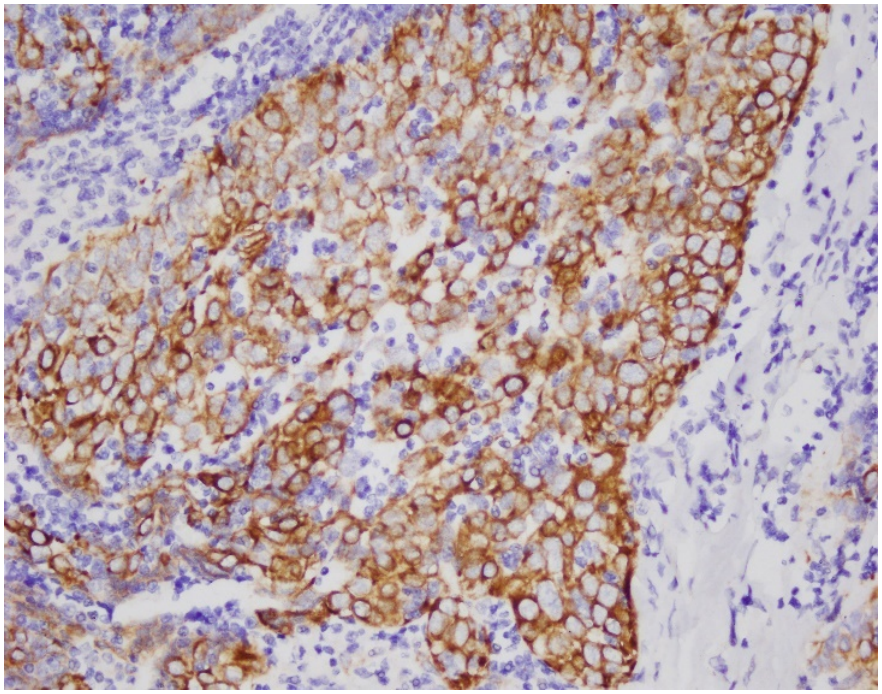

Fig 1. Programmed death ligand 2 (PD-L2) immunohistochemical staining in thymoma. In this case, the tumor cells had stained cell membranes, and this tumor was defined as PD-L2 positive. The proportion of PD-L1 positive tumor cells was 95% and staining intensity score 3.

(magnification, ×200).

Results

1, clinicopathologic characteristics

There were 70 patients enrolled in this study. As list in Table 1, the PD-L2 expression was positive in 41 patients (58.6%) and negative in 29 patients (41.4%). A positive expression was shown in Fig 1. The mean PD-L2 expression scores was 70.8. Table 1 shows the clinicopathologic characteristics of these patients. The mean age was 49. 60% of patients were male. And the mean tumor size was 5.35cm. B1 (17.1%) and B2 (44.3%) dominated the WHO pathologic type. Most of the patients were diagnosed with Masaoka-Koga stage I (71.4%). 33 patients (47.1%) were also MG patients.

Data	Data	No. of patients	percentage
Gender			
	male	42	60%
	female	28	40%
Age		49.0(18-76)	
Tumor size(cm)		5.35(1-15)	
Masaoka-Koga stage			
	I	50	71.40%
	IIA	11	15.70%
	IIB	1	1.40%
	IIIA	3	4.30%
	IIIB	3	4.30%
	IV	2	2.90%

Data	Data	No. of patients	percentage
WHO type			
	A	8	11.40%
	AB	10	14.30%
	B1	12	17.10%
	B2	31	44.30%
	B3	9	12.90%
Myasthenia gravis			
	negative	37	52.90%
	positive	33	47.10%
	MGFA class		
	I	10	14.30%
	IIA	4	5.70%
	IIB	17	24.30%
	IIIA	1	1.40%
	IIIB	1	1.40%
PDL2 expression			
	negative	29	41.40%
	positive	41	58.60%
	PDL2 expression intensity		
	0	29	41.40%
	1	16	22.90%
	2	11	15.70%
	3	14	20%
	PDL2 expression ratio	31.9+30.1%	0-98%
	PDL2 scores	70.8+102.7	0-294

Table 1 clinicopathologic characteristics and PD-L2 expression in 70 patients

2, correlation between PD-L2 expression and clinicopathologic characteristics

Table 2 shows the clinicopathologic characteristics stratified by PD-L2 and PD-L2 scores stratified by clinicopathologic characteristics. The PD-L2 expression was significantly associated WHO histology of type B2 and B3 thymoma (p=0.008). And the PD-L2 scores were significantly higher in patients with WHO type B2 and B3 (98.9 vs 33.4, p=0.007). The PD-L2 positive group also had a significantly higher proportion of the status of MG than without MG (p=0.002). The PD-L2 scores were significantly higher in MG positive group (124.1 vs 23.3, p=0.001). In addition, PD-L2 positive tumors showed a significantly smaller tumor size than the PD-L2 negative group (P=0.017). Ectopic thymus has been significantly more often seen in the PD-L2 positive group than the negative group (p=0.035). However, no significantly difference considering PD-L2 expression was observed in the age (p=0.275), gender (p=0.621) or Masaoka-Koga stage (p=0.791). The PD-L2 scores were not significantly different in gender (p=0.736), age (p=0.23), Masaoka-Koga stage (p=0.26), tumor size (p=0.111) or ectopic thymus (p=0.115). We conducted a logistic regression model to investigate PD-L2 expression (Table 3). The status of MG (OR 12.601, 95%CI 2.406-65.995, p=0.02) and age (OR 0.199, 95%CI 0.046-0.863, p=0.031) were significantly associated with the expression of PD-L2. WHO histology type, Masaoka-Koga stage, tumor size or ectopic thymus were not associated with PD-L2 expression.

Data	Data	PDL2 positive	PDL2 negative	p		PD-L2 scores	p
Gender	male	26	16	0.621		74.2±108.2	0.736
	female	15	13			66.7±95.6	
Age(years)	Age(years)	50.5±12.6	47.0±13.5	0.275	[?]49	85.2±109.3	0.230

Data	Data	PDL2 positive	PDL2 negative	p		PD-L2 scores	p
					>49	55.6±94.5	
Myasthenia gravis	positive	26	7	0.002		124.1±117.3	0.001
	negative	15	22			23.3±55.5	
WHO type	A+AB+B1	18	12	0.008		33.4±72.1	0.007
	B2+B3	29	11			98.9±113.7	
Masaoka-Koga stage	I	30	20	0.791		79.6±108.5	0.260
	IIA-IV	11	9			48.8±85.4	
Tumor size(cm)	Tumor size(cm)	4.7±2.4	6.2±2.8	0.017	[?]5cm	89.7±114.2	0.111
					>5cm	50.8±86.2	
Ectopic thymus	yes	11	2	0.035		111.4±124.4	0.115
	no	30	27			61.5±96.0	

Table 2 correlation between PD-L2 expression and clinicopathologic characteristics

Data		OR	95% CI	p
Gender	male	1.972	0.569-6.836	0.284
	female	1		
Age(years)	[?]49	0.199	0.046-0.863	0.031
	>49	1		
Myasthenia gravis	positive	12.601	2.406-65.995	0.003
	negative	1		
WHO type	A+AB+B1	0.407	0.115-1.437	0.163
	B2+B3	1		
Masaoka-Koga stage	I	1.728	0.414-7.211	0.453
	IIA-IV	1		
Tumor size	[?]5cm	0.594	0.156-2.263	0.446
	>5cm	1		
Ectopic thymus	yes	4.263	0.706-25.751	0.114
	no	1		

Table 3 Multivariate Logistic regression analysis of PD-L2 expression by clinicopathologic characteristics

3, MG and PD-L2 expression, clinicopathologic characteristics

Table 4 shows the compare of clinicopathologic characteristics between MG (+) group and MG (-) group. The patients with WHO histology of type B2 and B3 have a significantly higher incidence rate of MG (p=0.004). The patients with MG have smaller tumor size (p<0.001) and higher PD-L2 scores (p<0.001). No significantly difference were observed in age (p=0.052), gender (p=0.224), Masaoka-Koga stage (p=1) or ectopic thymus (p=0.357). Due to the positive association between MG and PD-L2 expression, we particularly analyzed the data of patients with MG to explore if the PD-L2 expression depended on the different features of MG (Table 5). 33 patients with MG were enrolled. There were no significantly difference of PD-L2 scores regarding gender, age, MGFA stage, WHO histology type, Masaoka-Koga stage, tumor size or ectopic thymus in patients with MG. We conducted a logistic regression model to predict PD-L2 expression in patients with MG. No feature listed above was associated with PD-L2 expression in patients with MG.

Data	Data	MG positive	MG negative	p
Gender	male	17	25	0.224
	female	16	12	

Data	Data	MG positive	MG negative	p
Age		45.8±12.8	51.9±12.7	0.052
WHO type	A+AB+B1	8	22	0.004
	B2+B3	25	15	
Masaoka-Koga	I	24	26	1
	IIA-IV	9	11	
Tumor size		4.1±2.3	6.5±2.4	0.001
Ectopic thymus	yes	8	5	0.357
	no	25	32	

Table 4 correlation between MG clinicopathologic characteristics

Data	Data	No. of patients	PD-L2 scores	PD-L2 scores	PD-L2 expression	PD-L2 expressio
			PD-L2 scores	p	OR	95% CI
Gender	male	17	141.0±125.7	0.402	2.488	0.234-26.444
	female	16	106.1±108.8		1	
Age(years)	[?]49	22	123.8±116.4	0.982	0.04	0.001-3.2
	>49	9	124.8±125.7		1	
MGFA class	I+IIA	13	121.6±127.4	0.92	0.151	0.011-2.009
	IIB-IV	18	125.9±112.8		1	
WHO type	A+AB+B1	8	61.6±92.3	0.061	0.198	0.016-2.514
	B2+B3	23	144.1±118.7		1	
Masaoka-Koga stage	I	23	135.8±119.5	0.356	6.215	0.238-162.494
	IIA-IV	8	92.8±111.6		1	
Tumor size	[?]5cm	21	128.0±123.6	0.774	0.283	0.01-7.879
	>5cm	10	115.0±106.9		1	
Ectopic thymus	yes	8	132.3±130.3	0.825	3.606	0.214-60.735
	no	23	121.5±115.6		1	

Table 5 PD-L2 scores and Multivariate analysis of PD-L2 expression by clinicopathologic characteristics in patients with MG

Discussion

There was little study investigating the PD-L2 expression in thymoma. And PD-L2 was less well studied. Human PD-L2 are expressed on dendritic cells and medullary thymic epithelial cells [10]. High PD-L2 expression may promote tumor metastasis and predict unfavorable prognosis in solid cancer patients after surgery[13]. Previous studies showed that PD-L2 exerts its main physiological and pathological function in immune tolerance via dampening and modulating T helper type 2 (Th2) response[14]. MG is an autoimmune disease associated with thymus pathologies. A preliminary investigation between the PD-L2 expression in thymoma and MG was shown in the results above.

In this study, the PD-L2 expression was positive in 41 patients (58.6%). However, Isabelle Rouquette et al found that PD-L2 antibody stained no thymic epithelial tumors [15]. Different antibody and cut-off values vary among studies. The positive expression of PD-L2 was associated with B2/B3 histologic type. Because there were few studies put attention in PD-L2 and thymoma, we referred PD-L1 expression. Numerous studies investigating the PD-L1 expression in thymoma indicated that a high expression of PD-L1 was significantly associated with B2/B3 histologic type [16,17]. Most studies played attention to the PD-L1/2 expression with prognosis. A meta-analysis showed high PD-L2 expression might predict unfavorable prognosis. We did not explore the prognosis in these patients but focus in the PD-L2 expression and MG.

Patients with B2 histologic type was the most in this study (44.3%). And the status of MG was associated with B2/B3 histologic types in this study. L. Maggi et al found that AB and B2 thymomas were most frequently observed histologic types in patients with MG[18]. Ströbel, P et al also observed that B2 was the most frequently [19]. However, other authors reported B1 and B2 histologic types as prevalent[20,21]. The positive expression of PD-L2 was also associated with a smaller tumor size. What's more, the tumor size was smaller in the patients with MG in this study. This can be explained by earlier diagnosis because of the status of MG[22]. The PD-L2 expression was associated with ectopic thymus in this study. The exist of ectopic thymus may play an important role in the pathogenesis of MG in patients with thymoma because of the TFH cells [6]. Furthermore, our group found that ectopic thymus was a poor prognosis factor in patients with MG [23]. Not only the Chi square test showed the positive relationship between PD-L2 expression and MG, but also the logistic regression demonstrated a significantly association between PD-L2 expression and MG in the patients with thymoma. Although age was associated with PD-L2 expression with the multivariate logistic regression analysis in the overall patients, but no significantly association was observed in patients with MG. Therefore, we do have sufficient reason to guess that the PD-L2 expression plays a crucial role in the pathogenesis of MG in patients with thymoma.

In this study, a status of MG in these patients with thymoma was associated with B2/B3 histologic types, but has no significantly associated with gender or Masaoka-Koga stage. There was tendency toward younger age in patients with MG (p=0.052). Kondo K et al found that a female dominance in their patients with thymoma and the patients with MG were significantly younger than those without MG[22]. While Jianfei Shen suggested that MG, WHO histology and Masaoka-Koga stage interrelate with one another. The inter-relationship drawn from their study was that MG was associated with early clinical stage and WHO histology of AB, B1 and B2-type thymomas [24]. The PD-L2 scores was much higher in the patients with MG than without MG (124.1 vs 23.3, $p<0.001$). We did an analysis of PD-L2 scores in patients with MG only. We found that there were no significantly difference in PD-L2 scores regarding MGFA stage, Masaoka-Koga stage, tumor size or ectopic thymus, but a tendency toward high PD-L2 scores in patients with B2/B3 types.

The different proportion of T-lymphocytes differing in WHO histologic types may contribute the different prevalence of MG. It is speculated that T-lymphocytes development is involved in the pathogenesis of MG in patients with thymoma [25]. And thymomas with MG are enriched for autoreactive T cells with specificity for AChR. And the export of autoreactive CD4+ T cells is of pathogenic relevance of MG[26]. These exporting T cells may carry special signal in the pathogenesis of MG. And there was a positive association between the percentage of CD4+ T cells and MG severity [4]. Furthermore, PD-1 is highly expressed in TFH, the PD-1 signaling involved decreased GC death and increase TFH cytokine production. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cell. [5] Our guess about thymoma related MG pathogenesis is that the PD-L2 co-stimulate PD-1 signal through exporting autoreactive T cells to help germinal center (GC) formation, B cell differentiation into plasma cells and memory cells, and antibody production in secondary lymphoid tissues. Furthermore, we will explore the potential mechanism of PD-L2 signal path in thymoma related MG pathogenesis with the techniques of immunofluorescence and flow cytometry.

However, several limitations associated with present study also warrant mention. First, the number of patients was small. Second, this was a retrospective analysis performed at a single institution. Third, we did not investigate the PD-L2 expression with survive.

In conclusions, a strong expression of PD-L2 in thymoma was significantly associated with thymomatous MG and WHO histologic type B2 and B3. On the basis of our results and analysis, PD-L2 may play a potential role in the pathogenesis of thymomatous MG.

There is no conflict of interest in whichever form at the submission of this manuscript

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