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Different pattern of PD-L1, IDO, and FOXP3 Tregs expression with survival in thymoma and thymic carcinoma

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ABSTRACT

Objectives: The expression of immune checkpoint ligand PD-L1 has been reported in various tumors. The expression of IDO and FOXP3 Tregs are considered to be associated with tumor-induced tolerance and poor outcome. Their prognostic role in surgically treated thymoma and thymic carcinoma, however, has not been investigated.

Materials and Methods: Tissue microarray (TMA) blocks comprised of 100 surgically treated thymomas and 69 surgically treated thymic carcinomas were conducted. Tissue sections were incubated with primary antibodies against PD-L1 (clone E1L3N, 1:100), IDO (clone 10.1, 1:50), and FOXP3 (clone 236 A/E7, 1:50). Comparisons for categorical variables were performed using χ^2 test and Fisher's exact test. Survival analysis was established using Kaplan-Meier method and log-rank test. Univariate and multivariate analyses were performed using Cox regression model.

Results and Conclusions: High expression of PD-L1, IDO, and FOXP3 Tregs were identified in 36 (36%), 13 (13%), and 16 (16%) thymoma patients, respectively. High expression of PD-L1, IDO, and FOXP3 Tregs was associated with higher grade of tumor histology ($P < 0.001$, $P = 0.007$, and 0.014, respectively). High expression of PD-L1 was also associated with advanced Masaoka staging ($P < 0.001$). In patients with thymic carcinoma, high expression of PD-L1, IDO, and FOXP3 Tregs were identified in 25 (36%), 10 (14%), and 20 (29%) patients, respectively. Complete resection, low expression of IDO, and high expression of FOXP3 Tregs were associated with better overall survival ($P = 0.001$, 0.004, and 0.032, respectively), and progression-free survival ($P < 0.001$, $P = 0.026$, and 0.047, respectively) in multivariate analysis.

In surgically treated thymoma, high PD-L1 expression was associated with advanced Masaoka staging. High PD-L1, IDO, and FOXP3 Tregs expression was associated with high grade histology. In surgically treated thymic carcinoma, significant survival benefit was noted in patients with complete resection, low IDO expression, and high FOXP3 Tregs expression.

1. Introduction

Thymic epithelial neoplasms are rare tumors with an incidence of less than 1% of all adult cancers. They are divided into thymomas and thymic carcinomas. Thymic carcinoma is relatively uncommon among thymic neoplasms, traditionally considered together with thymoma, comprising approximately 10% of these lesions [1], and presents frequently with locally advanced or distant metastatic disease. Thymic

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Table 1
Characteristics of 100 surgically treated thymoma with expression of PD-L1, IDO and FOXP3.

	PD-L1 low expression (N = 64)	PD-L1 high expression (N = 36)	P value	IDO low expression (N = 87)	IDO high expression (N = 13)	P value	FOXP3 low expression (N = 84)	FOXP3 high expression (N = 16)	P value
Age (Mean age ± SD, year)	51 ± 12	54 ± 14	0.305	52 ± 13	60 ± 10	0.025	53 ± 12	52 ± 17	0.806
≤60 (n, %)	47 (73.4)	20 (55.6)	0.068	62 (71.3)	5 (38.5)	0.019	58(69.1)	9 (56.3)	0.318
> 60 (n, %)	17 (26.6)	16 (44.4)		25 (28.7)	8 (61.5)		26(30.9)	7 (43.7)	
Sex, n (%)			0.770			0.761			0.964
Female	25 (39.1)	13 (36.1)		34 (39.1)	4 (30.8)		32 (38.1)	6 (37.5)	
Male	39 (60.9)	23 (63.9)		53 (60.9)	9 (69.2)		52 (61.9)	10 (62.5)	
Masaoka staging, n (%)			< 0.001			0.675			0.471
I	39 (60.9)	5 (13.9)		39 (44.8)	5 (38.5)		40 (47.6)	4 (25.0)	
II	17 (26.6)	10 (27.8)		24 (27.6)	3 (23.1)		22 (26.2)	5 (31.3)	
III	4 (6.3)	15 (41.7)		16 (18.4)	3 (23.1)		15 (17.9)	4 (25.0)	
IVA	2 (3.1)	5 (13.9)		5 (5.7)	2 (15.3)		5 (5.9)	2 (12.5)	
IVB	2 (3.1)	1 (2.7)		3 (3.5)	0 (0)		2 (2.4)	1 (6.2)	
Histology, n (%)			< 0.001			0.007			0.014
A	8 (12.5)	0 (0)		7 (8.2)	1 (7.7)		7 (8.3)	1 (6.3)	
AB	24 (37.5)	1 (2.8)		25 (28.7)	0 (0)		25 (29.8)	0 (0)	
B1	12 (18.8)	2 (5.6)		12 (13.7)	2 (15.4)		11 (13.1)	3 (18.7)	
B2	15 (23.4)	12 (33.3)		26 (29.9)	1 (7.7)		23 (27.4)	4 (25.0)	
B3	5 (7.8)	21 (58.3)		17 (19.5)	9 (69.2)		18 (21.4)	8 (50.0)	
Myasthenia gravis, n (%)	24 (37.5)	16 (44.4)	0.496	35 (40.2)	5 (38.5)	0.903	35 (41.8)	5 (31.3)	0.436
Radicality, n (%)			0.052			0.131			0.264
R0	59 (92.2)	27 (75)		77 (88.5)	9 (69.2)		73(86.9)	13 (81.3)	
R1	2 (3.1)	2 (5.6)		2 (2.3)	2 (15.4)		4(4.8)	0 (0)	
R2	3 (4.7)	7 (19.4)		8 (9.2)	2 (15.4)		7(8.3)	3 (18.8)	
Follow-up duration (median [range], month)	96 [1–228]	63.5 [1–206]		73 [1–228]	84 [1–206]		91 [1–228]	44.5 [1–135]	
Treatment modality, n (%)			0.032			0.929			0.008
Surgery	45 (70.3)	15 (41.7)		52 (59.7)	8 (61.5)		55 (65.5)	5 (31.2)	
Surgery + RT	13 (20.3)	15 (41.7)		25 (28.7)	3 (23.1)		19 (22.6)	9 (56.2)	
Surgery + CRT	4 (6.3)	6 (16.7)		8 (9.2)	2 (15.4)		9 (10.7)	1 (6.3)	
CRT + Surgery + RT	1(1.6)	0(0)		1 (1.2)	0 (0)		0 (0)	1 (6.3)	
CT + Surgery + RT	1(1.6)	0(0)		1 (1.2)	0 (0)		1 (1.2)	0 (0)	

RT, radiotherapy; CRT, chemoradiotherapy; CT, chemotherapy.

carcinomas are considered overtly malignant epithelial tumors, whereas thymomas exhibit at least low grade malignant behavior with the potential for invasion and metastasis. Surgery remains the mainstay of treatment for thymic epithelial tumors and complete resection provides the opportunity of long-term survival [1–6]. Despite that complete resection provides the best survival and opportunity of cure, approximately 10%–30% of patients with thymic epithelial tumors undergoing surgical resection developed recurrent diseases [1,3,7]. In patients with unresectable or recurrent disease, multiple treatment modalities have been advocated and investigated but the outcomes were inconclusive [8–11].

Many previous reports have stated that high programmed cell death 1 ligand (PD-L1) expression in tumor cells is correlated with clinical progression and worse overall survival in some tumor types. The poor prognosis in patients with high PD-L1 expression are thought to be attributable to the activity of the PD-L1 pathway, which induces T cell-mediated immune suppression in the tumor microenvironment. Indoleamine 2,3-dioxygenase (IDO) is responsible for tryptophan catabolism and immune tolerance to foreign antigens [12]. Studies on IDO expression have confirmed that IDO played an immunosuppressive role in carcinogenesis and served as a biomarker for poor prognosis in certain tumors [13–17]. Forkhead box P3 (FOXP3) is a transcriptional factor of CD4 +CD25+ regulatory T cells (Treg) and has an immunosuppressive function. Although tumor-infiltrating FOXP3 T cells have generally been regarded as having an adverse impact in the treatment outcome and survival because of the immunologic escape or evasion along with IDO [18–22], increased expression of FOXP3 Tregs has also been reported to associate with improved survival or treatment outcome [23–26]. Recently the development of immunotherapy has shed some light on the survival improvement of many different kinds of malignancy. We retrospectively analyze the expression of PD-L1, IDO, and FOXP3 Tregs, and its clinical significance in thymic epithelial tumors as a step toward comprehensive approach and pave the way to future immunotherapy in thymic epithelial tumors.

2. Materials and methods

2.1. Patient enrollment

A retrospective review of medical records between June of 1988 and March of 2013 was conducted among 169 patients who underwent intent-to-treat surgery for thymoma or thymic carcinoma. Informed consent was waived because the study was retrospective, and the review of medical records was approved by the Institutional Review Board of National Cheng Kung University Hospital (B-ER-102-443). The surgical pathologies, including the tumor histology and resection margin were microscopically confirmed by an experienced pathologist, who was blind to the clinical data, and the tumors were classified according to the newly published World Health Organization (WHO) classification. Clinical information was collected on sex, age, Masaoka staging, tumor histology, myasthenia gravis, surgical radicality, and the treatment modalities.

2.2. General management principles for thymic epithelial tumors

Computed tomography or magnetic resonance imaging was used to evaluate whether the lesions were resectable. Abdominal sonography and whole-body bone scan were routine preoperative metastatic evaluation modalities. Biopsy was performed for lesions that cannot be completely resected at initial presentation and evaluation. Preoperative cisplatin-based chemotherapy along with radiation therapy (3000–5000 cGy) were administered to patients whose imaging studies showed locally advanced and unresectable diseases, including superior vena cava syndrome, pericardial effusion, or encasement of the

Table 2
Characteristics of 69 surgically treated thymic carcinoma with expression of PD-L1, IDO and FOXP3.

	PD-L1 low expression (N = 44)	PD-L1 high expression (N = 25)	P value	IDO low expression (N = 59)	IDO high expression (N = 10)	P value	FOXP3 low expression (N = 49)	FOXP3 high expression (N = 20)	P value
Age (Mean age ± SD, year)	54 ± 10	56 ± 12	0.404	54 ± 12	56 ± 11	0.723	54 ± 11	56 ± 11	0.442
≤60 (n, %)	33 (75.0)	16 (64.0)	0.333	42 (71.2)	7 (70.0)	0.939	36 (73.5)	13 (65.0)	0.482
> 60 (n, %)	11 (25.0)	9 (36.0)		17 (28.8)	3 (30.0)		13 (26.5)	7 (35.0)	
Sex, n (%)			0.306			0.592			0.817
Female	25 (56.8)	11 (44.0)		30 (50.8)	6 (60.0)		26 (53.1)	10 (50.0)	
Male	19 (43.2)	14 (56.0)		29 (49.2)	4 (40.0)		23 (46.9)	10 (50.0)	
Masaoka staging, n (%)			0.868			0.641			0.280
I	1 (2.3)	0 (0)		1 (1.7)	0 (0)		0 (0)	1 (5.0)	
II	2 (4.5)	1 (4.0)		2 (3.4)	1 (10.0)		2 (4.1)	1 (5.0)	
III	28 (62.6)	17 (68.0)		39 (66.1)	6 (60.0)		30 (61.2)	15 (75.0)	
IVA	9 (20.5)	4 (16.0)		12 (20.3)	1 (10.0)		11 (22.5)	2 (10.0)	
IVB	4 (9.1)	3 (12.0)		5 (8.5)	2 (20.0)		6 (12.2)	1 (5.0)	
Histology, n (%)			0.123			0.406			0.422
Squamous cell caricnoma	26 (59.1)	20 (80.0)		40 (67.8)	6 (60.0)		31 (63.2)	15 (75.0)	
Lymphoepithelioma-like carcinoma	7 (15.9)	2 (8.0)		6 (10.2)	3 (30.0)		7 (14.3)	2 (10.0)	
Undifferentiated carcinoma	4 (9.1)	0 (0)		4 (6.8)	0 (0)		2 (4.1)	2 (10.0)	
Neuroendocrine carcinoma	5 (11.3)	3 (12.0)		7 (11.9)	1 (10.0)		7 (14.3)	1 (5.0)	
Adenocarcinoma	2 (4.6)	0 (0)		2 (3.4)	0 (0)		2 (4.1)	0 (0)	
Radicality, n (%)			0.835			0.697			0.106
R0	29 (65.9)	16 (64.0)		38 (64.4)	7 (70.0)		29 (59.2)	16 (80.0)	
R1	3 (6.8)	1 (4.0)		3 (5.1)	1 (10.0)		4 (8.2)	0 (0)	
R2	12 (27.3)	8 (32.0)		18 (30.5)	2 (20.0)		16 (32.6)	4 (20)	
Follow-up duration (median [range], month)	44 [1–249]	42 [1–218]		46 [1–249]	30 [1–219]		34 [1-249]	73 [3–219]	
Treatment modality, n (%)			0.660			0.595			0.601
Surgery + RT	26 (59.1)	13 (52.0)		32 (54.2)	7 (70.0)		29 (59.2)	10 (50.0)	
Surgery + CRT	12 (27.2)	8 (32.0)		19 (32.2)	1 (10.0)		12 (24.5)	8 (40.0)	
CRT + Surgery + RT	4 (9.1)	3 (12.0)		5 (8.5)	2 (20.0)		7 (14.3)	0 (0)	
CT + Surgery + CT	1 (2.3)	1 (4.0)		2 (3.4)	0 (0)		1 (2.0)	1 (5.0)	
RT + Surgery + CRT	1 (2.3)	0 (0)		1 (1.6)	0 (0)		0 (0)	1 (5.0)	

RT, radiotherapy; CRT, chemoradiotherapy; CT, chemotherapy.

pulmonary vessels or aorta. Operative procedures were subsequently performed if the tumor had been downstaged radiographically and evaluated as resectable. Radical resection, including extended thymectomy and thymomectomy was the surgical principle. Postoperative chemotherapy was given to patients who had residual or recurrent disease. Postoperative radiation therapy with a full dose (5000–6000 cGy) was routinely performed at the tumor bed and mediastinum for patients undergoing complete resection for high grade thymoma or thymic carcinoma if they were not preoperatively irradiated. For those with residual, recurrent disease or previously irradiated, the dose of radiotherapy was tailored accordingly so that it was limited to a total dose of 6000 cGy in the mediastinum.

2.3. Follow-up and evaluation of recurrence

All the patients were followed up until March of 2017 or until the time of their death. For patients with clinical appearance of new diseases on follow-up imaging after a complete resection, thoracoscopy was the option of diagnostic and therapeutic modality for intrathoracic lesions. Extrathoracic lesions were determined by biopsy and tissue proof whenever possible, or the consensus of a multidisciplinary panel discussion based on the RECIST guideline [27,28]. The overall survival was computed from the date of resection of primary tumor to the date of the last follow-up or death. The progression-free survival was calculated from the date of resection of primary tumor to the date of last follow-up or recurrence diagnosis.

2.4. Tissue microarray and immunohistochemical study

Tissue microarray (TMA) blocks comprised of 100 surgically treated thymomas and 69 surgically treated thymic carcinomas were conducted. The surgical specimens were fixed in 10% formalin, embedded in paraffin, and two samples (2 mm cores) were taken from each specimen. For immunohistochemistry, the TMA blocks were sectioned at a thickness of 5 μm, deparaffinized, rehydrated, incubated with 3% H_2O_2 in methanol for 15 min, and subjected to heat-induced antigen retrieval by means of boiling in 0.01 M citrate buffer for 10 min. Tissue sections were incubated with the primary antibodies against PD-L1 (clone E1L3N, 1:100, Cell Signaling Technology), IDO (clone 10.1, 1:50, Chemicon International), and FOXP3 (clone 236 A/E7, 1:50, Abcam), diluted in antibody diluent (DAKO Antibody Diluent, Background Reducing). For visualization of the antigen, DAKO HRP secondary antibodies and 3,3′-diaminobenzidine (DAB) were applied according to the manufacturer's instructions. Appropriate positive and negative controls were run for all of the antibodies.

The immunohistochemical slides were examined by an experienced pathologist in a blinded fashion. PD-L1 and IDO were calculated using a semiquantitative method based on the staining intensity [0–3] and the staining percentage [0–100%] of the tumor. The staining intensity was scored as follows: 0 = none, 1 = equivocal/weak, 2 = moderate, and 3 = strong. For the interpretation of PD-L1 and IDO, those cases with strong intensity in any percentage or moderate intensity in more than 50% of the tumor were categorized as high expression, and the remaining as low expression. The expression of FOXP3 Tregs was calculated according to the percentage of positive lymphocytes, and more than 1% was categorized as high expression.

2.5. Statistical analysis

Continuous variables were compared using Student's *t*-test, while categorical variables were compared using χ^2 test. When the expected numbers in a cell of comparison table were smaller than 5, Fisher's exact test was used for comparison. Survival analysis was performed using the Kaplan-Meier method, and the statistical difference was

Table 3
Univariate and multivariate analysis for overall survival and progression-free survival of 100 surgically treated thymoma.

	Overall survival		Progression-free survival	
Variable	HR (95% CI)	P value	HR (95% CI)	P value
Univariate analysis				
Sex	1.05	0.906	1.24	0.572
Male vs. Female	(0.46–2.42)		(0.59–2.58)	
Age	1.08	0.865	1.47	0.304
> 60 vs. ≤60	(0.44–2.63)		(0.70–3.09)	
Masaoka stage	1.49	0.028	1.43	0.012
IVA + IVB vs. III vs. I + II	(1.09–2.05)		(1.08–1.89)	
Histology B2 + B3 vs. A + AB + B1	2.97	0.021	2.33	0.027
	(1.18–7.48)		(1.10–4.94)	
Myasthenia gravis	0.87	0.744	0.66	0.271
Yes vs. No	(0.38–1.98)		(0.32–1.38)	
Radicality	2.65	0.031	2.93	0.007
R1 + R2 vs. R0	(1.09–6.41)		(1.35–6.38)	
PD-L1	1.94	0.111	2.21	0.024
High expression vs. Low expression	(0.86–4.38)		(1.09–4.49)	
IDO	0.52	0.382	1.22	0.682
High expression vs. Low expression	(0.12–2.24)		(0.47–3.19)	
FOXP3	2.43	0.090	2.12	0.107
High expression vs. Low expression	(0.87–6.80)		(0.85–5.28)	
Multivariate analysis				
Masaoka stage	1.14	0.553	1.09	0.704
IVA + IVB vs. III vs. I + II	(0.75–1.73)		(0.71–1.66)	
Histology	2.14	0.214	1.31	0.614
B2 + B3 vs. A + AB + B1	(0.65–7.10)		(0.46–3.70)	
Radicality	1.68	0.348	2.06	0.192
R1 + R2 vs. R0	(0.57–4.93)		(0.69–6.11)	
PD-L1	1.17	0.748	1.50	0.355
High expression vs. Low expression	(0.45–3.01)		(0.63–3.57)	
IDO	0.34	0.161	0.85	0.762
High expression vs. Low expression	(0.08–1.53)		(0.30–2.39)	
FOXP3	1.91	0.232	1.91	0.180
High expression vs. Low expression	(0.66–5.50)		(0.74–4.93)	

determined using the log-rank test. Univariate Cox regression analysis was initially carried out to identify the significant predictors of survival. All variables with $P < 0.05$ in univariate analysis as well as the PD-L1, IDO, and FOXP3 Tregs expression were entered into the multivariate model. Results were expressed as hazard ratios (HRs) with their 95% confidence intervals (CIs). All statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). P value < 0.05 were considered statistically significant.

3. Results

The staining results of PD-L1, IDO, and FOXP3 Tregs were illustrated in supplementary material Figs. S1 and S2. High expression of PD-L1 was identified in 36 (36%) thymoma patients, and high expression of PD-L1 was associated with advanced Masaoka staging ($P < 0.001$) and high grade tumor histology ($P < 0.001$) as illustrated. High expression of IDO was noted in 13 (13%) thymoma patients, and was associated with patients older than 60 ($P = 0.019$) and high grade tumor histology ($P = 0.007$). High expression of FOXP3 Tregs was observed in 16 (16%) thymoma patients, and was associated with high grade tumor histology ($P = 0.014$, Table 1). In patients with thymic carcinoma, high expression of PD-L1, IDO, and FOXP3 Tregs were identified in 25 (36%), 10 (14%), and 20 (29%) patients, respectively. They were not associated with Masaoka staging or tumor histology (Table 2).

In patients with thymoma, univariate analysis revealed that Masaoka staging (HR: 1.49, 95% CI: 1.09–2.05; $P = 0.028$), tumor histology (HR: 2.97, 95% CI: 1.18–7.48; $P = 0.021$), and surgical radicality (HR: 2.65, 95% CI: 1.09–6.41; $P = 0.031$) were associated with overall survival. Masaoka staging (HR: 1.43, 95% CI: 1.08–1.89; $P = 0.012$), tumor histology (HR: 2.33, 95% CI: 1.10–4.94; $P = 0.027$), and surgical radicality (HR: 2.93, 95% CI: 1.35–6.38; $P = 0.007$) were associated with progression-free survival in univariate analysis (Table 3). In multivariate analysis for patients with surgically treated thymoma, there was no independent prognostic factor for overall survival or progression-free survival. In patients with thymic carcinoma, surgical radicality (HR: 4.29, 95% CI: 1.88–9.79; $P = 0.001$), IDO expression (HR: 3.45, 95% CI: 1.47–8.10; $P = 0.004$), and FOXP3 Tregs expression (HR: 0.37, 95% CI: 0.15–0.92; $P = 0.032$) were associated with overall survival in multivariate analysis. Surgical radicality (HR: 4.28, 95% CI: 2.00–9.12; $P < 0.001$), IDO expression (HR: 2.57, 95% CI: 1.12–5.87; $P = 0.026$), and FOXP3 Tregs expression (HR: 0.43, 95% CI: 0.19–1.01; $P = 0.047$) were also associated with progession-free survival in multivariate analysis (Table 4).

The overall survival and progression-free survival of PD-L1, IDO, and FOXP3 Treg expression in thymoma and thymic carcinoma were demonstrated in Figs. 1 and 2. The overall survival and progression-free survival were further stratified according to the status of IDO and Foxp3 Treg expression. In patients with surgically treated thymoma, 12 had high expression of IDO and Foxp3 Tregs, 72 had low expression of IDO and Foxp3 Tregs, 15 had low expression of IDO and high expression of Foxp3 Tregs, and only 1 had high expression of IDO and low expression of Foxp3 Tregs. There was no significant difference in overall survival ($P = 0.234$) and progression-free survival ($P = 0.304$; Supplementary Fig. S3). In patients with surgically treated thymic carcinoma, 2 had high expression of IDO and Foxp3 Tregs, 41 had low expression of IDO and Foxp3 Tregs, 18 had low expression of IDO and high expression of Foxp3 Tregs, and 8 had high expression of IDO and low expression of Foxp3 Tregs. The overall and progression-free survival were significantly different ($P < 0.001$, respectively; Supplementary Fig. S3).

4. Discussion

It has been reported that PD-L1 expression is observed in more than 90% of the normal thymic epithelial cells [29]. The recent development of cancer immunotherapy relies heavily on the understanding of tumor microenvironment and the suppressive mechanisms. Some studies regarding the immunohistochemical (IHC) expression of PD-L1 in the context of thymic epithelial tumors have been reported [30–32]. Using tissue microarray for a substantial number of surgical patients, we found that high expression of PD-L1, IDO, and FOXP3 Tregs was associated with high grade histology but not survival in thymoma. In patients with surgically treated thymic carcinoma, survival benefit was noted in patients with high expression of IDO and low expression of FOXP3 Tregs in addition to complete resection.

PD-L1 is constitutively expressed on T and B cells, macrophages, and dendritic cells, and is upregulated in many human cancer cells [33]. Although inhibitors of PD-L1 in the form of monoclonal antibodies have been shown to promote significant and durable responses in cancer patients [34], their prognostic value in different tumor types is still undetermined and limited data for thymic epithelial neoplasms have shown conflicting results [31,32,35,36]. In 2015, Padda [32] et al evaluated PD-L1 expression in 69 cases using a tissue microarray, and more intense PD-L1 staining was associated with higher grade WHO histology, higher stage and worse clinical outcome. Katsuya [31] et al investigated PD-L1 expression in 101 thymomas and 38 thymic carcinomas in a tissue microarray, and 70% of thymic carcinomas and 23% of thymoma samples showed positive staining for PD-L1. It was concluded that although thymic carcinoma was significantly associated with PD-L1 expression, other variables including age at diagnosis, sex,

Table 4
Univariate and multivariate analysis for overall survival and progression-free survival of 69 surgically treated thymic carcinoma.

Variable	Overall survival HR (95% CI)	P value	Progression-free survival HR (95% CI)	P value
Univariate analysis				
Sex Male vs. Female	0.72 (0.38–1.35)	0.300	0.79 (0.43–1.44)	0.439
Age > 60 vs. ≤60	2.10 (1.04–4.21)	0.038	1.35 (0.70–2.59)	0.369
Masaoka stage IVA + IVB vs. III vs. I + II	2.47 (1.35–4.53)	0.003	3.05 (1.67–5.55)	< 0.001
Histology Non-SqCC vs. SqCC[a]	1.37 (0.71–2.63)	0.346	1.26 (0.67–2.35)	0.469
Radicality R1 + R2 vs. R0	5.03 (2.54–9.98)	< 0.001	5.47 (2.78–10.79)	< 0.001
PD-L1 High expression vs. Low expression	1.41 (0.75–2.65)	0.288	1.48 (0.81–2.69)	0.203
IDO High expression vs. Low expression	2.12 (0.97–4.61)	0.059	1.85 (0.86–3.99)	0.119
FOXP3 High expression vs. Low expression	0.32 (0.14–0.77)	0.011	0.34 (0.15–0.77)	0.009
Multivariate analysis				
Age > 60 vs. ≤60	2.01 (0.97–4.15)	0.060	1.08 (0.54–2.19)	0.825
Masaoka stage IVA + IVB vs. III vs. I + II	1.18 (0.65–2.15)	0.595	1.60 (0.89–2.88)	0.114
Radicality R1 + R2 vs. R0	4.29 (1.88–9.79)	0.001	4.28 (2.00–9.12)	< 0.001
PD-L1 High expression vs. Low expression	1.01 (0.52-1.96)	0.974	1.22 (0.66–2.25)	0.525
IDO High expression vs. Low expression	3.45 (1.47–8.10)	0.004	2.57 (1.12–5.87)	0.026
FOXP3 High expression vs. Low expression	0.37 (0.15–0.92)	0.032	0.43 (0.19–1.01)	0.047

[a] SqCC, squamous cell carcinoma.

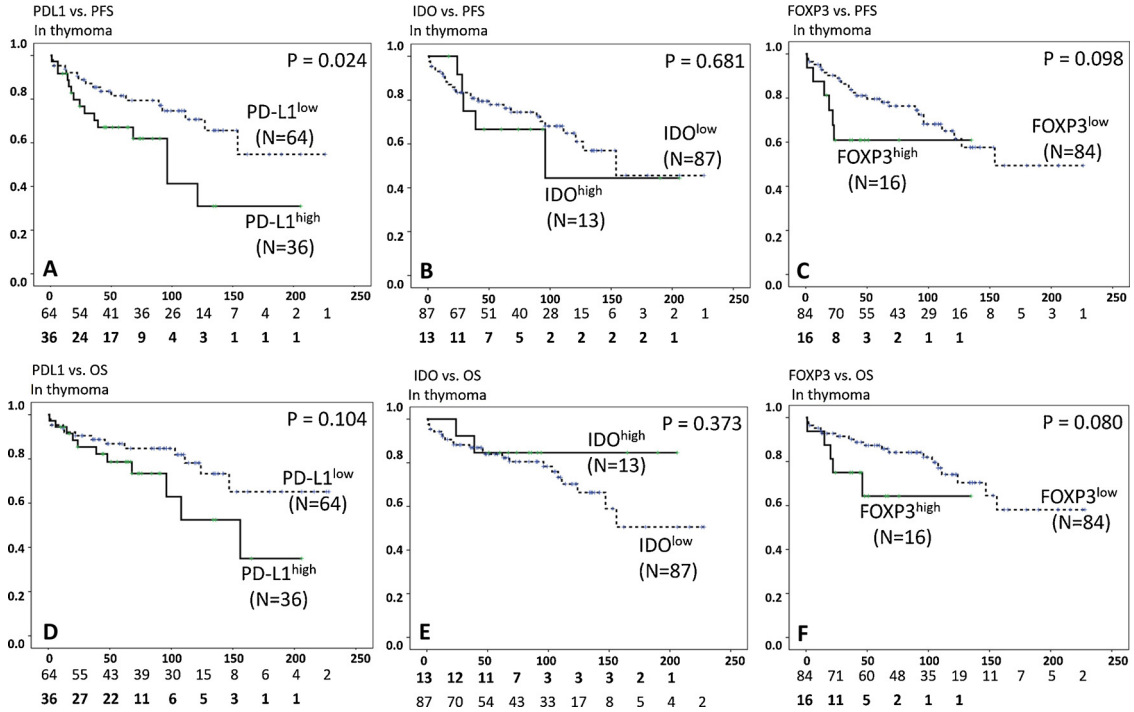

Fig. 1. The progression-free (A, B, C) and overall (D, E, F) survival curve of patients with surgically treated thymoma according to the expression of PD-L1 (A, D), IDO (B, E), and Foxp3 Tregs (C, F). High expression of PD-L1 was significantly associated with worse progression-free survival.

tumor stage, tumor size, neoadjuvant therapy, and overall survival did not show any association with PD-L1 expression status. In 2016 Yokoyama et al separately investigated thymoma [35] and thymic carcinoma [36]. While PD-L1 expression in thymoma was associated with high tumor stage, high grade WHO histology, worse disease-free survival, and increased rate of recurrence but not with overall survival, high PD-L1 expression was associated with a higher disease-free and overall survival in thymic carcinoma.

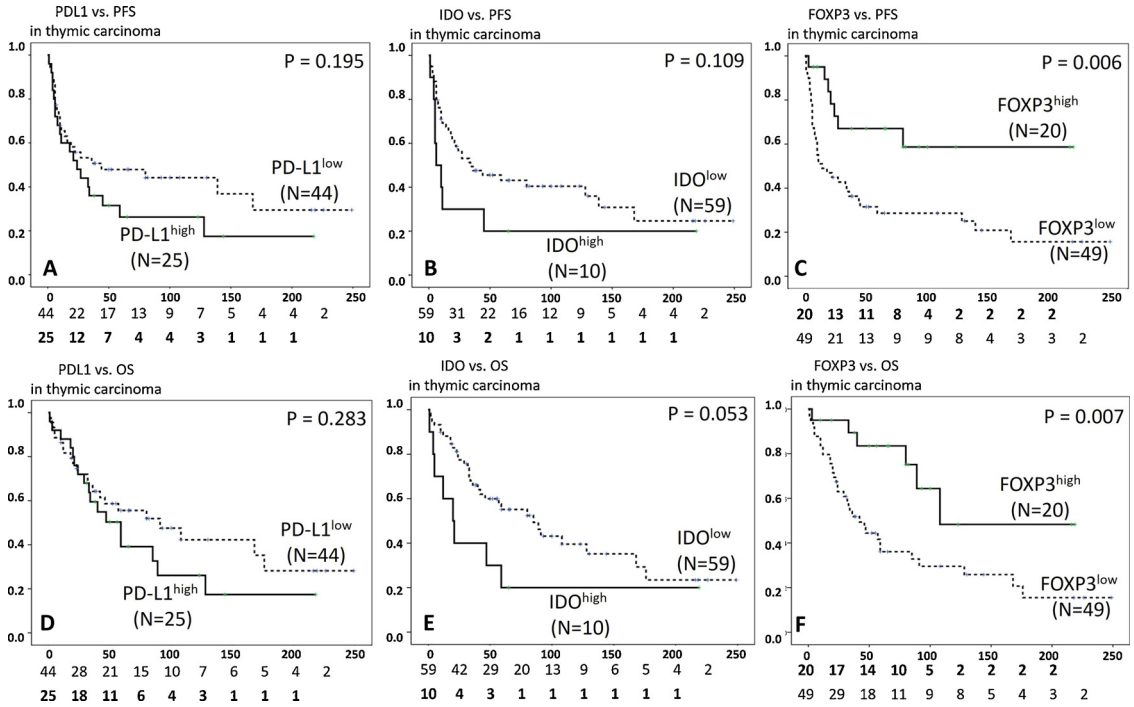

Fig. 2. The progression-free (A, B, C) and overall (D, E, F) survival curve of patients with surgically treated thymic carcinoma according to the expression of PD-L1 (A, D), IDO (B, E), and Foxp3 Tregs (C, F). High expression of Foxp3 Tregs was significantly associated with better progression-free and overall survival.

In a recently published trial, Giaccone et al found that patients with higher PD-L1 expression in recurrent thymic carcinoma has better response to pembrolizumab than those with lower PD-L1 expression in post-hoc analysis (mPFS; 24 vs 2.9 months and mOS ; not reached vs 15.5 months) [37]. However, six (15%) from all 40 patients suffered from multiple grade 3–4 immune-relates adverse effects (irAEs), which is higher than immunotherapy used for other cancer (6.10%) [38]. Other phase II trial also showed five of seven thymoma patients and four of 26 thymic cancer patients developed grade 3–4 irAE after receiving pembrolizumab treatment [39]. Our study showed that around 36% of thymic carcinoma were identified as PD-L1 high expression, which is compatible to the keynote 024 study (30.2%) in lung cancer [40]. It is a general belief that immunotherapy might be a new therapeutic strategy for thymic carcinoma since there is relative paucity of effective treatment and one third of these patients benefitted from immunotherapy in published clinical trial. Furthermore, methods to identify the risk factor of autoimmune toxicity are urgently needed to minimize the harmful effect. It is therefore critical to investigate the immunologic mechanism and then to evaluate the benefit or risk of immunotherapy in thymoma and thymic carcinoma.

With a substantial number of patients, the impact of PD-L1 expression on surgically treated thymoma could be influenced by its slow-growing nature. Because of the slow-growing nature of thymoma, debulking surgery for unresectable thymoma was reported to provide survival benefit [41]. Therefore, although the expression of PD-L1 as well as IDO and FOXP3 Tregs in thymoma was associated with high grade tumor histology, the impact on survival could be offset by the slow-growing nature and effective surgery in our study. Unlike thymoma, the heterogeneity of tumor histology in thymic carcinoma did not pose survival impact. Complete resection provided the opportunity of long-term survival [42,43], while the survival benefit in patients undergoing incomplete resection or debulking surgery was controversial [2,44]. In fact, patients with recurrent or residual thymic carcinoma have a rapidly deteriorating clinical course, implying the derangement of systemic immunity. Our study demonstrated that in surgically treated thymic carcinoma, high expression of IDO was associated with inferior overall and progression-free survival, while high expression of FOXP3 Tregs was associated with superior overall and progression-free survival. Indoleamine 2,3-dioxygenase (IDO) is responsible for the first enzymatic step of tryptophan catabolism and generated immune tolerance to foreign antigens in tissue microenvironment [12]. Studies have confirmed that IDO played an immunosuppressive role in carcinogenesis and served as a biomarker for poor prognosis in certain tumors [13–16]. FOXP3 is an immunosuppressive transcriptional factor of CD4 +CD25+Tregs. Although tumor-infiltrating FOXP3 T cells have been regarded as having an adverse impact in patients with certain hematologic or solid malignancies because of the immunologic escape or evasion along with (IDO) [18–22], increased expression of FOXP3 Tregs has also been reported to associate with improved survival or treatment outcome [23–26]. It has been found that at sites of inflammation or malignancy, FOXP3 Tregs could reprogram into helper-like cells without loss of the transcription factor FOXP3 [45–47]. These reprogrammed Treg cells were important in antigen-presenting in naive hosts [48]. It has also been reported that FOXP3 T cells could be strongly associated with CD8+ tumor-infiltrating lymphocytes and good outcome [49–52]. Compared with the rapidly deteriorating clinical course of patients with recurrent or residual thymic carcinoma, high expression of FOXP3 Tregs might suggest that the anti-tumor immunity in the host was relatively preserved, the immunity at sites of malignancy was elicited or mediated by a subset of reprogrammed FOXP3 Tregs, and hence the survival was prolonged.

There are some limitations in this study because of its retrospective nature. Different treatment modality before surgery might have changed the expression of PD-L1, IDO, and FOXP3 Tregs. Further biologic elucidation of the role of PD-L1, including in vivo experiments, should contribute to the development of better antitumor therapeutic strategies. It was reported that PD-L1 expression was observed in thymic epithelial cells by IHC staining in the normal fetal thymus and nonneoplastic thymic tissue [32]. Ordinary thymic cells may express PD-L1, IDO, and FOXP3 Tregs in a constitutive manner, and their expression does not correlate with protein expression. The advantage of using tissue microarray is reduce the influence of various staining conditions, especially the concentration and the reaction time of

reagents in each IHC study. The consistency of staining procedure makes the IHC result more objective and reliable in a comparative study. Indeed, IDO expression and FOXP3 Treg may be present in normal thymic tissue, and our study focused on the comparative expression in the tumor tissue. The IHC studies of IDO and FOXP3 Tregs were also utilized in the literature [13–16,18,19,22–26]. Nonetheless, additional evaluation of PD-L1 in both the normal thymic epithelium and thymoma are expected to contribute to understanding the causes of PD-L1 expression. Further studies comparing tissue microarray and small samples from biopsies, especially in unresectable cases, are needed.

In conclusion, high PD-L1 expression was associated with advanced Masaoka staging, and high expression of PD-L1, IDO, and FOXP3 Tregs was associated with high-grade histology but not survival in surgically treated thymoma. In surgically treated thymic carcinoma, significant survival benefit was noted in patients with complete resection, low expression of IDO, and high expression of FOXP3 Tregs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2018.09.002.

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