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PD-L1 and PD-1 expression in thyroid follicular epithelial dysplasia: Hashimoto thyroiditis related atypia and potential papillary carcinoma precursor

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Programmed cell death ligand (PD-L1)/PD-1 expression has been studied in a variety of cancers and blockage of PD-L1/PD-1 pathway is a cornerstone of immunotherapy. We studied PD-L1/PD-1 immunohistochemical expression in 47 thyroid gland specimens in groups of (1) Hashimoto thyroiditis (HT) only; (2) HT and follicular epithelial dysplasia (FED); and (3) HT, FED, and papillary thyroid carcinoma (PTC). PD-1 positivity was found in immune cells, namely in lymphocytes, macrophages, and plasma cells with mean values for lymphocytes and macrophages 9% in HT group, 4% in FED group, and 4% in PTC group. PD-L1 positivity was identified in both immune cells and in the normal epithelial cells. In the HT group, mean PD-L1 staining on immune cells was 6%, in FED group 5%, and in PTC group 7%. The mean PD-L1 staining on the epithelial cells in the inflammatory parenchyma was 11.7% in HT, 13.4% in FED, and 8.3% in PTC group. The mean PD-L1 staining of FED foci was 47.2% in FED group and 33.6% in PTC group. The mean tumor proportion score (TPS) was 10.4%, and the mean combined positive score (CPS) was 15.5. At the moment, PTC is not a target of immunotherapy. However, understanding the complex issue of concurrent inflammation and autoimmunity can importantly influence the cancer treatment in future.

Key words: PD-1; PD-L1; Hashimoto thyroiditis; follicular epithelial dysplasia; thyroid gland; papillary thyroid carcinoma.

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The intimate relation of immune system and the cancerogenesis has been recently extensively studied with several milestone discoveries. Cancer cells are recognized and eliminated by lymphocytes in the process of immune surveillance, but ultimately cancer cells escape immune control by lowering immunogenicity and resistance to immune effector cells and expand in a consequence [1, 2].

The father of modern pathology Rudolf Virchow already in 1863 proposed a link between inflammation and cancer when observed the presence of inflammatory cells in neoplasms [3–5]. In 1909, Ehrlich suggested the idea on continuous cancer cell mutation with immune system scan and eradication [6].

In thyroid gland, the association of autoimmune lymphocytic thyroiditis generally known as Hashimoto thyroiditis (HT) with papillary thyroid carcinoma (PTC) was reported with the frequency up to

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38% both in epidemiological and pathological studies [3, 7]. Despite the proposed link between HT and PTC, the immunologic link and mechanisms behind are under discussion and research [8].

HT-related epithelial changes with variable atypia have been observed in several studies [9, 10], and a term of follicular epithelial dysplasia (FED) was suggested by Chui et al. [11] and later on, endorsed by the others [10]. The FED lesions are groups of atypical epithelial cells within inflammatory infiltrate in the thyroid gland with following cellular characteristics: mild to moderate nuclear enlargement, grooves, membrane irregularities, chromatin margination, and crowding of nuclei with optical clearings. The foci of a diameter < 1.0 cm are irregular with formation of follicles, trabeculae, nests, and solid areas, but lacking papillae, infiltrative growth, and stromal desmoplasia [10, 11].

Recently, FED was suggested as a pathogenetic link between inflammation-related atypia in HT and PTC [10]. In various series, up to 42% of the FED-containing specimens contained concomitant PTC [10, 12]. In addition to the histopathological features, FED shares the immunoprofile with PTC [10–16].

Programmed Death Ligand 1 (PD-L1, CD274) is a co-regulatory molecule expressed on the surface of tumor and immune cells. It specifically bounds to its receptor PD-1 expressed on activated T cells, regulatory T cells (Treg), and other immune cells playing role in switching off their immune activity. PD-1 is a negative regulator, well-known as immune checkpoint, and its ligands include not only PD-L1 ligand, but also PD-L2. PD-L1/PD-1 expression has been studied in a variety of cancers and blockage of PD-L1/PD-1 pathway is a cornerstone of immunotherapy [17, 18].

In systematic reviews and meta-analyses, PD-L1 expression in non-medullary thyroid carcinomas showed significant association with poor survival and recurrence, tumor size, extrathyroidal extension, and unifocality [19, 20]. Nevertheless, the comparison of various studies faced the inconsistencies between used antibodies, staining procedures and platforms, as well as scoring methods and cut-offs [19, 21].

Of interest, several studies have reported the PD-L1/PD-1 expression in thyroiditis-related normal thyroid epithelium [22–26]. Lubin et al. [23] showed PD-L1 expression up to 90% in HT epithelium, but only 1-5% in chronic lymphocytic epithelium. On the other hand, Chowdhury et al. observed PD-L1 positivity both in HT and lymphocytic thyroiditis [24]. On the top of it, HT is an autoimmune entity, where immune system overreacted in contrast to cancerogenesis that is a consequence of lowered immune reaction.

In the present study, we aimed to determine PD-L1/PD-1 immunohistochemical expression in HT and FED in a small series of 1) HT only, 2) HT and FED, and 3) HT, FED, and PTC. PD-L1/PD-1 expression was studied both in inflammatory and epithelial cells.

MATERIALS AND METHODS

Study cohort

Study series consisted of 47 specimens. Median age of patients at the time of surgery was 57 (ranged 20-86) years. Data set included 8 (17.0%) males and 39 (83.0%) females. Surgical removal was either total thyroidectomy (n = 22 (46.8%)), or lobectomy (n = 25 (53.2%)). The specimens were divided into three groups: specimens with Hashimoto thyroiditis (HT) only, specimens with HT and follicular epithelial dysplasia (FED), and specimens with HT, FED, and papillary thyroid carcinoma (PTC). HT variants (IgG4 positive, fibrous) were distributed in all three groups with no dominance in any group ($p = 0.529$ and $p = 0.531$).

The HT group consisted of 17 patients. HT was defined microscopically by the presence of diffuse lymphoplasmacytic inflammation with germinal centers, epithelial damage and atrophy, variable fibrosis, and oncocytic or squamous metaplasia. Median age at the time of surgery was 61 (ranged 20–86) years, including two males (11.8%) and 15 females (88.2%).

The FED group was formed by 16 samples, with median age at the time of surgery being 58 (ranged 23–70) years. The group included three males (18.8%) and 12 females (81.3%). In this group, the patients were diagnosed with both HT and FED. In addition to previously collected data, we collected the size of FED and FED multifocality. FED was defined as previously published [10, 11].

The PTC group consisted of 14 samples. Median age at the time of surgery was 55 (ranged 35–74) years. Data set included three males (21.4%) and 11 females (78.6%). The size, PTC variant, and TNM staging of the tumor were collected additionally to the basic epidemiological data.

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded whole-tissue sections from the 47 cases were stained for PD-L1 and PD-1. Staining was conducted with anti-PDCD1 (SP269, 1:50; Spring Bioscience, Pleasanton, CA, USA) and anti-CD274 (E1L3N, 1:100; Cell Signaling, Technology, Danvers, MA, USA) antibodies using a BOND III stainer (Leica Biosysteme, Buffalo Grove, IL, USA) as previously described [27, 28].

Slides were digitally scanned with NanoZoomer-XR (Hamamatsu Photonics, Hertfordshire, UK) and then analyzed manually at ×20 magnification using NDP.view2 - software (Hamamatsu Photonics, Hertfordshire, UK).

PD-1 positive inflammatory cells (namely lymphocytes, macrophages, and plasma cells) in the thyroid parenchyma

were analyzed based on morphology with 1% interval, when the staining was <5% of immune cells, and with 5% interval when staining was considered >5%. The counted percentages of positive cells were analyzed in two groups, first one only with positive lymphocytes and macrophages, and second one also including plasma cells.

PD-L1 positive inflammatory cells in inflammatory thyroid parenchyma were analyzed with the same percentage interval as PD-1. In PD-L1 analyses, only lymphocytes and macrophages were taken into the account. Membranous and cytoplasmic staining as well as only partial membranous staining were considered positive.

PD-L1 staining of normal epithelial cells in inflamed thyroid parenchyma was analyzed considering only membranous staining of any intensity as positive. The percentage of stained cells was listed with 1% interval, when the staining was <5% of the follicular epithelium and with 5% interval, when staining was considered >5%.

The dysplastic cells in FED and PTC categories were analyzed by counting the PD-L1 positive and negative stained cells, and then, calculating the percentage of staining. The dysplastic lesions were examined with the help of the original hematoxylin eosin-stained slides.

In PTC group, tumor cells were analyzed using combined positive score (CPS) and tumor proportion score (TPS). Ten fields at 20× magnification from each sample were analyzed. In CPS, both PD-L1 stained immune cells (IC) and vital tumor cells (TC) were counted, and then, divided with the number of all vital tumor cells. In TPS, only positively stained vital cancer cells were counted, and then, divided with the number of all vital tumor cells. Membranous staining of any intensity was considered positive.

The study was performed in accordance with the ethical standards of the Helsinki Declaration and approved by the Ethical Committee of the Pirkanmaa Hospital District (R13168, 2.12.2013, extension approval 1.12.2020).

The use of tissue blocks was approved by Valvira. After approval, the consent of individual patients was not requested.

Statistical analyses

All statistical analyses were performed with IBM SPSS Statistics (version 22.0; SPSS IBM, Armonk, NY, USA). The data were analyzed using Fisher's exact test, Mann–Whitney U test and Kruskal–Wallis test. We used Spearman correlation method for counted correlation. p-values <0.05 were considered statistically significant in all statistical analyses.

RESULTS

PD-1 positivity was found in immune cells, namely in lymphocytes, macrophages, and plasma cells (Fig. 1). When analyzed as percentages, the mean values for PD-1 expression were 9% (ranged 2–65%) in HT, 4% (ranged 1–10%) in FED, and 4% (ranged 3–5%) in PTC group. PD-1 staining in inflammatory cells did not differ between the three histological groups ($p = 0.184$). PD-1 expression of lymphocytes correlated with PD-1 expression in macrophages only as well as with PD-1 expression in all inflammatory cells ($rs = 0.571$, $p \leq 0.001$). When PD-1 staining was analyzed among the thyroiditis variants (IgG4 positive, fibrous), statistical difference was not found ($p = 0.885$ and $p = 0.472$, respectively). PD-1 results are summarized in Table 1.

PD-L1 positivity was identified both in immune and epithelial cells (Fig. 2). When PD-L1 expression was analyzed in normal epithelial cells, only membranous staining of any intensity was considered positive. In the HT group, mean PD-L1 staining in immune cells was 6% (ranged 2–15%), in FED group 5% (ranged 3–10%) and in PTC group 7% (ranged 4-15%). PD-L1 staining in inflammatory cells did not differ between the three histological groups ($p = 0.081$) nor between thyroiditis variants (IgG4 positive, fibrous) ($p = 0.621$ and $p = 0.825$, respectively).

The mean PD-L1 expression in the epithelial cells of the inflammatory parenchyma was 11.7% (ranged 0–65%) in HT, 13.4% (ranged 0–55%) in FED and 8.3% (ranged 0–70%) in PTC group with no significant differences between the groups ($p = 0.658$). Furthermore, differences were not found among subgroups of thyroiditis variants (IgG4 positive, fibrous) ($p = 0.174$ and $p = 0.634$, respectively).

The results from PD-L1 immunohistochemical analyses in all three groups are summarized in Table 2.

The statistical tests were repeated with the study cohort divided into two groups. The HT and FED groups were combined into HT + FED group and compared with the PTC group based on the clinical management. The epithelial PD-L1 staining in the normal epithelial cells was the same in both groups ($p = 0.620$). In PD-1 staining, and PD-1 staining including plasma cells, no differences were found ($p = 0.673$ and $p = 0.988$). However, the PD-L1 staining of immune cells was significantly higher in PTC group compared with HT+FED group ($p = 0.030$). Mean staining of immune cells was 7.1% (ranged 4–15%) in the PTC group and 5.4% (ranged 2–15%) in HT+FED group. Thyroiditis variants (IgG4 positive, fibrous) had no effect on any of the results.

The mean PD-L1 staining of dysplastic foci was 47.2% (ranged 0–100%) in FED group and 33.6% (ranged 0–100%) in PTC group. The difference was not statistically significant ($p = 0.377$).

When PTC variants were examined as individual subgroups, the Warthin-like variant showed more PD-L1 staining in immune cells and FED compared with other studied variants, namely follicular,

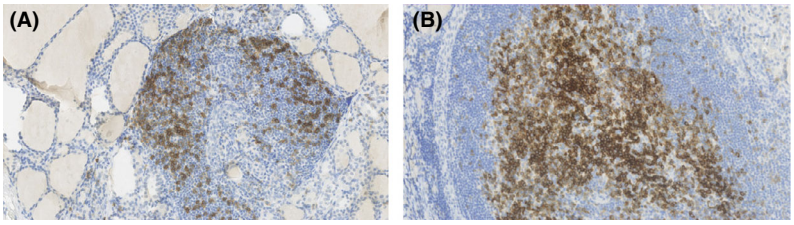

Fig. 1. PD-1 immunohistochemical stainings. (A) Positive PD-1 staining in inflammatory cells, mainly lymphocytes in follicular epithelial dysplasia group with 5% positivity (200x magnification). (B) Positive PD-1 staining in plasma cells in follicular epithelial dysplasia group with 10% positivity (200× magnification)

Table 1. Summary of the PD-1 positivity expressed in percentages in all three study groups

		HT	FED	PTC
PD-1 immune cells	Mean	9.0	4.0	4.4
	Median	5.0	4.0	5.0
	Minimum	2.0	1.0	3.0
	Maximum	65.0	10.0	5.0
PD-1 immune cells including plasma cells	Mean	11.9	8.0	8.1
	Median	10.0	10.0	9.8
	Minimum	4.0	2.0	4.0
	Maximum	55.0	10.0	15.0

classic, and papillary microcarcinoma (Fig. 3). In the Warthin-like variant, the mean PD-L1 staining of immune cells was 12.5% (ranged 5–10%), in papillary microcarcinoma 9.0% (ranged 5–15%), in follicular 6.3% (ranged 4–10%), and in classical variant 5.0% in all cases without a range. Respectively, mean PD-L1 epithelial staining in the studied PTC variants were 36.0% (ranged 2–70%) in Warthin-like variant, 2.6% (ranged 0–5%) in papillary microcarcinoma, 5.0% (ranged 0–10%) in follicular, and 3.3% (ranged 0–10%) in classical variant. Mean FED staining was 90.0% (ranged 80–100%) in Warthin-like, 16.0% (ranged 0–50%) in papillary microcarcinoma, 23.3% (ranged 0-50%) in follicular variant, and 35.0% (ranged 15–75%) in classical variant of PTC.

TPS and CPS scores were calculated in all cases in PTC group. The mean TPS score was 10.4% (ranged 0–43%), and the mean CPS score was 15.5 (ranged 0–49). When analyzing the PTC variants individually, the mean TPS and CPS scores in Warthin-like variant were 17.5% (ranged 5–30%) and 24.0 (ranged 11–37), in papillary microcarcinoma 4.6% (ranged 0–10%) and 8.8 (ranged 0–18), in follicular variant 6.0% (ranged 1–10%) and 9.0 (ranged 2–15), and in classical variant 17.5% (ranged 5–43%) and 24.5 (ranged 8–49), respectively.

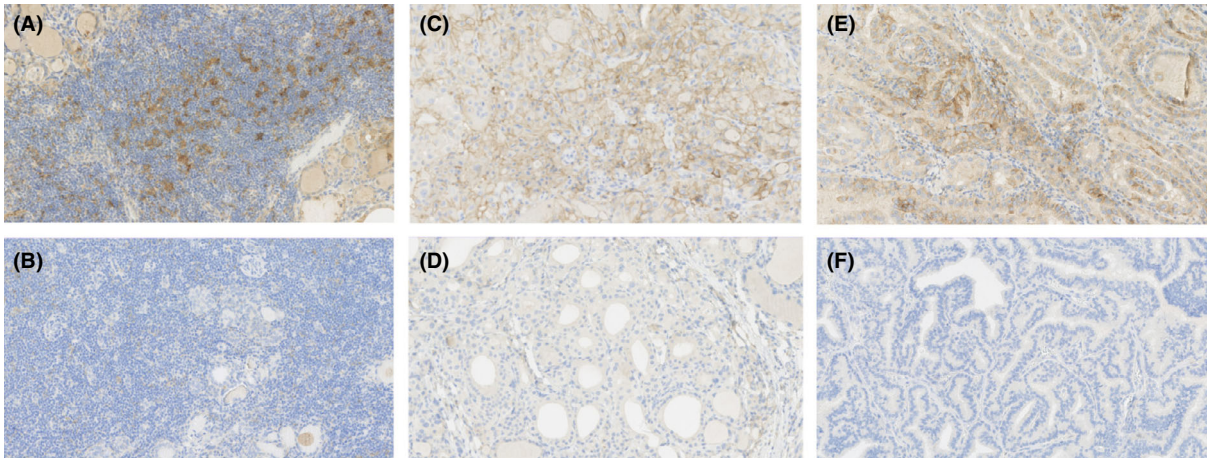

Fig. 2. Immunohistochemical PD-L1 staining in the three histological groups: (A) Positive PD-L1 staining of the inflammatory cells in Hashimoto thyroiditis case with 10% (200× magnification). (B) Negative PD-L1 staining of the inflammatory cells in Hashimoto thyroiditis (200× magnification). (C) Positive PD-L1 staining in follicular epithelial dysplasia case with 50% positivity (200× magnification). (D) Negative PD-L1 staining in follicular epithelial dysplasia case (200× magnification). (E) Positive PD-L1 staining of papillary thyroid carcinoma case with TPS score 43% and CPS score 49 (200× magnification). (F) Negative PD-L1 staining in papillary thyroid carcinoma case (200× magnification)

Table 2. Summary of the PD-L1 positivity expressed in percentages in all three study groups

		HT	FED	PTC
PD-L1 epithelial cells	Mean	11.7	13.4	8.3
	Median	3.0	4.5	4.0
	Minimum	0.0	0.0	0.0
	Maximum	65.0	55.0	70.0
PD-L1 immune cells	Mean	6.0	5.0	7.1
	Median	5.0	5.0	5.4
	Minimum	2.0	3.0	4.0
	Maximum	15.0	10.0	15.0

Data of PTC variants TPS and CPS scores are summarized in Table 3.

DISCUSSION

Expression of immunomodulatory molecules PD-L1/PD-1 is a cornerstone of immunotherapy in a variety of cancers. In the present study, we studied PD-L1/PD-1 immunohistochemical expression in thyroid HT, FED, and PTC with the emphasis on dysplastic epithelium and inflammatory cells. PD-L1 was expressed both in inflammatory cells and epithelial cells in inflamed thyroid gland, FED and PTC with the differences among PTC variants. PD-1 was found in immune cells, namely in lymphocytes, macrophages, and plasma cells.

Interestingly, thyroiditis-related epithelium has been shown to express PD-L1 in contrast to normal thyroid epithelium in several studies [23, 24] including the present series with 0-70% range in PD-L1 epithelial positivity and 2-15% range in PD-L1 inflammatory cells positivity. Some studies have shown PD-L1 epithelial positivity only in HT, but not in chronic lymphocytic thyroiditis [23]. Inflammatory environment is rich in various chemokines and cytokines such as interferons and interleukins that can cause upregulation of PD-L1 [29]. In pathology practice, only neoplastic epithelium should be evaluated for PD-L1 scoring. Therefore, the knowledge of the potential pitfall because of the normal epithelium embedded within the inflammatory infiltrate is of paramount practical importance.

Many PTC are accompanied by HT and chronic lymphocytic thyroiditis [3, 7]. The local environment may thus play role in the cancer development. In meta-analysis of 906 non-medullary thyroid carcinomas, PD-L1 positivity was significantly associated with concurrent thyroiditis with odds ratio (OR) 1.65 [19]. In another meta-analysis, concurrent chronic lymphocytic thyroiditis was shown to be associated with PD-L1 positivity with OR 2.19 ($p = 0.479$) [21] and HR = 1.41 ($p = 0.007$) [20]. Furthermore, both PD-L1 and PD-1 positivity in PTC were shown to correlate with chronic lymphocytic thyroiditis [22]. On the other hand, it has been speculated that PD-L1 negative cases with HT may benefit from protective autoimmune reaction [30].

Nevertheless, clinical outcome of PD-L1 positive PTC patients was not significantly different compared with patients with or without concomitant HT [30]. Deeper analysis of inflammatory clusters of differentiation revealed that lower levels of CD3- and CD8-positive lymphocytes correlate with extrathyroidal extension and lymph node metastases [26]. However, the only 2 metastatic PTCs in our study had no PD-L1 epithelial positivity in inflammatory areas, but TPS was 7% and 11% and CPS 13 and 15, respectively.

Unfortunately, there is no consistency in defining HT and chronic lymphocytic thyroiditis in the studies, and thus, the comparison of the results is limited. Furthermore, also PD-L1 analyses in HT cases faced methodological discrepancies, namely membranous vs. cytoplasmic expression, tumoral vs. tumoral and inflammatory expression, cutoffs of intensity and extent of immunopositivity [25]. In our study, only HT cases were included with strict diagnostic criteria and in 29.8% of cases, there was no epithelial PD-L1 positivity in epithelium adjacent to inflammatory infiltrate including 28.6% of PTC cases and 21.4% of FED cases. We studied PD-L1 both in inflammatory and epithelial cells

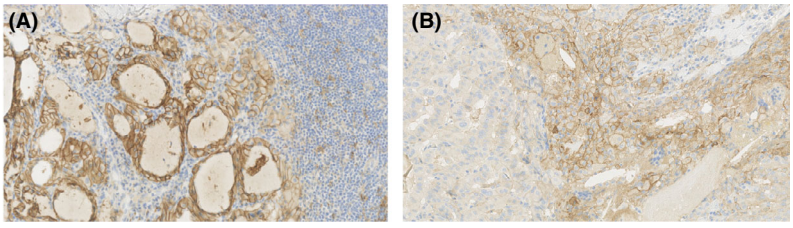

Fig. 3. Immunohistochemical PD-L1 staining in the Warthin-like variant of papillary thyroid carcinoma. (A) Positive PD-L1 staining in follicular epithelial dysplasia with 100% positivity (200x magnification). (B) Positive PD-L1 staining in tumor cells, TPS score 30% and CPS score 37 (200× magnification)

Table 3. Summary of TPS (in percentages) and CPS scores in different PTC variants

		TPS (%)	CPS
PTC all variants	Mean	10.4	15.5
	Median	8.0	12.0
	Minimum	0.0	0.0
	Maximum	43.0	49.0
Warthin-like variant	Mean	17.5	24.0
	Median	17.5	24.0
	Minimum	5.0	11.0
	Maximum	30.0	37.0
Papillary microcarcinoma	Mean	4.6	8.8
	Median	3.0	5.0
	Minimum	0.0	0.0
	Maximum	10.0	18.0
Follicular variant	Mean	6.0	9.0
	Median	4.0	6.0
	Minimum	1.0	2.0
	Maximum	10.0	15.0
Classical variant	Mean	17.5	24.5
	Median	11.0	20.5
	Minimum	5.0	8.0
	Maximum	43.0	49.0

with distinction of various cell types as lymphocytes, macrophages, plasma cells and normal, dysplastic, and tumoral epithelial cells, which we think is important for further studies and analyses.

PD-1/PD-L1 pathway is activated in HT as well as in another autoimmune thyroid entity; Grave's disease as shown by combined flow cytometry, immunofluorescence, and cell line culture analyses [31]. PD-L1/PD-1 blocking is accompanied in 10% with various endocrinopathies, of which 1-2% are high-grade leveled. Hypothyroidism as the most common complication is often preceded by thyroiditis. [32] Accordingly, thyroid autoimmunity as a consequence of PD-1/PD-L1 immunotherapy may be the reaction of pre-existing inflammation that predisposes to thyroid parenchyma destruction by anti PD-L1/PD-1 therapy [31, 33]. Thus, autoimmune diseases may be a target for PD-1/PD-L1 immunotherapies in the future [31, 34]. In lung parenchyma, experimental PD-L1 blocking resulted in reduced lung damage and neutrophiles infiltration [35]. Consequently, thyroid parenchyma damage in HT might be rescued via PD-1/PD-L1 pathway blocking.

FED has been morphologically defined as dysplastic foci related to HT [10, 11]. Recently, several studies on PD-L1 expression in various dysplastic tissues have emerged: such as oral mucosa [36, 37], respiratory epithelium [38], Barrett's esophagus [39], and anal epithelium [40]. PD-L1 expression both in dysplastic epithelium and accompanying lymphocytes has been higher in high-grade lesions (12% and 25% positivity) in comparison with low-grade dysplastic anal lesions (6% and 6% positivity) [40]. Nevertheless, comparison of various dysplastic lesions should take into account the variety of cancerogenesis in different localizations and cancer types, that is, virus and tobacco etiologies [38].

FED epithelium has morphologically the features of dysplasia and share morphological and immunohistochemical features with PTC [10–16]. In our study, 86.7% of FED lesions expressed PD-L1 in dysplastic epithelium and in 53.3% of cases, the expression PD-L1 was found in more than 50% of epithelial cells. The positivity was found on average in 47.2% of dysplastic cells (range 0–100% with only three negative cases and three cases with 100% positivity). In contrast to thyroid inflamed parenchyma, pulmonary squamous cell carcinoma-related dysplastic epithelium expressed PD-L1, but no expression was seen in normal and metaplastic epithelium [38].

In our study, accompanying inflammatory cells were PD-L1 positive in all cases with at least 3% of positive cells in agreement with anal intraepithelial lesion analysis in accompanying inflammation (6–25% PD-L1 positivity according to low or high-grade dysplasia) [40]. PD-1 positive cells in more than 3% of cells were also found in all cases except one in FED group. In concurrent HT epithelium, only 3 (20%) negative cases were observed in FED group in our study. In oral leukoplakia, increased levels of both PD-1 and PD-L1 were shown to relate with malignant transformation in 5 years showing the putative role of immunosuppressive microenvironment [36].

Due to the limited size of FED, the lesions are often cut away and multiple immunohistochemical stainings are impossible to perform. Therefore, an application of multiplex immunohistochemistry or immunofluorescence could solve the obstacle with limited tissue sections in the near future [39, 41, 42].

CONCLUSIONS

PD-L1 was expressed both in inflammatory cells and normal, dysplastic and tumoral epithelial cells in HT, FED, and PTC groups with only mild qualitative nonsignificant differences among groups. PD-1 was found in immune cells, namely in lymphocytes, macrophages, and plasma cells. At the moment, PTC is not a target of immunotherapy. However, understanding the complex issue of concurrent inflammation and autoimmunity can importantly influence the cancer treatment in future. Further studies focusing on cancer

microenvironment and accompanying pathological changes need to be performed in the future.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

E.P. and I.K. involved in conceptualization, writing—original draft preparation, visualization, and project administration. E.P., I.K., and M.A. involved in methodology. E.P., M.A., and D.K. involved in validation. E.P. and D.K. involved in formal analysis. E.P., I.K., M.A., D.K., T.K., and M.L. involved in investigation and writing—review and editing. I.K. involved in resources, supervision, and funding acquisition. E.P., I.K., and D.K. involved in data curation. All authors have read and agreed to the published version of the manuscript.

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