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Original Article

Evaluation of PD-L1 antigen expression using immunohistochemistry technique in medullary thyroid carcinoma samples

Behnaz Karkheiran[1], Behnaz Jahanbin[2], Golnoosh Shahverdi[3], Vahid Soleimani[2]

[1]Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran; [2]Cancer Institute, Assistant Professor of Pathology, Tehran University of Medical Sciences, Tehran, Iran; [3]School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

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Abstract: Background: Markers related to the mechanism of tumoral cell escape from the immune system have received more attention. The PD-L1 molecule encoded by the "CD274" gene binds to T lymphocytes and can inhibit these cells. Therefore, increasing the expression of this marker on inflammatory or tumor cells can indicate tumor progression invasiveness and long-term consequences. The present study aimed to determine the expression of the PD-L1 marker in thyroid medullary tumors and to evaluate its role in predicting long-term outcomes after cancer. Methods: This retrospective longitudinal study was performed on pathology samples of patients with medullary thyroid carcinoma referred to the Cancer Institute of Imam Khomeini Hospital from 2015 to 2020. Slides related to medullary thyroid tumors were examined. A tissue microarray was used to evaluate the immunohistochemistry of PD-L1. Patients were followed up to assess the occurrence of recurrence. Out of 207 patients evaluated in the present study, histopathological information of 144 patients was available. Results: The expression rate of PD-L1 in our community was 14.6% in lymphocyte cells, 35.4% in tumor cells, and 12.5% in both cells. The presence of metastasis at the time of diagnosis was reported in 35 cases (72.9%), and the occurrence of tumor recurrence was reported in 38 cases (79.2%). There was no relationship between the expression of this marker and the sex and age of patients. In addition, PD-L1 expression was unrelated to the two main characteristics of this cancer, namely tumor size and its focality. The presentation of tumor PD (L1) (but not lymphocytic) was a prognostic marker for synchronous metastasis at cancer diagnosis but could not predict tumor recurrence. Conclusion: PD-L1 tumor marker expression is predictable in 14.6% of lymphocyte cells, 35.4% of tumor cells, and 12.5% in the selected Iranian population with medullary thyroid cancer. The expression of this marker is not related to the morphological characteristics of the tumor, such as tumor size or focality.

Keywords: Immunohistochemistry, antigen expression, medullary thyroid carcinoma

Introduction

Cancer is one of the leading causes of death and thyroid cancer is the most common endocrine malignancy [1]. This malignancy is 40 cases per million people, equivalent to 1% of all cancers [1, 2]. Typically, thyroid cancer doesn't trigger any signs or symptoms in its early stages. Clinical presentations of thyroid cancer could mimic hyperthyroidism. A nodule could also be detected in the thyroid gland in patients [3, 4]. A definite diagnosis of thyroid cancer is made via fine needle aspiration (FNA) or core biopsy and pathologic studies [4]. Relative or actual contraindications for thyroidectomy include a history of head & neck surgery, history of head, neck, or upper mediastinal irradiation, inability to tolerate general anesthesia, evidence of clinical hyperthyroidism, preoperative recurrent laryngeal nerve palsy, lymph node metastasis, extrathyroidal extension [5].

Thyroid cancer includes different types based on pathological manifestations such as differentiated thyroid carcinoma (DTC), poorly DTC thyroid carcinomas, anaplastic thyroid carcinomas (ATC) and medullary thyroid carcinoma (MTC) [6, 7]. In the MTC type, carcinoma origi-

nates mainly from parafollicular cells, while other types of cancer arise from follicular thyroid epithelial cells. Medullary thyroid carcinoma (MTC) is a rare malignancy of parafollicular cell origin that is different from other types of thyroid carcinomas [8, 9]. Surgical resection is the only treatment for most patients with MTC [10].

Among thyroid cancers, the prevalence of MTC is estimated at 1 to 2% [8]. Routine treatment for thyroid cancer includes hormone inhibitor therapy, surgery, and radiotherapy. 10-year survival of patients is estimated at 19% [11, 12]. Determining a method for predicting disease prognosis has always been emphasized. Currently, some molecular markers such as programmed death-ligand 1 or PD-L1 are used as prognostic markers in some cancers such as melanoma, small cell lung carcinoma, prostate cancer, breast carcinoma, and other carcinomas evaluated [13-15]. This molecule is widely expressed in various cells such as dendritic cells, B and T lymphocytes, macrophages, vascular endothelial cells, islet cells, and tumor cell types [16, 17].

The number one cell death programmed receptor, or PD-1, is actually as immunologically essential as the PD-L1 receptor in a variety of immune cells such as monocytes, CD4+ T cells, CD8+ T cells, B cells, NKC cells, cells Dendritic and antigen-expressing cells are abundant [18]. Some studies have also shown that PD-L1 expression is present in various malignant tumors. This process is also related to the pathophysiological status of the tumor and its prognosis [19]. In this regard, even monoclonal anti-PD-1/PD-L1 antibodies have been used to treat a variety of cancers, such as melanoma, lung carcinoma, and urethral cancer [20, 21].

Studies have shown that the PD-1/PD-L1 molecular pathway has adverse effects on the tumor immune response, allowing tumor cells to escape from the immune system, and resulting in an unfavorable prognosis. However, the relationship between PD-L1 expression and thyroid cancer prognosis remains unclear. Some studies have concluded that PD-L1 expression has been significantly associated with even extra-thyroid proliferation and concomitant thyroiditis [22]. Of course, studies show the relationship between PD-L1 expression and patient survival only in some tumor subtypes such as DTC and DPTC. Still, few studies have been performed on the relationship between PD-L1 expression and MTC [23].

Due to these results and the need for further studies to evaluate the prevalence and prognostic value of PD-1/PD-L1 expression in the population of medullary thyroid carcinoma, we decided to evaluate the PD-L1 antigen expression using the immunohistochemistry (IHC) technique in medullary thyroid carcinoma samples.

Methods and material

Study design

This retrospective longitudinal study was performed from 2015-2020 in Imam Khomeini hospital, affiliated with the Tehran University of Medical Science. The current study was conducted on all pathologic samples of patients with MTC. The study protocol was approved by the Research Committee of Tehran University of Medical Sciences and the Ethics committee has confirmed it (Ethics code: IR.TUMS.IKHC.REC.1400.288).

Inclusion and exclusion criteria

The inclusion criteria were diagnosis of MTC based on the pathology report, diagnosis by two expert pathologists by microscopic examination, and confirmation by positive IHC report for calcitonin and carcinoembryonic antigen (CEA) in any stage. Tissues obtained from thyroid or other organs involved with MTC, admission in our medical center from 2010 to 2020, and informed consent of the patients to participate in this study. The exclusion criteria were insufficient or unsuitable block for IHC staining and lack of access to patients' histopathological information.

Study population

Out of 207 patients evaluated in the present study, histopathological information of 144 patients was available. In this study, out of 144 cases, 96 (66.7%) were male, and 48 (33.3%) were female. The mean age was 47.44±17.15 years ranging from 17 to 77 years.

Tissue preparations

This study was performed on pathology specimens of patients with MTC. Tissues for MTC were re-examined to determine the stage of the

disease based on the latest version of the CAP Cancer Reporting and Biomarker Reporting Protocols for MTC. Also, a suitable paraffin block was selected for the work. In the next step, a paraffin block with a suitable tumor volume was selected for sections at the IHC. 5m sections were made of paraffin block and prepared for IHC.

IHC was performed for the PD-L1 marker. A pathologist interpreted IHC results. Only membrane staining of any intensity that stained more than 1% of tumor cells was considered a positive expression of PD-L1 in tumor cells. Membrane and cytoplasmic staining were considered positive expressions in tumor infiltration immune cells. Standard tonsil tissue samples were used as a positive control.

Immunohistochemistry staining technique

We first evaluated all available pathologic slides in this study to obtain appropriate formalin-fixed, paraffin-embedded tissue blocks. We thoroughly examined each block, chose the best representative area from each slide, and prepared a 5 mm tissue array sample with punch biopsy for the subsequent IHC study. We followed manufacturers' recommended methods for preparing tissues for IHC. The deparaffinization and rehydration processes were performed using sequential concentrations of xylene and ethanol. Then, we washed samples using PBS buffer and performed the antigen retrieval process using the heat-mediated epitope retrieval (HIER) approach with Tris-EDTA buffer at pH 9. We used hydrogen peroxide 0.3% as the blocking solution and administered PD-L1 (Master-Diagnostica) on samples, followed by overnight incubation at 4°C. We administered anti-rabbit IgG was used as the secondary antibody and stained samples (Master-Diagnostica). A pathologist interpreted IHC results. Only membrane staining of any intensity that stained more than 1% of tumor cells was considered a positive expression of PD-L1 in tumor cells. Membrane and cytoplasmic staining were considered positive expressions in tumor infiltration immune cells. Standard tonsil tissue samples were used as a positive control.

PD-L1 antibodies

Rabbit anti-human PD-L1 monoclonal antibodies were used to assess PD-L1 expression in tumor cells and tumor-infiltrated immune cells. PD-L1 expression was found in tumor-infiltrated immune cells by rabbit monoclonal antibodies. Tumor samples fixed in paraffin were removed from the paraffin and released with ethanol. Heat-induced antigen recovery was achieved by boiling at 100°C for 30 min. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 5 min. Tissue sections were then incubated with antibodies against PD-1 and PD-L1 at 37°C for 16 min, followed by incubation with secondary antibodies to Horsadides peroxidase conjugate. An immune reaction was observed by diaminobenzidine. Tissue sections were counterstained by hematoxylin.

Statistical analysis

The data were entered into the Statistical Package for Social Sciences (SPSS) (version 24, SPSS Inc., Chicago, IL). Classified data were analyzed based on chi-square analysis, and their information was written in abundance and with related percentages. First, the normality of the data distribution was assessed using the Kolmogorov-Smirnov test. Score variables were measured with a t-test if they followed a normal distribution and the Mann-Whitney test was used if they did not have a normal distribution. Pearson or Spearman correlation test was used to examine the correlation between the data. Qualitative variables were compared using the Chi-square test or Fisher's exact test. *P*-value <0.05 was considered as a significance threshold.

Results

Tumor samples

The tumor size was initially 3.74±1.58 cm in terms of tumor characteristics. A total of 108 cases (75%) were unifocal masses, and 36 patients (25.0%) were multifocal. According to the sampling location, in 60 cases (41.7%), the site was the thyroid gland; in 72 patients (50.0%) lymph node; and in 12 cases (8.3%), the location was the liver. The tumor recurrence was reported in 114 cases (79.2%). Forty-two patients (29.1%) died four years after diagnosis. Patient survival was estimated at 70.9% within two years of diagnosis. These data are shown in **Table 1**.

Table 1. Background characteristics of patients

Variable		Frequency/mean	Percent/SD
Gender	Male	96	66.7%
	Female	48	33.3%
Age (years)		47.44	17.15
Tumor size (cm)		3.74	1.58
Tumor size more than 5 cm		30	20.8%
Focality	Unifocal	108	75%
	Multifocal	36	25%
Sampling site	Thyroid	60	41.7%
	Lymph node	72	50%
	Liver	12	8.3%
Metastasis		105	72.9%
Recurrence		114	79.2%

Table 2. Relationships between PDL-1 and patient's characteristics

Variable		PD-L1 in lymphocytes	*P*-value	PD-L1 in tumor cells	*P*-value
Gender	Male	12.5%	0.672	34.4%	0.831
	Female	18.8%		37.5%	
Age	≤60 years	11.8%	0.400	35.3%	0.978
	<60 years	21.4%		35.7%	
Tumor size	≤5 cm	13.2%	0.625	39.5%	0.252
	<5 cm	20%		20%	
Focality	Unifocal	13.9%	0.813	33.3%	0.731
	Multifocal	16.7%		41.7%	

PD-L1 expression in lymphocyte cells and tumor features

Based on immunohistochemical assessments, PD-L1 expression in lymphocyte cells was reported in 21 cases (14.6%). In between, the staining intensity was weak in nine cases and moderate in 12 cases. Accordingly, the frequency of PD-L1 expression in men and women was 12.5% and 18.8%, respectively, with no significant difference between the sexes (P=0.672). The mean age in the two groups with and without PD-L1 lymphocyte expression was 52.86±17.09 years and 46.51±17.20 years, respectively, indicating no relationship between PD-L1 expression and patients' age (P=0.173). PD-L1 expression in the two groups of unifocal and multifocal tumors was 13.9% and 16.7%, respectively, which was not different between the two groups of tumors (P=0.813). The mean tumor size in the two groups with and without PD-L1 lymphocyte expression was 4.34±0.90 cm and 3.63±1.66 cm, respectively, indicating a lack of relationship between PD-L1 expression and tumor size (P=0.278). Therefore, the expression of the PD-L1 marker in lymphocyte cells was not related to patient's demographic characteristics or tumor characteristics such as size, focality (**Table 2**). **Figure 1** shows thyroid tissue with tumor infiltrating lymphocytes. This figure indicates expression of PD-L1 in lymphocytes between the tumor cells and cytoplasmic and membranous expression of PD-L1 in lymphocytes between the tumor cells.

PD-L1 expression in tumor cells and tumor features

Based on immunohistochemical evaluations, PD-L1 expression was reported in 51 cases (35.4%) of tumor cells, among which the intensity of staining was weak in 24 cases and moderate in 27 cases. Based on the above evaluation, simultaneous expression of PD-L1 in lymphocyte and tumor cells was also reported in 18 cases (12.5%). The frequency of tumor PD-L1 expression in men and women was 34.4% and 37.5%, respectively, with no significant difference between the sexes (P=0.831). The mean age in the two groups with and without PD-L1 tumor expression was 48.71±14.64 years and 46.74±18.58 years, respectively, indicating a lack of relationship between PD-L1 expression and patients' age (P=0.709). PD-L1 expression in the two groups of unifocal and multifocal tumors was 33.3% and 41.7%, respectively, which was not different between the two groups (P=0.731). The mean tumor size in the two groups with and without PD-L1 tumor expression was 3.83±0.82 cm and 3.68±1.89 cm, respectively, indicating no relationship between PD-L1 expression and tumor size (P=0.765). The frequency of PD-L1 expression in the lymph node, thyroid, and liver samples was 25%, 33.3%, and 40%, respectively, which did not show a significant difference (P=0.811).

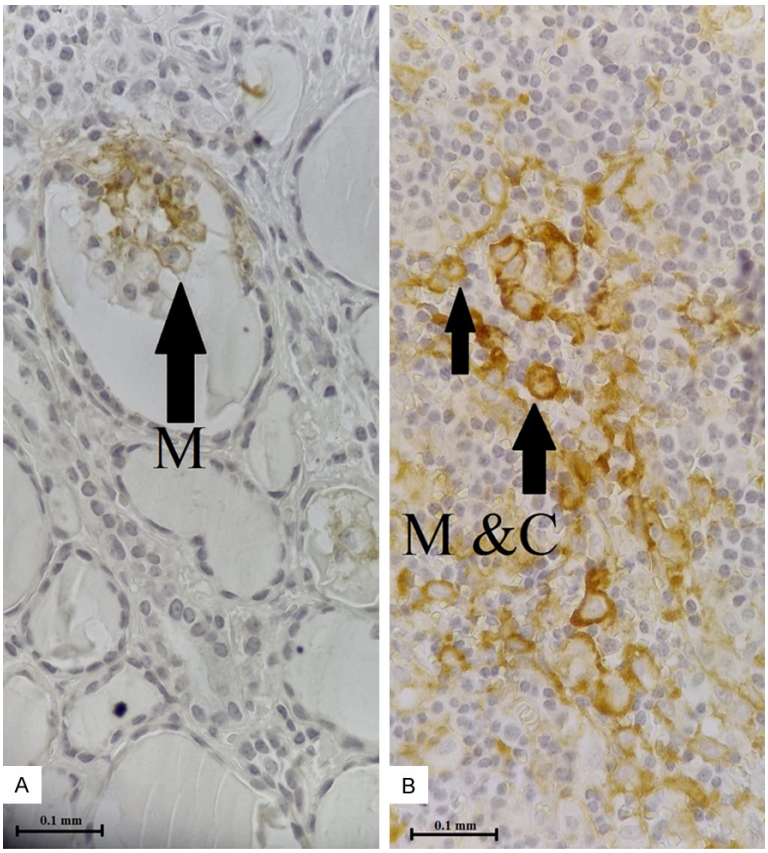

Figure 1. Thyroid tissue with tumor infiltrating lymphocytes (TIL). A: Membranous expression of PD-L1 in lymphocytes between the tumor cells (M: membranous staining). B: Cytoplasmic and membranous expression of PD-L1 in lymphocytes between the tumor cells (M&C: membranous and cytoplasmic staining) (High power ×400).

Therefore, the expression of the PD-L1 marker in tumor cells was unrelated to the patient's demographic characteristics or tumor characteristics such as size and focality (**Table 2**). **Figure 2** shows membranous expression of PD-L1 in tumor cells of MTC. B: Membranous expression of PD-L1 in tumor cells of MTC.

PD-L1 expression in lymphocyte cells and tumor outcome

The frequency of metastasis in cases with and without PD-L1 expression in lymphocyte cells was estimated to be 100% and 68.3%, respectively, which was not statistically significant (P=0.166). Also, the frequency of tumor recurrence in cases with and without PD-L1 expression in lymphocyte cells was 85.7% and 78.0%, respectively, which was not statistically significant (P=0.644) (**Table 3**). According to the Cox Proportional Hazard Model, PD-L1 in lymphocyte cells and other underlying and tumor features did not predict tumor recurrence in patients (HR=0.335, 95% CI: 0.046-2.448, P=0.281).

PD-L1 expression in tumor cells and tumor outcome

The frequency of metastasis at the time of diagnosis in cases with and without PD-L1 expression in tumor cells was estimated to be 94.1% and 61.3%, respectively, which was significantly higher in patients with metastasis (P=0.018). The frequency of tumor recurrence in cases with and without PD-L1 expression in tumor cells was 82.4% and 77.4%, respectively, which was not statistically significant (P=0.687) (**Table 3**). According to Cox Proportional Hazard Model, PD-L1 expression in tumor cells and other underlying tumor characteristics did not predict tumor recurrence in patients (HR=0.831, 95% CI: 0.158-4.366, P=0.827).

Discussion

Despite significant advances in treating patients with advanced thyroid cancer, the initial diagnosis of the disease and the prediction of its adverse consequences, especially the occurrence of metastasis and tumor recurrence, is still a significant challenge. In this regard, the use of some molecular and genomic markers in predicting the survival of these patients has been considered. In particular, markers related to the escape mechanism of this cancer from anti-inflammatory processes have received more attention. The PD-L1 molecule encoded by the "CD274" gene binds to T lymphocytes and can inhibit these cells and therefore increasing the expression of this marker on inflammatory or tumor cells can be an indicator of tumor progression, invasiveness and long-term consequences. However, studies have yielded conflicting results regarding how well this marker predicts tumor characteristics and extent [24-26].

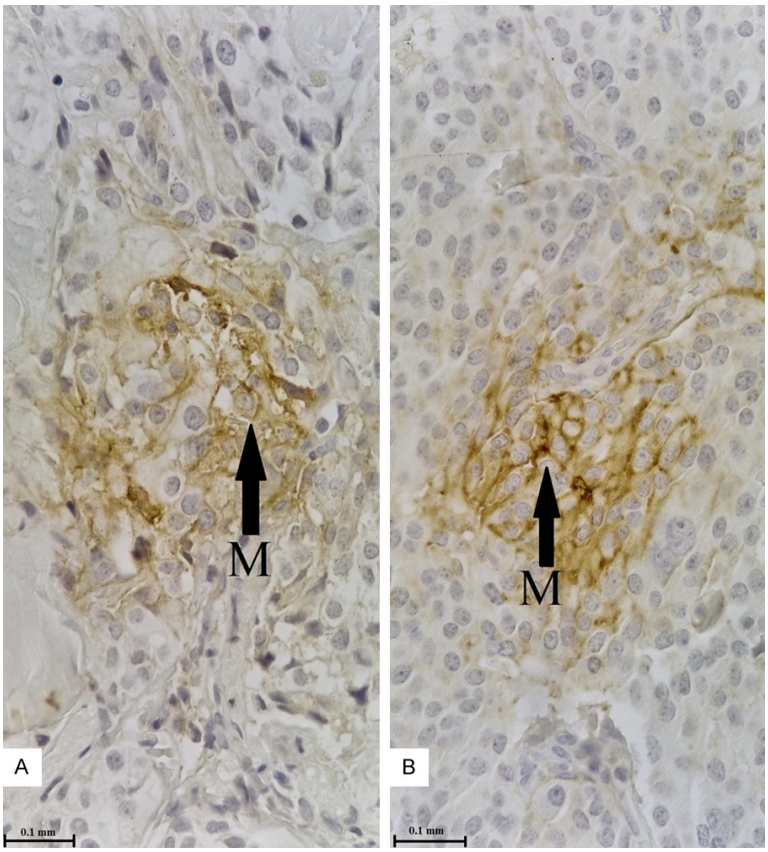

Figure 2. Medullary tumor cells. A and B: Membranous expression of PD-L1 in tumor cells of MTC (M: membranous staining) (High power ×400).

Table 3. Relationships between PDL-1 and tumor outcome

Variable		PD-L1 in lymphocytes	*P*-value	PD-L1 in tumor cells	*P*-value
Metastasis	Positive	100%	0.166	94.1%	0.018
	Negative	68.3%		61.3%	
Recurrence	Positive	85.7%	0.644	82.4%	0.687
	Negative	78%		77.4%	

We conducted the present study to determine the expression of PD-L1 marker in thyroid medullary tumors and to evaluate its role in predicting long-term outcomes after cancer. In the first place, we achieved that the expression rate of the relevant molecule in patients with thyroid medullary tumors in our community was 14.6% in lymphocyte cells, 35.4% in tumor cells, and 12.5% in tumor cells in both. It was a group of cells. This rate of expression is very different in different studies. Therefore, in the first conclusion, the expression of this tumor marker can be different in other communities, which will be descriptive in achieving differences in its prognostic role.

In Shi and c colleagues' study, cases of PD-1 expression were detected in 13.5% of cancer patients, which was lower than in our study [27]. In another study by Shi and others, PD-L1 expression was reported in 14.4% of patients [28]. In a study by Yalan Bi and others, PD-L1 was positive in 19 of 87 patients (21.8%), which was again lower than our reported rate [29]. In a study by Soomin Ahn and colleagues in Seoul, South Korea, PD-L1 was expressed in 6.1% of papillary tumors, 7.6% of follicular carcinomas, and 22.2% of anaplastic carcinomas. Also, the PD-L1 tumor cell infiltration rate was 28.5% in papillary carcinoma, 9.1% in follicular carcinoma and 11.1% in anaplastic carcinoma [30]. Studies show that PD-L1 expression will have a wide range among different types of thyroid cancers [31]. In this regard, the frequency of PD-L1 positivity in various studies varies from 6.1% to 82.5% in patients with papillary thyroid cancer and from 22.2% to 8.2% in patients with anaplastic thyroid carcinoma [32].

In the study's second phase, we investigated the relationship between PD-L1 expression and tumor features. First, we showed no connection between the expression of this marker and the sex and age of patients, and almost all similar studies had achieved the same result. But we also showed that the expression of PD-L1 is unrelated to the two main characteristics of this cancer, namely tumor size and its focality. However, the results of studies in this regard have been completely contradictory. In the study of Wan and colleagues, PD-L1 expression was associated with tumor size greater than 2 cm and focal ion tumor [33], which is not consistent with our study. In the study by Shi and colleagues, PD-L1 expression was primarily associated with larger

tumor size, lymph node metastasis, and higher tumor stage [28].

In 2017, Soomin Ahn and colleagues in Seoul, South Korea, observed no significant differences between pathological and clinical findings, course of the disease and oncological mutations, and PD-L1 expression [30]. Therefore, the claim that the evaluation of the expression of this marker is representative of the morphological features of the tumor was not confirmed in our study. As a third finding, in our study, the expression of tumor PD (L1) (but not lymphocytic) was a prognostic marker regarding metastasis at the time of cancer diagnosis. Still, it could not predict tumor recurrence; again, the results were different in different studies. In a study by Wan and others in a systematic review of 13 articles, an increase in PD-L1 expression was associated with a 3.37-fold reduction in optimal disease outcome and a 2.5-fold decrease in survival. PD-L1 expression was also associated with tumor recurrence [33]. In the study by Shi and others, increased PD-1/PD-L1 expression was associated with decreased disease-free survival [27]. In the study by Shi and others, PD-L1 expression was inversely related to long-term survival of patients. 40% of patients with the long-term cancer recurrence expressed PD-L1 [28]. In the study of Yalan Bi and colleagues, the rate of PD-L1 positive results showed a statistically significant relationship with distant metastasis during surgery [34].

Therefore, evaluation of PD-L1 expression in thyroid tumor cells can provide a reliable prediction of the occurrence of cancer metastasis and consequent long-term survival of the disease. However, the results of our study were affected by some potential limitations, including the limited sample size of patients. One of the limitations of this study was that we evaluated only one group of patients with thyroid cancer, and our results were not compared to a control group. It is recommended that further studies compare these data with other groups.

Conclusion

PD-L1 tumor marker expression is predictable in 14.6% of lymphocyte cells, 35.4% of tumor cells, and 12.5% in the selected Iranian population with medullary thyroid cancer. The expression of this marker is not related to the morphological characteristics of the tumor, such as tumor size or focality. Although a significant association was found between PD-L1 marker expression and the simultaneous occurrence of metastasis at the time of diagnosis in patients, it may not have predictive value for predicting cancer recurrence.

Disclosure of conflict of interest

None.

Address correspondence to: Vahid Soleimani, School of Medicine, Tehran University of Medical Sciences, No. 8, Daneshgah Blvd, Tehran, Iran. Tel: +989132688726; Fax: +983137265207; E-mail: vsoleimani@sina.tums.ac.ir

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