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Original Article

Combined Use of PD-L1 and HBME-1 in the Assessment of Follicular Thyroid Lesions

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Abstract

Background: Follicular lesions of thyroid represent a heterogeneous group of cases with varying malignant potential. Immunohistochemical (IHC) markers could be used to distinguish between equivocal, benign and malignant thyroid lesions, but no single marker is sensitive or specific enough to be used alone for this purpose. Thus, a combination of IHC panel consisting of 2 or more markers may be required**.**

Aim of Study **:** This study aimed to assess IHC expression of PD-L1 and HBME-1 in follicular thyroid lesions. The sensitivity and specificity of each marker alone and in combination were assessed to achieve more accurate diagnosis.

Patients and Methods: This study included 70 cases of benign and malignant thyroid lesions were tested for PD-L1 and HBME-1 positivity scores based on the percentage positivity and staining intensity. A total score was obtained by adding the percentage positivity scores and intensity scores for each section.

Results: PD-L1 expression was detected in 50/70 (71.4%) of all studied cases. HBME-1 expression was detected in 44/70 (62.9%) of studied cases. the combined use of both markers for diagnosis of thyroid carcinoma compared to other thyroid lesions revealed that; the sensitivity was 85.7%, specificity was 85.7%, positive predictive value (PPV) was 90% and negative predictive value (NPV) was 80%. The overall diagnostic accuracy was 85.7%,

Conclusion: The IHC evaluation of PD-L1 and HBME1 expression in follicular thyroid lesions demonstrates a gradual decrease in their expression from malignant follicular lesions to low-risk lesions and finally to benign lesions. The combined use of PD-L1 and HBME-1 increases the sensitivity and specificity of diagnosis of thyroid carcinoma.

Abbreviations: IHC; Immunohistochemical, PPV; positive predictive value, NPV; negative predictive value, FA; follicular adenomas, FC; follicular carcinoma, FVPTC; follicular variant of papillary thyroid carcinomas, H&E; hematoxylin and eosin, PTC; papillary thyroid carcinoma, PDTC; poorly differentiated thyroid carcinoma, ATC; anaplastic thyroid carcinoma, NIFTP; non-invasive follicular thyroid neoplasm with papillary-like nuclear features, WDTUMP; well-differentiated tumor of uncertain malignant potential, NH; nodular hyperplasia. DAB; diaminobenzidine.

Key words: Follicular thyroid lesions, PD-L1, HBME-1.
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Introduction

Follicular lesions represent a heterogeneous group of cases with varying malignant potential. Follicular patterned thyroid lesions are; adenomatous (hyperplastic, adenomatoid) nodules, follicular adenomas (FA), follicular carcinomas (FC), follicular variant of papillary thyroid carcinomas (FVPTC). Due to the difficulties in assessment of malignant potential with certainty, thyroid lesions with follicular pattern are sometimes termed as thyroid tumors of uncertain or indeterminate malignant potential; UMP **(Arpaci et al., 2017).** [(2)]

Histological evaluation of routine hematoxylin and eosin (H&E) stained-tissue sections is still the cornerstone for categorizing thyroid lesions. However, because subjective histo-morphological criteria, diagnostic dilemma may arise, especially in lesions having a follicular growth pattern **(Palo and Biligi, 2017).** [(10)]

Immunohistochemical IHC markers could be used to distinguish between equivocal, benign and malignant thyroid lesions, but no single marker is sensitive or specific enough to be used alone for this purpose. Thus, a combination of an IHC panel consisting of two or more markers may be required **.** [(14)]

PD-L1; a ligand for programmed cell death 1 (PD-1/B7-H1) receptor is functioning as a negative immune regulator. PD-L1 overexpression in malignant neoplasms prevents malignant cells from being attacked by the immune system due to suppression of cytotoxic T cells. Thus, higher expression of PD-L1 in tumors will interfere with anti-tumor immunological attack and facilitate tumor growth and metastasis **(Boussiotis, 2016).** [(4)]

HBME-1, a mesothelioma marker, is a promising antibody for identifying thyroid malignancy **(Palo and Biligi, 2017).** [(10)]

It is a monoclonal antibody which reacts with uncharacterized antigen in microvilli of mesothelial cells. HBME-1 has been assessed in the thyroid with the aim to help in differentiation between benign and malignant lesion as it is more expressed in malignant lesions compared to benign lesions **(Jang et al., 2015).** [(8)]

Patients and methods:

This mixed prospective and retrospective study included 70 patients with clinical and radiological findings of thyroid nodules. All cases were obtained from specimens referred to the Pathology Lab from cases admitted to the Surgery Department; Sohag University Hospitals at the period from 2019 to 2022. This study was approved from the Institutional Ethics Research Committee of Sohag Faculty of Medicine (October 2020).

The 70 studied cases of thyroid lesions were classified into three groups according to 5th edition of the WHO Classification of thyroid neoplasms **(Baloch et al., 2022).** [(3)]

Malignant lesions (42 cases) included 20 cases of papillary thyroid carcinoma (PTC), 10 cases of FVPTC, 7 cases of FC, 3 cases of poorly differentiated carcinoma (PDTC), one case of medullary thyroid carcinoma (MTC) and one case of anaplastic thyroid carcinoma (ATC).

Low risk lesions of the thyroid (8 cases) included 4 cases of non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), 4 cases of well-differentiated tumor of uncertain malignant potential (WDT-UMP). Benign lesions (20 cases) included 10 cases of FA and 10 cases of nodular hyperplasia (NH).

Three sections of 4-μm thickness were cut from formalin-fixed paraffin-embedded tumor blocks. One tissue section for H&E staining for histopathological reassessment. The following parameters were assessed: Presence of true papillae, capsular and vascular invasion. Nuclear scoring was assessed for PTC, FVPTC and NIFTP cases.

Immunohistochemical interpretation:

Tissue sections were stained with PD-L1 and HBME1 antibodies and their expression was evaluated in the studied specimens. Reagents used were; Rabbit monoclonal PD-L1 antibody (clone QR001) with Catalog number; Cat# P-P001-30 (BIOCYC GmbH & Co. Kg. postdam, Germany). Mouse monoclonal HBME-1 antibody with Cat# GTX22383 (GENE TEX. Inc. North America).

A biotinylated goat anti-polyvalent secondary antibody (Universal Staining Kit) (UltraTek HRP Anti-Polyvalent Lab Pack; #UHP125; ScyTek Laboratories, Inc.). It contains: Hydrogen peroxide block, Biotinylated goat anti-polyvalent, Streptavidine peroxidase, diaminobenzidine (DAB) chromogen, DAB substrate. Mayer's Hematoxylin and mounting media: DPX.

Assessment of PD-L1expression:

PD-L1 expression appeared as brownish cytoplasmic staining. PD-L1 expression was assessed semi-quantitatively as a percentage and intensity. Percentage positive scores were assigned according to the following scale: 0 ≤ 10%; 1 ≥ 11-30%; 2 ≥ 31-50%; 3 ≥ 51-70%; and 4≥ 71%. Staining intensity was scored as follows: 0 (none); 1 (mild); 2 (moderate) and 3 (intense). A total score was then obtained (ranging from 0 to 7) by adding the percentage positivity scores and intensity scores for each section**.** [6,7]

Assessment of HBME-1 expression:

HBME1 expression was detected as brownish membranous staining with characteristic apical accentuation. The percentage of positive stained cells was determined for 5×400 fields and it was categorized on a scale of 0=no positive cells; 1=1~25 % positive cells, 2= 26~50 % positive cells, 3=51~75 % positive cells, and 4 = 76~100 % positive cells. The intensity was scored as: 0 = absence of staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. The final score was determined by adding the above two scores together, namely 0 = negative (−), 2-3= weak positive (+), 4-5 = moderate positive (++), 6-7 = strong positive (+++). [5]

Statistical analysis: Data was analyzed using Statistical Software Package version 27 (IBM SPSS version 27.0, SPSS Inc., Chicago, IL, USA). Descriptive analysis was performed. Qualitative variables were presented as frequencies and percentages. Quantitative variables are presented as mean ± standard deviation (SD), median and range.

Parametric continuous data were compared by using independent t-test for the two tested groups and One Way ANOVA test for the 3 or more tested groups. Nonparametric data were compared by using Mann-Whitney Test for the two tested groups and Kruskal-Wallis Test for the 3 or more tested groups.

Qualitative variables were compared by using the Fisher exact test and Chi-square test. The Spearman rank correlation analysis was performed to determine the relationships. Sensitivity, specificity, PPV, NPV and diagnostic accuracy tests were determined by using contingency tables, and were assessed for combination of both markers. All used statistical analysis tested p-value was considered significant if it was less than 0.05.

Results

This study included 70 patients with thyroid nodules. The age range of the studied patients was 21-75 years, with mean ± SD of 45.9±13.6 and median (min-max) of 45 (21-75). The majority of the patients were females (52/70), whereas 18/70 cases were males, and the male: female ratio was 1: 2.89. The size of studied thyroid lesions ranged from 1 to 6 cm in diameter with mean ± SD of 2.99 ± 1.3 and median of 3 cm. Tissue specimens were obtained by total thyroidectomy in 54/70 cases and by hemithyroidectomy in the remaining 16/70 cases.

In the current study, true papillae were found in 20/70 (PTC cases). Capsular and vascular invasion were detected in 7/70 cases (FC cases). There were 4 cases of WDT-UMP with equivocal capsular and vascular invasion. There were 20 cases of follicular adenoma and 4 cases of NIFTP without any evidence of capsular and/or vascular invasion. Nuclear scoring was assessed in cases of PTC, FVPTC and NIFTP and no significant difference in this score was found (p=0.64) between these groups

Immunohistochemical expression of PDL1

Positive expression of PD-L1 was detected in 50/70 (71.4%) of all the studied cases (Table 1).

Table (1): The difference in PD-L1 expression between the three studied groups

		Final histopathological type			Total	p-value <
		Malignant	Low risk	Benign		
PDL1 expression	Positive	41	5	4	50	**0.0001***
	Negative	1	3	16	20	
Total number of cases		42	8	20	70	

Assessment of the sensitivity, specificity, PPV, NPV and diagnostic accuracy of PDL1:

The sensitivity of PD-L1 in assessment of thyroid carcinoma was 97.6%, specificity was 67.9 %, PPV was 82%, NPV was 95 % and diagnostic accuracy was 85.7%. Assessment of the sensitivity, specificity, PPV, NPV and diagnostic accuracy of PD-L1 between each two studied groups was shown in table (2).

Table (2): Assessment of the sensitivity, specificity and accuracy tests of PD-L1:

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
Malignant vs other thyroid lesions	97.6%	67.9%	82%	95%	85.7%
Malignant vs benign	97.6%	80%	91.1%	94.1%	91.9%
Malignant vs low risk	97.6%	37.5%	89.1%	75%	88%
Low risk vs benign	62.5%	80%	55.6%	84.2%	75%

Immunohistochemical Expression of HBME-1:

Positive expression of HBME-1 was detected in 44/70 (62.9%) of the studied cases, while 26/70 (37.1%) cases showed negative expression Table (3).

Table (3): The difference in HBME1 expression between the three studied groups

		Histopathological type			Total	p-value
		Malignant	Low risk	Benign		
HBME1 expression	**Positive**	36	4	4	44	**<0.0001***
	Negative	6	4	16	26	
Total number of cases		42	8	20	70	

Assessment of the sensitivity, specificity, PPV, NPV and diagnostic accuracy of HBME1 in the studied cases:

The sensitivity of HBME1 in assessment of thyroid carcinoma was 85.7%, specificity was 71.4%, PPV was 81.8%, NPV was 76.9% and diagnostic accuracy was 80%. Assessment of the sensitivity, specificity, PPV, NPV and diagnostic accuracy of HBME-1 between each two studied groups was shown in table (4).

Table (4): Assessment of the sensitivity, specificity and diagnostic accuracy of HBME1in three studied groups

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
Malignant vs other thyroid lesions	85.7%	71.4%	81.8%	76.9%	80%
Malignant vs benign lesions	85.7%	80%	90%	72.7%	83.9%
Malignant vs low risk lesions	85.7%	50%	90%	40%	80%
Low risk vs benign lesions	50%	80%	50%	80%	71.4%

Assessment of the sensitivity, specificity and diagnostic accuracy of both PDL1 and HBME1 in combination in diagnosis of thyroid carcinoma:

To test the best way to use PDL1 and HBME1 in diagnosis of thyroid carcinoma we combined the results of their use once in test in series (which means that if both markers were positive this was considered positive and if any of them was negative this was considered negative) and once in test in parallel (which means that if either PDL1 or HBME1 was positive this was considered positive and negativity of both was required to consider it negative). Number of positive and negative cases according to these tests were illustrated in table (5).

Table (5): Number of positive and negative cases according to test in series and test in parallel in the studied cases

		Histopathological type			Total
		Malignant	Low risk	Benign	
Test in series	**Positive**	36	3	1	40
	Negative	6	5	19	30
Test in parallel	**Positive**	41	6	7	54
	Negative	1	2	13	16
Total		42	8	20	70

Combined use of PDL1 and HBME1 in differentiating thyroid carcinoma from other follicular thyroid lesions

By using test in series, the sensitivity of combination of PDL1 and HBME1 in diagnosis of thyroid carcinoma versus (vs) other thyroid lesions was 85.7%, the specificity was 85.7%, PPV was 90%, NPV was 80% and diagnostic accuracy was 85.7% Table (6).

By using test in parallel, the sensitivity of combination both markers in diagnosis of thyroid carcinoma vs other thyroid lesions was 97.6%, the specificity was 53.6%, PPV was 75.9%, NPV was 93.8% and diagnostic accuracy was 80% Table (7).

Table (6): Test in series of PDL1 and HBME1 in combination in the diagnosis of thyroid carcinoma vs other thyroid lesions

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
Thyroid carcinoma vs other thyroid lesions	85.7%	85.7%	90%	80%	85.7%

Table (7): Test in parallel of PDL1 and HBME1 in combination in the diagnosis of thyroid carcinoma vs other thyroid lesions

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
Thyroid carcinoma vs other thyroid lesions	97.6%	53.6%	75.9%	93.8%	80%

The combined use of PDL1 and HBME1 in differentiating malignant thyroid lesions from benign thyroid lesions.

Using test in series, the sensitivity of PDL1 and HBME1 in combination in diagnosis of thyroid carcinoma vs benign lesions was 85.7%, specificity was 95%, PPV was 97.3%, NPV was 76% and diagnostic accuracy was 88.7% (Table 8).

Using test in parallel, the sensitivity of both markers in combination in diagnosis of thyroid carcinoma vs benign lesions was 97.6%, specificity was 65%, PPV was 85.4%, NPV was 92.9% and diagnostic accuracy was 87.1% (Table 9).

The combined use of PDL1 and HBME1 in differentiating thyroid carcinoma from low risk cases

Using test in series, the sensitivity of combination of PDL1 and HBME1 in diagnosis of thyroid carcinoma vs low risk lesions was 85.7%, specific-ity was 62.5%, PPV was 92.3%, NPV was 45.5% and diagnostic accuracy was 82% Table (8).

Using test in parallel, the sensitivity of combination of PDL1 and HBME1 in diagnosis of thyroid carcinoma vs low risk lesions was 97.6%, the specificity was 25%, PPV was 87.2%, NPV was 66.7% and diagnostic accuracy was 86% Table (9).

The combined use of both markers in differentiating low risk follicular thyroid lesions from benign lesions

Using the test in series, the sensitivity of combination of PDL1 and HBME1 in diagnosis of low risk lesions vs benign lesions was 37.5%, specificity was 95%, PPV was 75%, NPV was 79.2% and diagnostic accuracy was 78.6% Table (8).

Using test in parallel, the sensitivity of combination of both markers in diagnosis of low risk lesions vs benign lesions was 75%, specificity was 65%, PPV was 46.2%, NPV was 86.7% and diagnostic accuracy was 67.9% Table (9).

Table (8): Test in series of PDL1 and HBME1 in combination for diagnosis of thyroid lesions:

Diagnoses	Sensitivity	Specificity	PPV	NPV	Accuracy
Malignant VS Benign	85.7%	95%	97.3%	76%	88.7%
Malignant VS low risk	85.7%	62.5%	92.3%	45.5%	82%
low risk VS Benign	37.5%	95%	75%	79.2%	78.6%

Table (9): Test in parallel of both markers in combination for diagnosis of thyroid lesions:

Diagnoses	Sensitivity	Specificity	PPV	NPV	Accuracy
Malignant vs benign	97.6%	65%	85.4%	92.9%	87.1%
Malignant vs low risk	97.6%	25%	87.2%	66.7%	86%
Low risk vs benign	75%	65%	46.2%	86.7%	67.9%

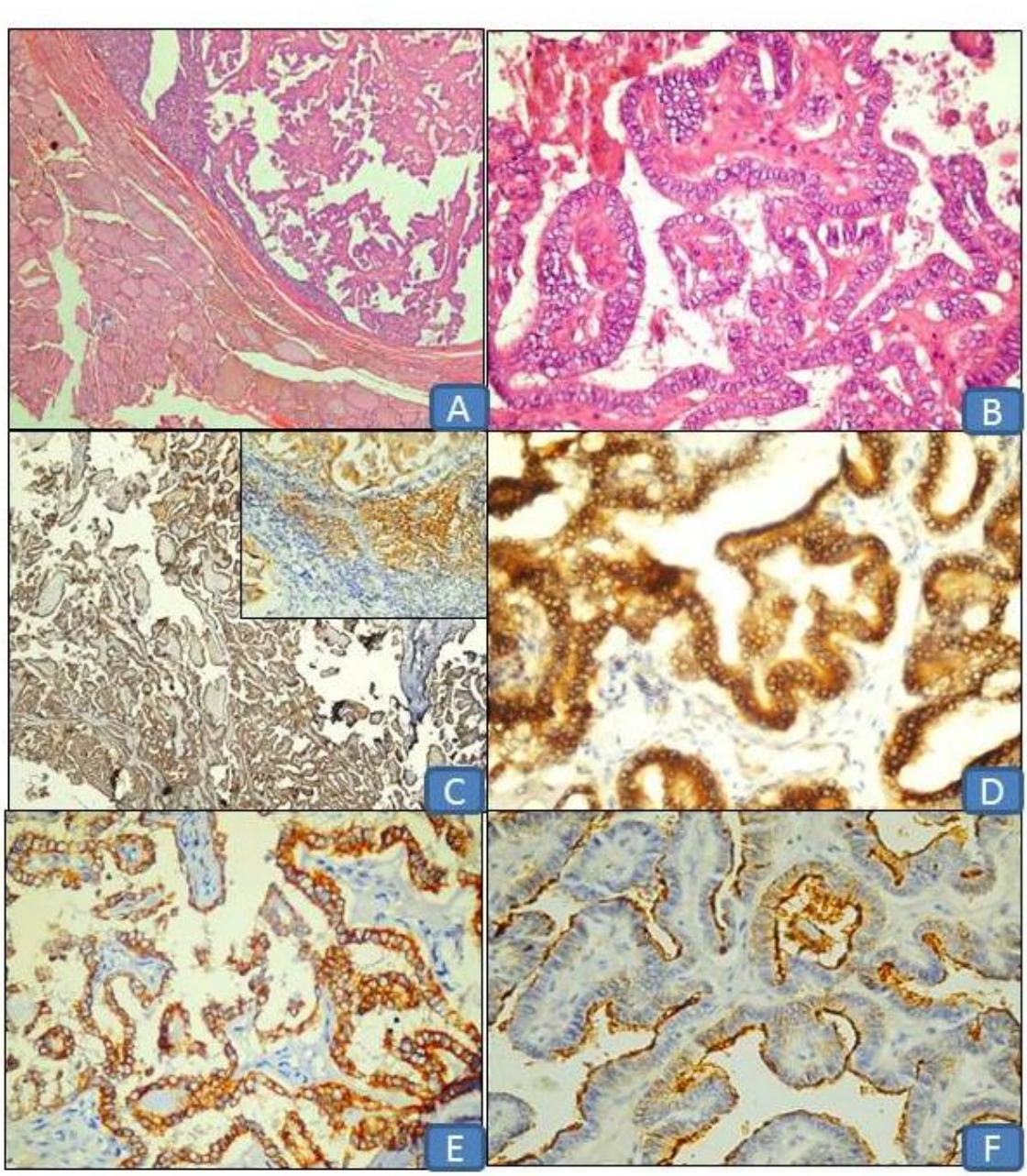

Figure (1): (A, B) showing characteristic nuclear features (enlargement, overlapping, clearing, grooving) (H&E x100, x400). (C&D) PD-L1 showing positive cytoplasmic expression in PTC, score 7 (IHCX100, X400), positive PD-L1 expression in lymphocytes (in set). (E&F) HBME-1 showing strong positive membranous HBME-1 expression and characteristic apical accentuation in PTC (IHC X400)

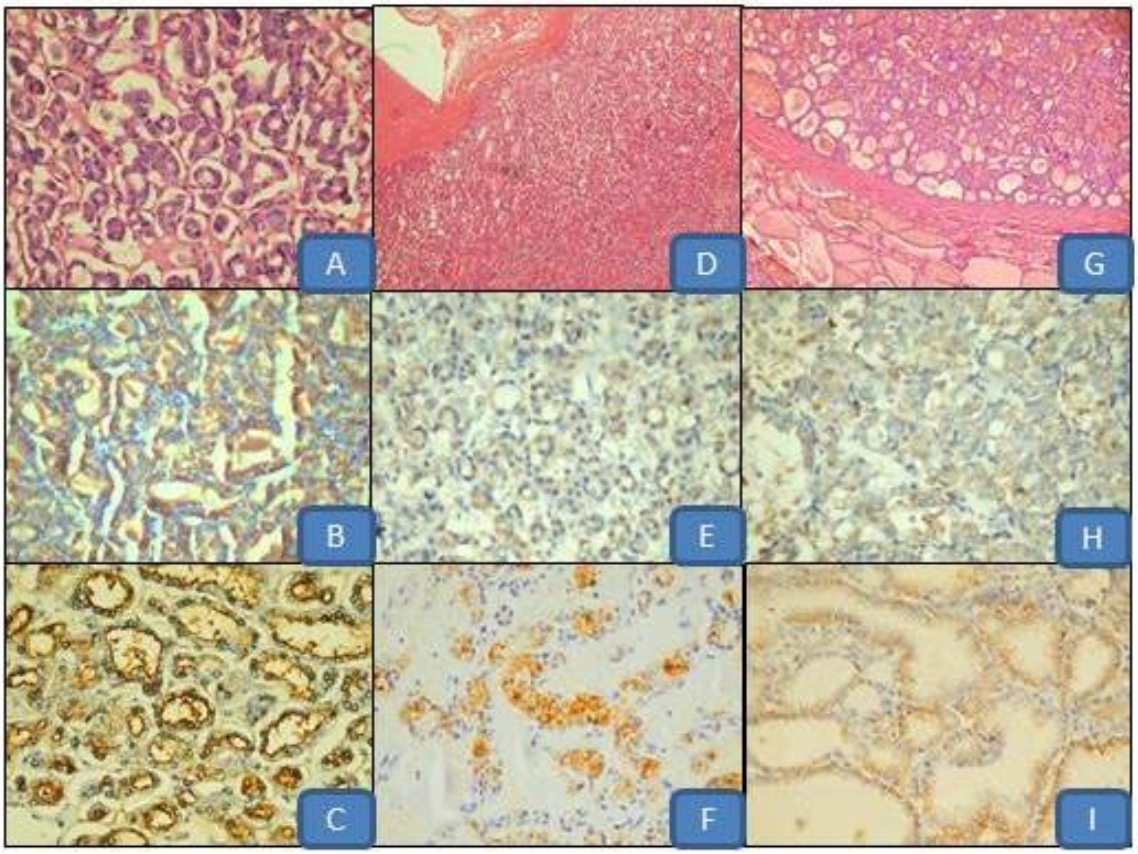

Figure (2): This figure highlight the difference of PD-L1 and HBME-1 expression in FVPTC, WDTUMP and NIFTP. (A) FVPTC (H&E X400). (B) PD-L1 showing positive cytoplasmic expression in FVPTC, score 6 (IHC X 400). (C) HBME-1 showing strong positive membranous expression with characteristic apical accentuation in FVPTC (H&E X 400). (D) WDTUMP (H&E X100). (E) PD-L1 showing positive cytoplasmic expression in WDTUMP score 1 (IHC X 400). (F) HBME-1 showing moderate positive membranous expression in WDTUMP (IHC X 200). (G) NIFTP showing absence of capsular invasion and PTC like nuclear features (H&E X400). (H) PD-L1 showing positive cytoplasmic expression in NIFTP , score 2 (IHC X 400). (I) HBME-1 showing weak positive membranous expression in NIFTP (IHC X 400).

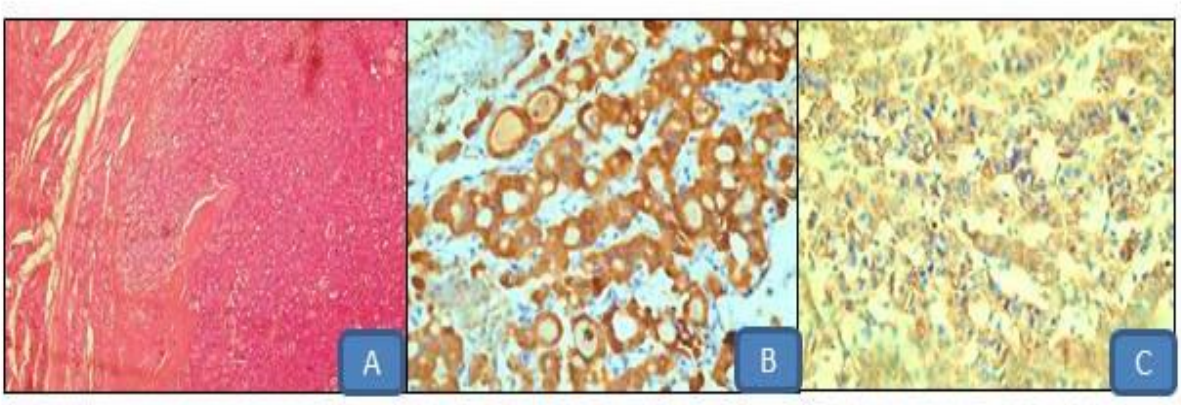

Figure (3): FC showing capsular invasion (H&E x100). (B) PD-L1 showing positive cytoplasmic PD-L1 expression in FC, score 7 (IHC X400). (C) HBME-1 showing moderate positive membranous expression in FC (IHC X 400).

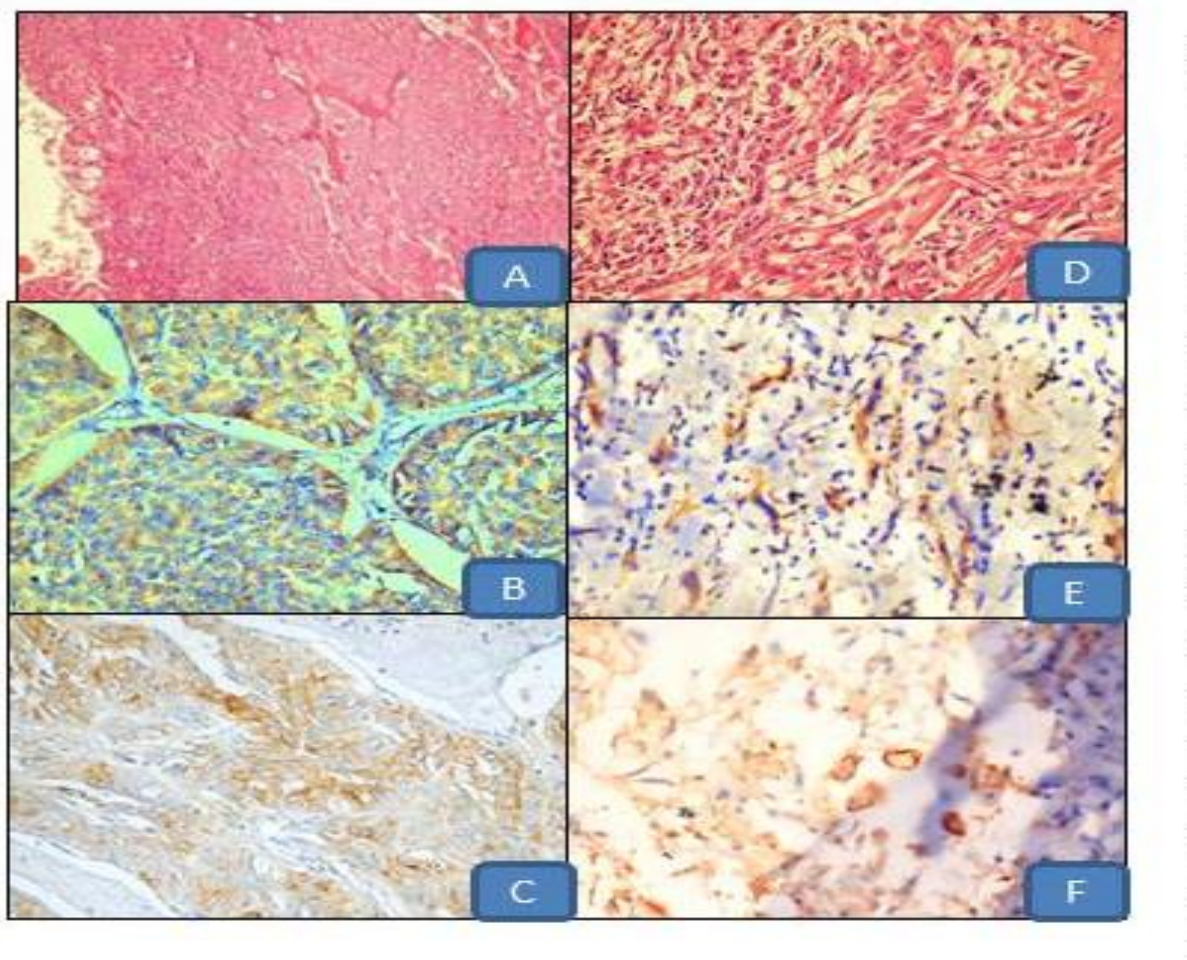

Figure (4): (A) PDTC, insular pattern (H&E X 100). (B) PD-L1 showing positive cytoplasmic expression in PDTC, score 5 (IHC X400). (C) HBME-1 showing moderate positive membranous expression in PDTC (IHC X400). (D) AC showing malignant spindle shaped cells (H&E X400). (E) PD-L1 showing positive cytoplasmic expression in AC score 3 (IHC x400). (F) HBME-1 showing moderate positive membranous expression in AC (IHC X400).

Discussion

The current study assesses the expression of PD-L1 and HBME-1 in follicular thyroid lesions. The level of PD-L1 expression may be useful in guiding management of thyroid lesions by improving the accuracy of histopathological diagnosis of low risk thyroid nodules "grey zone" and pre-surgical diagnosis thereby allowing a better selection of patients requiring surgery to prevent overtreatment and patient's distress. ([7])

The IHC expression of PD-L1 is sometimes a prerequisite for the establishment of checkpoint inhibitor therapy and has a prognostic value in several types of malignant tumors**.** [(15)]

In the present study PD-L1 expression was positively expressed in 50/70 (71.4%) and absent in 20/70 (28.6%) of the studied cases. Positive PD-L1 expression was detected in 41/42 (97.5%) of malignant, in 5/8 (62.5%) of low risk and in benign 4/20 (20%) studied lesions. This difference in PD-L1 expression regarding histopathological type was highly significant ($p<0.0001$).

These results slightly different from that of **Khalifa et al.** [(9)] who reported that 33/40 (82.5%) of studied benign and malignant thyroid lesions showed positive PD-L1 expression while 7/40 cases (17.5%) showed negative PD-L1 expression. About 22/28 (78.5%) of the studied malignant lesions and 11/12 (91.5%) of studied benign lesions were positive for PD-L1. This difference may be due to their use of a different PD-L1 antibody clone (CAL10) compared to the clone used in the present study (QR001).

Ahn et al. [1] reported that PD-L1 expression was detected in only 27 out of 407 (6.6%) cases of thyroid carcinoma. The discrepancies between their findings and those of the present study may be due to the larger sample size (407 cases) that have been used in their study, different methodology (they use tissue microarray versus whole slide sections in the present study), and the use of a different PD-L1 antibody clone (clone SP142) in their study.

It was found that the sensitivity of PD-L1 in diagnosis of thyroid carcinoma was 97.6%, the specificity was 67.9 %, PPV was 82%, NPV was 95 % and diagnostic accuracy was 85.7%.

These results contradict those reported by **Chowdhury et al.** [6] who found that PD-L1 had a sensitivity of 91% and a specificity of 85% for the diagnosis of thyroid carcinoma, with a PPV of 92% and a NPV of 82%.

In the current study, positive HBME1 expression was detected predominantly in malignant lesions as it was expressed in 34/42 (80.9%) of malignant, 4/8 (50%) of low risk and 4/20 (20%) benign lesions.

These results are consistent with **Zargari and Mokhtari** [14] who found HBME-1 positivity in 84% of malignant cases. However, their findings were different regarding benign lesions, as only 2.2% of the studied benign lesions were positive. **Saleh et al.,** [13] reported that 87% of studied thyroid carcinoma showed positive expression of HBME-1, but their results were not similar regarding benign lesions as 56.5% of FA and (17.3%) of NH cases were positive for HBME-1.

They found that carcinomas showed strong and diffuse HBME-1 staining, but benign thyroid lesions had focal and weaker staining positivity. They stated that HBME-1 was not a good IHC marker to differentiate between thyroid adenomas and carcinomas because half of adenomas showed immunoreactivity with this marker. The difference in HBME-1 expression in benign lesions may be due to their use of large number of benign lesions (98 cases compared vs 20 cases in the present study.

Diagnostic power of HBME1 in assessment of thyroid carcinoma revealed that; the sensitivity was 85.7%, specificity was 71.4%, PPV was 81.8%, NPV was 76.9% and diagnostic accuracy was 80%. **Rikhotso** [12] reported that HBME-1 had an overall specificity and sensitivity for thyroid malignancy of 82.1% and 78.8% respectively.

In the current study, the sensitivity of HBME1 in differentiating malignant from benign lesions were 85.7%, specificity was 80%, PPV was 90%, NPV was 72.7% and accuracy was 83.9%.

These results are near to that reported by **Prasad et al.** [11] who found that the sensitivity and the specificity of HBME-1 in distinguishing malignant from benign thyroid lesions were 80% and 84% respectively.

Similarly, **Palo and Pelligi** [10] found that HBME-1 is highly sensitive and specific marker for distinguishing benign from malignant thyroid lesions, with a sensitivity of 86.1%, specificity of 87.5%, PPV of 91.2%, and NPV of 80.8%.

Our results are inconsistent with **Rikhotso** [12] who reported that HMBE-1 have sensitivity and specificity values of 74% and 72% respectively for distinguishing benign from malignant thyroid tumors.

The current study revealed that the sensitivity of HBME1 in differentiating thyroid carcinomas from low risk lesions was high (85.7%) and the specificity was low (50%), However the sensitivity of HBME1 in differentiating low risk from benign thyroid lesions was low (50%) and the specificity was high (80%).

To the best of our knowledge, this is the first study in which a combination of PD-L1 & HBME1 was used to differentiate between different thyroid lesions. There was a highly significant strong positive correlation between the score of HBME1 and PDL1 in all studied thyroid lesions (Correlation Coefficient was $r = 0.66$ and $p < 0.0001$).

Using a combination of both markers, the sensitivity and the specificity for diagnosis of thyroid carcinoma compared to other thyroid lesions was the same for each (85.7%). PPV was 90% and NPV was 80%, and the overall diagnostic accuracy was 85.7%. This indicates that using both markers together in diagnosis of thyroid carcinoma improves sensitivity, specificity and overall diagnostic accuracy.

Conclusion

PD-L1 or HBME-1 can be used for differentiation between benign and malignant thyroid nodules, and between malignant and low risk thyroid lesions but can't be used for differentiation between benign and low risk thyroid lesions. The combined use of PD-L1 and HBME-1 enhances the sensitivity and specificity of diagnosis of thyroid carcinoma. Increased expression of PD-L1 in thyroid carcinoma cases suggests that these patients may benefit from immunotherapy by checkpoint inhibitors.

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