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Clinical significance of immunohistochemistry to detect BRAF V600E mutant protein in thyroid tissues

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Abstract

This study investigated the feasibility of using immunohistochemistry (IHC) instead of PCR to detect BRAF V600E mutant protein in papillary thyroid carcinoma (PTC), and to determine the value of using preoperative BRAF V600E mutant protein by IHC to assist in the diagnosis of thyroid nodule patients with Hashimoto's thyroiditis (HT).

The expression of BRAFV600E mutant protein was measured in 23 cases of HT+PTC, 31 cases of PTC, and 28 cases of HT by IHC, followed by PCR in the same samples for validation. SPSS 19.0 software was used for statistical analysis.

The sensitivity and specificity of IHC to detect BRAF V600E mutation were 100% and 42.86%, respectively. In addition, the mutation rate of BRAF V600E protein in the HT+PTC group (34.78%, 8/23) was lower than that in the PTC group (80.65%, 25/31).

The application of IHC to detect BRAF V600E mutant protein has good sensitivity but not specificity to diagnose PTC. IHC can be used as a preliminary screening method to detect BRAF V600E mutation. The strongly positive (+++) staining of IHC potently indicated BRAF V600E gene mutation. For suspicious thyroid nodules combined with HT, the detection of BRAF V600E mutant protein with IHC alone is not of great significance for differentiating benign and malignant nodules.

Abbreviations: FNA = fine needle aspiration, FNA = fine needle aspiration, HT = Hashimoto's thyroiditis, IHC = immunohistochemistry, PTC = papillary thyroid carcinoma.

Keywords: BRAF V600E, Hashimoto's thyroiditis, papillary thyroid carcinoma

1. Introduction

Thyroid cancer is the most common type of endocrine cancer, and papillary thyroid carcinoma (PTC) is the most common thyroid cancer, accounting for approximately 90% of all cases. With the development of ultrasonography, fine needle aspiration (FNA) has been gradually applied in clinical practice. Moreover, American Thyroid Association guidelines have recommended FNA as the most sensitive and specific method to preoperatively differentiate benign and malignant thyroid nodules.[1] However, it is difficult to make a clear diagnosis if the cell number of FNA specimens is too small or when papillary nucleus characteristics are not obvious in clinical practice.

In this molecular diagnostic era, molecular detection has been used to assist clinical diagnosis, prognostic assessment, and targeted therapy guidance in various fields such as breast, ovarian, and lung cancers. For PTC, BRAF V600E mutation can be potentially used to further evaluate malignant risk in samples without clear preoperative FNA diagnosis. It has been proposed that preoperative FNA combined with BRAF V600E mutation detection of PTC can further improve diagnostic sensitivity.[2] Genetic testing is expensive, with high-level laboratory requirement, which is not available in many primary hospitals, whereas immunohistochemistry (IHC) is economical, simple, and feasible. Therefore, further investigations are needed on the feasibility of IHC instead of PCR to detect BRAF V600E mutations.

The combination of Hashimoto's thyroiditis (HT) and thyroid cancer is becoming increasingly common, of which the most common pathological type is PTC. For FNA samples, which are thought to have HT background by preoperative ultrasonography and serum antibody result, it also requires further investigations of whether BRAF mutation detection is of the same diagnostic significance.

In this study, IHC was used to measure the expression of BRAF V600E mutant protein in PTC tissues with and without HT. Meanwhile, PCR was performed on the same tissues to evaluate the feasibility of IHC instead of genetic testing, and to determine the value of preoperative BRAF V600E detection in FNA tissues with PTC and HT.

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Ethics approval: The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the second hospital of Dalian Medical University, China.

Informed consent: All participants signed written informed consent.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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2. Materials and methods

2.1. Sample collection

In total, 82 paraffin-embedded thyroid surgical samples with complete clinicopathological data were selected from The Second Affiliated Hospital of Dalian Medical University (Dalian, China) between 2014 and 2019. Among these cases, 23 were PTC combined with HT (PTC + HT group), 31 were common PTC (PTC group), and the remaining 28 were HT (HT group). The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Second Hospital of Dalian Medical University. All participants provided written informed consent.

3. Methods

3.1. Detection of BRAF V600E mutant protein expression by IHC

A total of 82 paraffin-embedded tissues were cut into 4 μm thick sections, and placed in an oven at 40°C for 1 h. Then the sections were incubated with rabbit anti-human BRAF V600E monoclonal antibody (clone number: VE1) working solution (Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

3.2. Interpretation of IHC staining of BRAF V600E

BRAF V600E protein was expressed in the cytoplasm. Yellow particles in the cytoplasm of the thyroid follicular epithelial cells indicated positive staining, while no staining indicated a negative result. For staining intensity, three views with the strongest staining intensity were randomly selected and observed (400x magnification) while avoiding necrotic and marginal areas. A semi-quantitative approach was used to score the proportion of positive cells and staining intensity of positive cells. The percentage of positive cells was scored as follows: 0 points, <5%; 1 point, 5–25%; 2 points, 26–50%; 3 points, 51–75%, and >75% for 4 points. The staining intensity was scored as 0, 1, 2 and 3 points for no staining, light yellow, brownish yellow, and brown, respectively. Finally, the scores representing the proportion of positive cells and staining intensity were multiplied to determine the degree of staining as follows: 1–4 points indicated weakly positive (+), 5–8 points indicated moderately positive (++), and ≥9 points indicated strongly positive (+++).

3.3. Detection of BRAF V600E mutation by PCR

The DNA concentration was measured by an ultraviolet spectrophotometer. The DNA of all 82 patients was isolated from the formalin-fixed paraffin-embedded samples and centrifuged, followed by PCR with 2 μL sample, 1 μL BRAF positive control P, and 1 μL BRAF negative control P. After the PCR reaction, the baseline and threshold were manually or automatically adjusted according to the instructions of the PCR instrument and the fluorescence curve. The starting point of the baseline was generally set between 5 and 8, the end point between 12 and 15, and the threshold line between 1000 and 20000. The Ct value of each sample was obtained from the Reports window by clicking the analysis button.

3.4. Quality control

Two conditions needed to be simultaneously met within the same experiment: no S-type amplification curve of the target gene in the negative control or S-type amplification curve of the target gene in the positive control; and Ct < 30. Otherwise, the results were invalid.

3.5. Interpretation of the results

The Ct value of mutation (CtM) and Ct value of wild-type (Ctw) were determined in each reaction. When the value of △Ct (CtM-Ctw) was ≤8 and the Ctw value was ≥30, the sample was considered mutant (mutation content 1–100%); when the value of △Ct was >8 and the Ctw value was ≥30, the sample was considered wild-type (mutation content 0–1%).

3.6. Statistical analysis

SPSS software (version 19.0) was employed for the statistical analyses. Data are shown as the mean ± standard deviation, and the independent samples *t*-test and analysis of variance were used for comparison between groups. Categorical data, which are shown as numbers (n) and percentages (%), were compared between groups using the chi-square test. $P < .05$ was considered statistically significant.

4. Results

4.1. BRAF V600E protein expression

IHC was used to determine BRAF V600E protein expression in 82 tissue samples, which revealed that BRAF V600E protein was localized in the cytoplasm. Moreover, the positive expression rate was lowest in the PTC + HT group (43.48%) compared to the PTC and HT groups ($P < .01$; Table 1), with weak to moderate staining (the average score, 1.65 ± 2.23 points; range: 1–6 points). The positive expression rate of BRAF V600E protein was highest in the PTC group (96.77%), with moderate to strong

Table 1

Comparison of BRAF V600E protein expression in three groups by IHC.

Group	Number of cases	BRAF V600E (number)		Positive rate	χ^2	*P*
		−	+			
PTC+HT	23	13	10	43.48%	30.724	.000*
PTC	31	1	30	96.77%	13.443	.000†
HT	28	7	21	75.00%	15.791	.000‡

* Comparison between PTC+HT group and PTC group.
† Comparison between PTC+HT group and HT group.
‡ Comparison between PTC group and HT group.

staining (average score, 7.61 ± 3.35 points; range: 3–14 points). The positive expression rate in the HT group was 75.00%, with weak to moderate staining (average score, 3.14 ± 2.43 points; range: 2–8 points). BRAF V600E protein expression was significantly different among the three groups ($P < .05$; Table 1, Fig. 1).

4.2. PCR detection of BRAF V600E mutation

The BRAF V600E mutation in 82 thyroid tissue samples was detected by PCR. The mutation rate of BRAF V600E in the PTC + HT group (34.78%) was significantly lower than that in the PTC group (80.65%), and the △Ct value of the PTC + HT group (3.26) was significantly higher compared with the PTC group

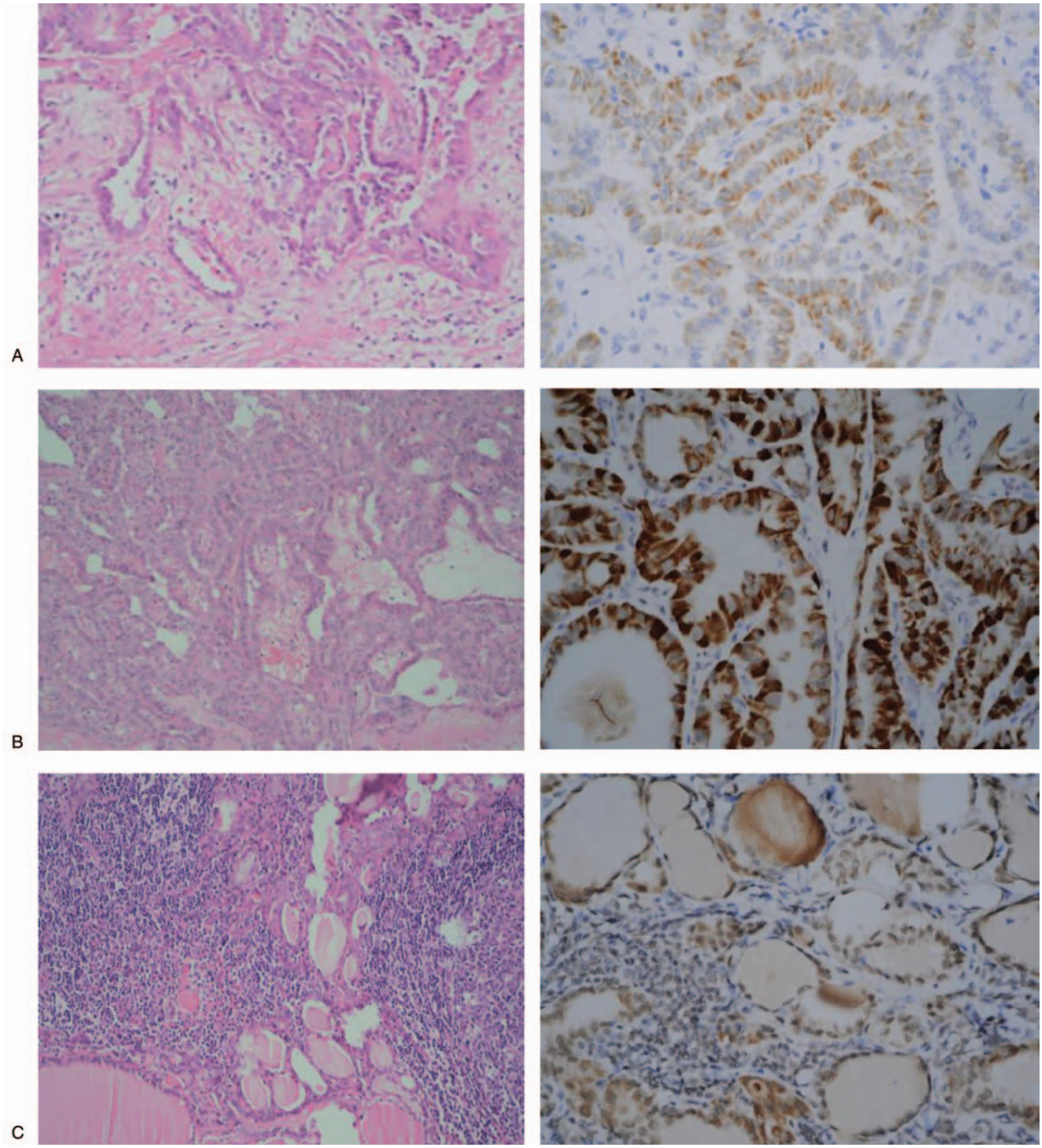

Figure 1. Representative images of H&E staining of thyroid tissues and IHC of BRAFV600E protein in three groups. (A) PTC+HT group. Left: H&E, 200x, Right: IHC, 400x, showing medium positive in the cytoplasm. (B) PTC group. Left: H&E, 200x, Right: IHC, 400x, showing strong positive in the cytoplasm. (C) HT group. Left: H&E, 200x, Right: IHC, 400x, showing medium positive in the cytoplasm.

Table 2

BRAF V600E mutation results in three groups by PCR.

Group	Number of cases	BRAFV600E		Positive rate %	χ^2	*P*	△Ct value $\bar{x}$	Z	*P*
		–	+						
PTC+HT	23	15	8	34.78	11.686	.000*	3.26	2.897	.031§
PTC	31	6	25	80.65	11.551	.000†	1.72		
HT	28	28	0	0.00	39.184	.000‡			

* Comparison between PTC+HT group and PTC group.
† Comparison between PTC+HT group and HT group.
‡ Comparison between PTC group and HT group.
§ Statistically significant.

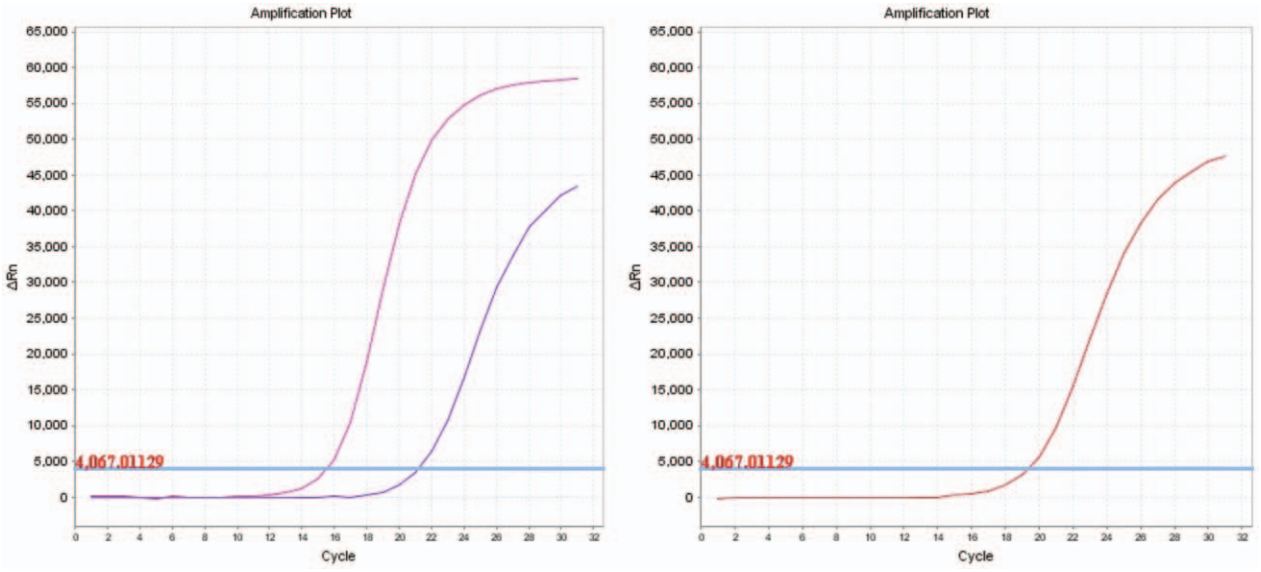

Figure 2. PCR detection of BRAF V600E mutation. Left: Positive, Right: Negative.

(1.72) ($P < .05$). BRAF V600E mutation was not detectable in the 28 cases of HT (Table 2, Fig. 2).

4.3. Sensitivity and specificity of IHC for detection of BRAF V600E mutation

Of the 82 samples, there were 61 cases of positive protein expression of BRAF V600E determined by IHC, and 33 cases of positive gene expression of BRAF V600E determined by PCR (Table 3). Compared with PCR, the sensitivity and specificity of IHC for detecting BRAF V600E (VE1) was 100% (33/33) and 42.86% (21/49), respectively.

4.4. Comparison of BRAF V600E mutations by IHC and PCR

Of the 82 samples, there were 61 cases of positive protein expression of BRAFV600E determined by IHC, and 33 cases of positive gene expression of BRAF V600E determined by PCR. In addition, the 33 cases with positive expression for BRAF V600E gene by PCR were also revealed as positive by IHC. While 28 cases were positive by IHC but negative by PCR (2 cases in PTC + HT group, 5 cases in PTC group, and 21 cases in HT group), whose staining intensity was weak or moderate (Table 4).

Table 3

Comparison of BRAF V600E mutation detection in thyroid tissue by PCR and IHC.

PCR	IHC		Total	sensitivity	specificity
	+	–			
+	33	0	33	100%	42.86%
–	28	21	49		
Total	61	21	82		

Table 4

IHC staining intensity of BRAF V600E in cases with positive IHC results but negative PCR results.

Group	IHC staining intensity %(n/N)		
	+	++	+++
PTC+HT	0 (0/2)	100 (2/2)	0 (0/2)
PTC	60 (3/5)	40 (2/5)	0 (0/5)
HT	57.14 (12/21)	42.86 (9/21)	0 (0/21)

Table 5

Comparison of positive degree by IHC and △Ct value by PCR in detecting BRAF V600E.

Positive degree of IHC	Average △Ct value	R	P
+	2.73	−0.464	.040*
++	2.66		
+++	1.23		

* Statistically significant.

4.5. Correlation between positive degree of BRAF V600E protein expression and expression of BRAF V600E mutation

In our study, 33 cases were positive for BRAF V600E mutation detected by both IHC and PCR (8 in PTC + HT group and 25 in PTC group). The correlation between the positive degree by IHC and the △Ct value by PCR was compared, revealing that the positive degree by IHC was negatively correlated with the △Ct value by PCR (r = −0.464, $P = .04$; Table 5).

5. Discussion

The BRAF gene, located on chromosome 7q23 and encoding a 95 kDa protein, belongs to the tryptophan/serine kinase RAF family. T to A mutation at 1799 of the BRAF gene can cause glutamic acid to valine (V600E) point mutation in the encoded protein, activating MEK and ERK tumor genes in the MAPK pathway,[3] leading to tumorigenesis. The mutation rate of BRAF V600E in PTC tissues ranges from 29% to 83%.[4]

Multiple methods are used to detect gene mutations including probe amplification refractory mutation system,[5,6] sequencing,[7] high-resolution melting curve analysis technology,[8] and denaturing high performance liquid chromatography.[9] Quantitative PCR has high sensitivity and specificity and can detect 101 copies/per reaction, which can screen mutant alleles when tumor samples contain relatively few mutant cells, with good application prospects.[10] However, due to the expensive equipment, strict quality control and professional knowledge of molecular detection technology of PCR, it is not conducive to clinical application. Therefore, it is urgent to explore a fast, simple and cheap approach to detect gene mutations. As a commonly used method in clinical pathological diagnosis, the process of IHC is relatively simple, which is convenient for clinical application.

BRAF V600E gene mutation is highly specific in PTC tissues, which is rarely detectable in benign thyroid disease and follicular and medullary carcinoma tissues.[11] At present, the incidence of HT combined with PTC is increasing annually.[12] As early as the 1950s, Dailey et al [13] proposed the close relationship between HT and PTC, followed by studies by numerous scholars; however, there is still no definitive conclusion. Studies on BRAF V600E mutation in PTC combined with HT have shown that the mutation rate is significantly lower in PTC combined with HT than simple PTC.[14]

In this study, BRAF V600E protein-specific monoclonal antibody (VE1) was used for IHC staining in the three groups (PTC + HT, PTC and HT groups). Subsequently, IHC staining was compared to PCR assay in BRAF V600E mutation, to investigate the feasibility of inference of BRAF V600E gene mutation by using IHC staining, and to assess the significance of using preoperative BRAF V600E gene testing to assist diagnosis in thyroid nodule patients with HT. Among the 82 samples, 61 cases were positive for BRAF V600E protein expression by IHC and 33 cases were positive for BRAF V600E gene mutation. Additionally, 33 cases with positive expression for BRAF V600E gene by PCR were also revealed as positive by IHC. While 28 cases were positive by IHC staining but negative by PCR (2 cases in PTC + HT group, 5 cases in PTC group, and 21 cases in HT group), whose staining intensity was weak or moderate. The overall sensitivity and specificity of IHC to detect BRAF V600E was 100% and 42.86%, respectively, whose specificity was lower than foreign reports (96.8% for sensitivity and 86.3% for specificity).[15]

Therefore, further analysis of the positive degree of IHC staining of the three groups in our study showed that the positive degree of IHC was negatively related to the △Ct value of the PCR assay (r = −0.464, $P < .05$). Notably, when IHC was strongly positive (+++), the results from IHC and PCR were totally consistent, indicating that the protein expression level of BRAF V600E was positively correlated with the amount of mutation copy of BRAF V600E gene. In other words, the higher positive degree of IHC staining suggested the greater reliability. Of the 21 HT cases with focal expression of BRAF V600E protein by IHC (positive staining was mostly located in the eosinophilic follicular epithelium, with the positive degree all less than (++), BRAF V600E gene mutation was not detected in these 21 HT cases by PCR assay. The above findings proved that the IHC results of BRAF V600E in HT group were false positive, which might be due to the eosinophilic change of the follicular epithelium of HT, causing non-specific staining in IHC. To further validate the deduction, eight cases of thyroid eosinophilic adenoma were selected and subjected to immunohistochemistry (IHC) for BRAF V600E detection. As a result, there were two cases of (+++), four cases of (++), one case of (+) and one case of negative. In addition, PCR assay was used to detect BRAF V600E gene mutation, which revealed negative outcomes of all eight cases (data not shown). The above findings further show that the positive IHC results in detecting BRAF V600E in thyroid eosinophilic adenoma should be cautiously interpreted, which should be confirmed by PCR assay or sequencing if necessary.

Here, we showed that BRAF V600E mutation rate in the HT + PTC group was 34.78% (8/23), which was significantly lower than that in PTC group (80.65%, 25/31) ($P < .05$). And our results are consistent with previous literature.[14] Although BRAF V600E mutation is the most important form of gene mutation in PTC, it may not be the main molecular genetic alteration of PTC combined with HT. In a study of 262 PTC patients, the incidence of RET/PTC rearrangement in PTC+HT group was 76.47%, which was significantly higher than that of PTC group without HT (53.33%).[16] In addition, the PI3K/AKT pathway has been proposed as the main carcinogenic mechanism of PTC combined with HT,[17] and this study has reported that the expression of PI3K/AKT pathway is significantly higher in PTC+HT group than that in PTC group. The abnormal activation of PI3K/AKT pathway has been revealed to be associated with carcinogenesis and tumor progression in multiple types of malignancies. Therefore, it is speculated that excessive activation of the PI3K/AKT pathway may be correlated with the occurrence of PTC combined with HT.

In summary, the application of IHC to detect BRAF V600E mutation has good sensitivity but not specificity in the diagnosis of PTC. IHC can be used as a preliminary screening method to detect BRAF V600E mutation. The strongly positive (+++) staining of IHC potently indicates BRAF V600E gene mutation.

However, when IHC staining is weakly or moderately positive (+ or ++), re-examination is recommended. When preoperative ultrasonography and serological tests suggest HT, it is not of obvious diagnostic significance to detect BRAF V600E mutation simply by IHC, while multi-gene tests are required for comprehensive considerations before diagnosis.

Author contributions

Conceptualization: Lifen Wang.
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Supervision: Lifen Wang.
Validation: Yini Zhang.
Visualization: Yini Zhang.
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