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ORIGINAL ARTICLE

Clinical significance of HBME-1, Galectin-3, and CK19 expression and the status of BRAF mutation in papillary thyroid carcinoma

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Abstract

Objective The aim of this study was to explore the clinical significance of the expression of proteins human bone marrow endothelial cell markers (HBME-1), Galectin-3, and cytokeratin19 (CK19), as well as the status of v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutation in papillary thyroid carcinoma (PTC).

Methods Immunohistochemical staining was performed in 82 specimens each of PTC and papillary benign lesions to detect the expression of HBME-1, Galectin-3, and CK19. Polymerase chain reaction (PCR) and gene sequencing were performed on 60 specimens each of PTC and papillary benign lesions to detect the status of *BRAF* mutation.

Results The positive expression ratios of HBME-1, Galectin-3, and CK19 in PTC were 98.8%, 97.6% and 100% respectively, which were significantly higher than the expressions in papillary benign lesions ($P < 0.05$). No significant relationship was observed between the expression of these makers and the clinicopathological features of PTC. The sensitivity of co-expression of HBME-1 and CK19 or HBME-1 and Galectin-3 as diagnostic criteria of PTC was 99.9%, with a specificity of 95.4%. *BRAF* mutation was detected in 40 of 60 PTC (66.7%) specimens. There was a statistical difference in *BRAF* mutations between PTC and papillary benign lesions ($P < 0.05$); there were no associations between *BRAF* mutation and the clinicopathological features of PTC.

Conclusion Combined immunohistochemical staining of HBME-1, Galectin-3, and CK19 can further improve the sensitivity and specificity of differential diagnosis of PTC. *BRAF* mutation is a significant genetic event, which may have diagnostic value for PTC.

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Key words papillary thyroid carcinoma (PTC); human bone marrow endothelial cell markers (HBME-1); Galectin-3; cytokeratin19 (CK19); v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*)

The pathological diagnosis of papillary carcinoma thyroid (PTC) relies mostly on its complex papillary structure and typical nuclear features. Some benign thyroid lesions may also be accompanied by "real papillary structure," which is similar to PTC and hence difficult to distinguish from it. Nuclear features of follicular type of papillary carcinoma are unconspicuous and easily confused with follicular adenoma. In the last few years, the molecular markers Galectin-3, cytokeratin19 (CK19), and human bone marrow endothelial cell markers (HBME-1), or their combinations, have been suggested for differential diagnosis of PTC, but there remain larger controversies regarding the reliability of these markers. In addition, the relationship between the gene v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) and PTC has been increasingly implicated. Thus, our study attempts to explore the practical value of detecting and analyzing the proteins Galectin-3, CK19, and HBME-1, as well as the status of *BRAF* mutation in the diagnosis of PTC and their correlations with clinicopathological features.

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Materials and methods

Patients and specimens

The collected cases of thyroid surgical specimens and thyroid fresh resection specimens were archived within two phases in the Department of Pathology at the First Affiliated Hospital of Anhui Medical University, China.

The cases of thyroid surgical specimens included 82 cases each of PTC and thyroid benign lesions, which were collected from January 2009 to December 2010. The cases of PTC included 20 males and 62 females, with ages ranging from 13 to 78 years and a median age of 46 years, of which 10 were that of lymph node metastasis. The cases of thyroid benign lesions were distributed as follows: 20 follicular adenoma, 47 nodular goiter, and 15 Hashimoto's Thyroiditis, including 20 male and 62 females at the ages of 15 to 75 years, with the median age of 46 years.

The cases of thyroid fresh resection specimens included 60 cases each of PTC and thyroid benign lesions, which were collected from January 2011 to October 2012. The cases of PTC included 10 males and 50 females at ages of 16–79 years, with a median age of 49 years, of which 10 were that of lymph node metastasis. The cases of thyroid benign lesions also included 10 males and 50 females, at ages of 15–78 years, and a median age of 48 years.

Main reagents

Anti-human Galectin-3 McAb (ready-to-use), anti-human CK19 McAb (ready-to-use), anti-human HBME-1 McAb (ready-to-use), and the reagent kits for detection, named Envision, were purchased from Fuzhou New Biotechnology Corporation. Ltd. (China). The reagent kits for DNA extraction and PCR reagents were purchased from Shanghai Biological Engineering Corporation. Ltd. (China).

Immunohistochemical staining for Galectin-3, CK19, and HBME-1

Following the two-step method of Envision, the positively stained specimens were compared with a known positive biopsy specimen, and the negatively stained specimens compared with specimens treated with phosphate-buffered saline instead of the antibody. We scored the percentage of positively-stained cells and intensity of the staining per biopsy in five fields, at 400 × magnification, by manual counting. The percentage of positive cells in the area of the field was scored on a 5-point scale as follows: 0, no staining; 1, less than 25% positive; 2, between 26% to 50% positive; 3, between 51% to 75% positive; and 4, more than 75% positive.

The intensity of the positive staining in the area of the field was scored as follows: Score 0: without staining, Score 1: with pale yellow staining, Score 2: with brown-yellow staining, and Score 3: with sepia-yellow staining.

The results were evaluated by combining two different scores as follows: -, negative-score 0; 1+, weakly positive-scores 2 and 3; 2+, moderately positive-scores 4 and 5; and 3+, strongly positive-scores 6 and 7.

PCR and DNA sequencing for *BRAF* gene mutation

After DNA was extracted from tissue specimens according to the directions of the DNA extraction kits, the designed primers (upstream, 5′-TCATAATGCTTGCTCTGATAGGA-3′; downstream, 5′-GGCCAAAAATT—TAATCAGTGGA-3′) were used to amplify the DNA sequence containing *BRAF* gene mutation hot spot (exon 15, T1799A) by PCR. The amplified products (224 bp) were verified by agarose gel electrophoresis, after which they were sent to Sangon Biotech (China) Co. Ltd. For sequencing. The mutation was verified by sequence alignment between the *BRAF* gene and products of DNA sequencing.

Statistical analysis

Statistical evaluations were performed by χ^2-test and Fisher's exact test, using the SPSS 13.0 software. A $P < 0.05$ was considered statistically significant. Specificities of independent and joint detection of different protein markers were also calculated and compared for differential diagnosis of thyroid papillary carcinoma.

Results

Expression of Galectin-3, CK19 and HBME-1 and the mutation of *BRAF* gene in benign lesions and PTC

Galectin-3 protein was mainly located in the cytoplasm with nuclear occurrences observed occassionally. The positive ratio of Galectin-3 in PTC was 97.6%, with the main expression of medium seen to be above intensity. The positive ratio of Galectin-3 in thyroid benign lesions was 25.6%, with its main expression being weakly positive. The expression of Galectin-3 revealed a statistically significant difference between PTC and thyroid benign lesions ($P < 0.05$; Fig. 1a and Table 1).

The positive signal of CK19 was located in the cytoplasm and occassionally at the cellular membrane. The positive expression ratio of CK19 was 100%, and 81 cases showed expression with above medium intensity, and the positive expression ratio in benign lesions was 50%, with weakly positive expression. The expression of CK19 revealed a statistically significant difference between PTC and thyroid benign lesions ($P < 0.05$; Fig. 1b and Table 1).

The positive signal of HBME-1 was mainly located at the cellular membrane, with occassional staining at the edge of glandular lumens. The positive expression ratio in

Table 1 Expression of Galectin-3, CK19, HBME-1 in PTC and thyroid benign lesions (*n*)

	n	Galectin-3				%	CK19				%	HBME-1				%
		–	+	++	+++		–	+	++	+++		–	+	++	+++	
PTC	82	2	4	11	65	97.6	0	1	11	70	100.0	1	4	26	51	98.8
TBL	82	61	18	3	0	25.6#	41	39	2	0	50.0*	77	4	1	0	6.1△
FA	20	15	4	1	0	25.0	11	8	1	0	45.0	18	1	1	0	10.0
NG	47	31	14	2	0	34.0	22	24	1	0	53.2	45	2	0	0	4.3
HT	15	15	15	0	0	0	8	7	0	0	46.7	14	1	0	0	6.7

Compared with markers of corresponding to PTC: # $P = 0.000$, * $P = 0.000$, △ $P = 0.000$, PTC = Papillary thyroid carcinoma; TBL = Thyroid benign lesions; FA = Follicular adenoma; NG = Nodular goiter; HT = Hashimoto's thyroiditis

Table 2 The relationships between the clinicopathological features and the expressions of Galectin-3, CK19, HBME-1

Items	*n*	Galectin-3		*P*	CK19		*P*▲	HBME-1		*P*
		n	%		*n*	%		*n*	%	
Sex										
Male	20	20	100.0	1.000	20	100.0	–	20	100.0	1.000
Female	62	60	96.8		62	100.0		61	98.4	
AGE (years)										
< 45	46	46	100.0	0.190	46	100.0	–	46	100.0	0.439
≥ 45	36	34	94.4		36	100.0		35	97.2	
Diameter (cm)										
< 2	23	23	100.0	0.169	23	100.0	–	23	100.0	0.397
2–4	30	28	93.3		30	100.0		30	100.0	
> 4	29	29	100.0		29	100.0		28	96.5	
LNM										
Positive	10	10	100.0	1.000	10	100.0	–	10	100.0	1.000
Negative	72	70	97.2		72	100.0		71	98.6	

▲ The positive expression ratio of CK19 was 100% in all PTC which showed no statistically comparability in the different clinicopathological features. LNM = Lymph node metastasis

PTC was of 98.8%, mostly expressed with above-medium intensity, and the positive expression ratio in benign lesions was 6.7%, all of which expressed weakly, except for one case that was moderately positive. The expression of HBME-1 revealed a statistically significant difference between PTC and thyroid benign lesions ($P < 0.05$; Fig. 1c, and Table 1).

Relationships between the clinicopathological features and expression of Galectin-3, CK19, and HBME-1 in PTC

As depicted in Table 2, the expression of the three protein markers showed no statistically significant differences in different sexes, ages, tumor sizes, and with or without lymph node metastasis.

Comparisons of sensitivity and specificity of protein markers in diagnosis of PTC

Comparisons of the sensitivity and specificity of Galectin-3, CK19 and HBME-1 in differential diagnosis as well as combining HBME-1 (the highest specificity) with other expressions revealed that the results of combined Galectin-3, CK19, and HBME-1, and co-expression of HBME-1 and CK19 or HBME-1 and Galectin-3 had the best specificity and sensitivity in PTC diagnosis (Table 3).

BRAF gene mutation status in PTC and papillary benign lesions

The *BRAF* gene T1799A mutation in exon 15 was studied in 60 cases with PTC by sequencing, and heterozygous mutation was detected in 40 out of 60 cases, yielding a mutation ratio of 66.7% (Fig. 2), whereas in the benign lesions, the ratio was 0% (Fig. 3; $\chi^2 = 24.000$, $P < 0.05$). In addition, according to the Table 4， the *BRAF* gene mutation in PTC showed no statistically significant differences

Table 3 The sensitivity and specificity of Galectin-3, CK19, HBME-1 and combinations in PTC diagnosis

Expressions of markers	Sensitivity (%)	Specificity (%)
Galectin-3 (+)	97.6	74.4
CK19 (+)	100.0	50.0
HBME-1 (+)	98.8	93.9
HBME-1 (+), Galectin-3 (+)	96.4	98.4
HBME-1 (+), CK19 (+)	98.8	97.0
HBME-1 (+), Galectin-3 (+), CK19 (+)	96.4	99.2
HBME-1 (+), CK19 (+) or HBME-1 (+), Galectin-3 (+)	99.9	95.4

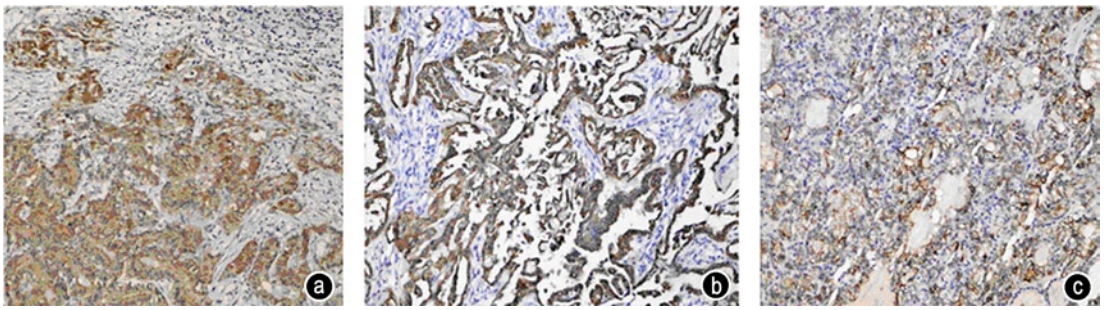

Fig. 1 Expression of Galectin-3, CK19、HBME-1 in PTC (Envision × 100). (a) Galectin-3; (b) CK19; (c) HBME-1

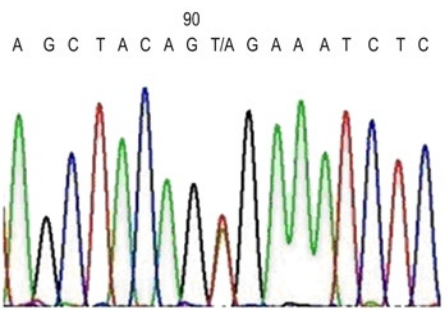

Fig. 2 Heterozygous mutation of *BRAF* gene in PTC

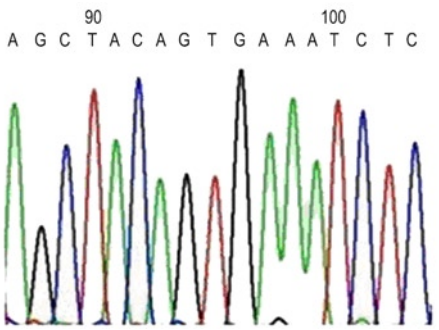

Fig. 3 Nodular goiter without *BRAF* gene mutation

in different sexes, ages, tumor sizes, and with or without lymph node metastasis.

Discussion

Galectin-3 is a β-galactoside-binding protein, which may be involved in cell growth, cell adhesion, inflammation, immune regulation, and cell apoptosis [1], and some studies had shown that Galectin-3 is also involved in processing of many neoplastic formations and transformation, such as colon cancer [2] and PTC [3–5]. In this study, the positive expression ratio of Galectin-3 in PTC was significantly higher than in thyroid benign lesions, which was consistent with previous reports [6–7]. However, Galectin-3 had low specificity in distinguishing PTC and thyroid benign lesions, making it difficult to diagnose PTC independently, while the intensity of positive expression in thyroid benign lesions is very weak, so the observed above-medium intensity of the positive expression has important significance. Other studies [4, 8] have shown that Galectin-3 negatively regulates metastasis and invasion of PTC cases, and lymph node metastasis was the primary means for metastasis of thyroid carcinoma. In our study of 10 cases of lymph node metastasis in PTC, we observed that there was no distinct difference with the group without lymph node metastasis, and majority of Galectin-3 still expressed positively.

Table 4 The relationships between *BRAF* mutation and the clinicopathological features

Items	*n*	Positive expression	%	*P*
Sex				
Male	10	6	60.0	0.903
Female	50	34	68.0	
Ages (years)				
< 45	24	7	58.3	0.264
≥ 45	36	33	72.2	
Diameter (cm)				
< 2	35	27	77.1	
2–4	20	10	50.0	0.114
> 4	5	3	60.0	
LNM				
Positive	10	7	70.0	1.000
Negative	50	33	66.0	

CK19 is a low-molecular-weight keratin that is expressed in many epithelial tissues. Some studies [6–7, 9] have reported stronger CK19 expression in PTC, with positive ratios from 70% to 100% compared to the inconformity reports in thyroid benign lesions. Our study revealed stronger CK19 expression in PTC than in thyroid benign lesions, with a sensitivity for diagnosing PTC of 100%, but much lower specificity. This low specificity imposes limits on its diagnostic use for PTC. The independent positive expression cannot diagnose PTC, however, the above-medium intensity of positive expression has a good

vigilance for PTC, whereas strong sensitivity of CK19 imparts a unique value to differential diagnoses for PTC, implying that negative CK19 expression can be used as an exclusive index for PTC diagnosis.

HBME-1 was widely reported [10–11] to be expressed in thyroid tumors, especially in PTC. Its use has been suggested for differential diagnosis between benign thyroid lesions and malignant thyroid tumors, but there were few domestic reports with different opinions. In our study, HBME-1 shows statistically significant differences between thyroid benign lesions and PTC with higher sensitivity and specificity. It was thus concluded that HBME-1 is a better marker for differential diagnosis of PTC.

Besides, we found that the results of pathological diagnosis can be more reliable if we combined the highest specificity of HBME-1 with Galectin-3 and CK19, and compared the co-expression of HBME-1 and CK19 or HBME-1 and Galectin-3 as a diagnostic criteria of PTC.

BRAF is a member of the *RAF* gene family location at 7q34, and plays an important role in the MAPK signaling pathway. Several studies [12–13] have reported that the *BRAF* T1799A mutation may be involved in thyroid carcinoma, and that the most mutated locus for this gene lies in exon 15 T1799A. This mutation causes the 600th valine of the protein to be replaced by glutamic acid, leading to abnormality in mediating the downstream signaling pathway. Studies have also implicated the mutation in PTC alone and in some undifferentiated carcinomas that may originate from PTC, with the mutation ratio of PTC ranging from 30% to 45%. However, such mutations have been rarely documented in domestic reports of China. Our study was similar to other studies [13–15] that revealed many cases of *BRAF* T1799A in PTC compared to no case in thyroid benign lesions. *BRAF* mutation is a significant event in PTC, which may be useful for early diagnosis of the disease; however, the *BRAF* mutation ratio in our study was slightly higher than that reported previously. This discrepancy can be attributed to the different backgrounds in the studies. There remain some controversies [14] to the relationship between *BRAF* gene mutation and the clinicopathological features of PTC, however, our study did not find any significant relationship between *BRAF* gene mutation and patients' sexes, ages, tumor sizes as well as presence or absence of lymph node metastasis.

Overall, our study suggests that combined immunohistochemical staining of HBME-1, Galectin-3, and CK19 can further improve the sensitivity and specificity of PTC diagnosis, with co-expression of HBME-1 and CK19 or HBME-1 and Galectin-3 proving to be a more potent diagnostic criteria. *BRAF* mutation is a significant genetic event, which may have diagnostic importance in PTC.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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