Comparison of diagnostic methods for the detection of a BRAF mutation in papillary thyroid cancer

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Abstract. The most common genetic alteration identified in papillary thyroid cancer (PTC) encodes a valine to glutamic acid change at position 600 (V600E) in the BRAF proto-oncoprotein. The most accurate and reliable method for detecting this BRAF mutation has not yet been determined. In the present study, the sensitivity, specificity and feasibility of diagnostic methods for BRAF mutations were assessed. BRAF mutational analysis was performed by Sanger DNA sequencing, using the Cobas® 4800 BRAF V600 test and by immunohistochemistry (IHC). A total of 185 tumor tissues samples were analyzed using the three assays. BRAF mutations were identified in 76.2% of samples by Sanger sequencing, 78.9% of samples by Cobas 4800 BRAF V600 test and 76.8% of samples by IHC. Complete concordance for the three methods was observed in 92.4% of samples. Sensitivity and specificity of Sanger sequencing were 97.2 and 95.2%. Sensitivity and specificity of the Cobas 4800 BRAF V600 test were 99.3 and 90.5%. Sensitivity and specificity of IHC were 98.6 and 97.6%. Furthermore, the presence of a BRAF mutation was significantly associated with extrathyroid extension and multifocality ($P<0.05$), but not associated with age, sex, lymph node metastasis, central node metastasis, lateral node metastasis, Tumor-Node-Metastasis stage or tumor size in patients with PTC. These results suggest that a combination of IHC and the Cobas 4800 BRAF V600 Test kit for V600E mutation analysis is the most efficient and reliable method in routine practice. Accurate screening for BRAF mutation may contribute to improving the risk stratification of PTC.

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Introduction

Papillary thyroid cancer (PTC) is a common type of endocrine malignancy, and its incidence has increased substantially in recent decades (1). Despite PTC generally being a highly curable disease, a minority of PTC cases follow a more aggressive clinical course, characterized by local invasion, distant metastasis or recurrent disease and, rarely, to mortality (2). The revised American Thyroid Association guidelines indicate that thyroid cancer should be treated according to risk stratification, assessed on the basis of disease stage and genetic testing (3). Therefore, accurate risk stratification is vital for clinical decision-making, and may decrease the rate of disease recurrence.

The BRAF proto-oncogene is an important genetic factor in patients with PTC. The V600E mutation in the BRAF protein, where a valine residue has been replaced with glutamic acid at position 600, is the most common alteration identified in PTC cases, and it promotes tumorigenesis by aberrantly activating the mitogen-activated protein kinase signaling pathway (4). The V600E mutation is exhibited in 45-80% of PTC cases (5,6). However, the reported incidence of this mutation is markedly affected by the diagnostic method used.

Previous studies have demonstrated that the BRAF V600E mutation is associated with aggressive clinicopathological characteristics of PTC (7,8). Previously, a number of methods have been used to detect BRAF mutations (9-17). The ideal assay should be highly accurate and sensitive to ensure the detection of the BRAF mutant. Although BRAF mutations have a marked clinical effect in patients with PTC, the methods have been compared in a small number of studies (18,19). Thus, the aim of the present study was to compare the different methods, including Sanger sequencing, the Cobas® 4800 BRAF V600

Test kit and IHC, for the detection of BRAF mutations with regard to their sensitivity, specificity, feasibility and limitations.

Materials and methods

Samples and DNA isolation. Clinical data of patients who underwent thyroidectomy at Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) between August 2015 and December 2015 were retrospectively reviewed. Eligibility criteria were as follows: Patients' acceptance of thyroidectomy plus neck lymph node dissection; absence of a history of neck surgery on the thyroid; and absence of other types of head and neck cancer. These patients were diagnosed with PTC during pathological examination. A total of 185 patients with PTC were included in the present study. All PTC samples were screened for BRAF mutation by IHC, Sanger sequencing and using the Cobas 4800 BRAF V600 Test kit. The present study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital, and informed consent was obtained from all patients.

Sections were cut from formalin-fixed paraffin-embedded (FFPE) tissues, with 10 μm thickness for DNA extraction and 4 μm thickness for IHC. All samples were stained with hematoxylin and eosin at room temperature, and evaluated for tumor content prior to proceeding to molecular analysis. DNA was extracted from unstained 10-μm-thick sections using the QIAmp DNA FFPE tissue kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. Quality and quantity of DNA samples were evaluated using a SPECTROstar Nano plate reader (BMG Labtech GmbH, Ortenberg, Germany).

Sanger sequencing. Sanger sequencing for BRAF mutations was performed as described previously (17). The primers used to amplify BRAF exon 15 were as follows: 5'-TCATAA TGCTTGCTCTGATAGGA-3' (forward) and 5'-GGCCAA AAATTTAATCAGTGGA-3' (reverse). Following denaturation at 95˚C for 10 min, 38 amplification cycles at 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 45 sec were performed. Samples were then extended at 72˚C for 10 min. Sequencing was performed using a BigDye Terminator Sequencing kit version 1.1 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol, and sequence reactions were electrophoresed on an ABI 3100 genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sequence data were analyzed using an ABI 3100XL DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Cobas 4800 BRAF V600 Test kit. The Cobas 4800 BRAF V600 Mutation Test kit (Roche Molecular Diagnostics, Pleasanton, CA, USA) was used to detect the V600 mutation by real-time polymerase chain reaction. A set concentration of DNA was analyzed on the Cobas® z 480 analyzer (Roche Molecular Diagnostics) according to the manufacturer's protocol. All runs and specimen validation were performed using the Cobas z 480 software (version 2.0; Roche Molecular Diagnostics). The results were generated automatically as a report using the Cobas z 480 software.

IHC. IHC was performed using anti-BRAF V600E (VE1) mouse monoclonal primary antibody (cat. no. 790-4855; Ventana Medical Systems; Roche Diagnostics, Basel, Switzerland), which is designed to specifically detect the BRAF V600E mutation (16). BRAF V600E-positive melanoma served as a positive control and a section without incubation in primary antibody served as a negative control. Tissue sections (4 μm) were dried, deparaffinized and rehydrated. Immunoreactions were performed using the BenchMark XT immunostainer (Roche Diagnostics). The staining protocol using the OptiView DAB IHC Detection kit (cat. no. 760-700; Roche Diagnostics) included pretreatment with cell conditioner 1 (pH 8.5) for 64 min, followed by incubation with VE1 antibody at 37˚C for 16 min. Antibody incubation was followed by counterstaining with hematoxylin II and bluing reagent at room temperature for 4 min each. Subsequently, slides were removed from the immunostainer, washed in water with a drop of dishwashing detergent and mounted. The stained slides were examined using an Olympus DP70 light microscope (Olympus Corporation, Tokyo, Japan) by two experienced pathologists blinded to clinical data. The intensity of staining was scored from 0 to 3+. Positive staining results for V600E staining (1+, 2+ and 3+) were considered when tumor cells showed positive cytoplasmic staining. Negative staining results (0) were determined when there was no or only slight staining in tumor cells.

Statistical analysis. All 185 samples were analyzed with three different methods. To analyze the specificity and sensitivity of each method, the reference response of each case was determined, as presented in Fig. 1. Statistical analysis was performed using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). The association between BRAF mutation and clinicopathological features was analyzed using a χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sanger sequencing. Of the 185 samples, mutations in the BRAF gene at V600E were detected in 141 (76.2%) samples using Sanger sequencing. The sensitivity and specificity of Sanger sequencing were 97.2 and 95.2%, respectively (Table I).

Cobas 4800 BRAF V600 test. Mutations in BRAF were detected in 146 (78.9%) samples using the Cobas 4800 BRAF V600 test. The sensitivity and specificity of the test were 99.3 and 90.5%, respectively (Table I).

IHC with VE1 antibody. Of the 185 cases, 142 cases were positive for BRAF V600E staining (76.8%) (Fig. 2). The sensitivity and specificity of IHC with VE1 antibody were 98.6 and 97.6%, respectively (Table I).

Comparison of the three methods. The Cobas 4800 BRAF V600 test proved to be the most sensitive method (99.3%), whereas the other techniques revealed a sensitivity of 98.6% for IHC and 97.2% for Sanger sequencing.

The properties of the three methods were also estimated (Table II). IHC was the cheapest and least time-consuming technique, and its specificity was higher compared with that of Sanger sequencing and the Cobas 4800 BRAF V600 test. Sanger sequencing was a reliable analysis, although

Table I. Performance of the three diagnostic methods for BRAF detection.

Method	Detection of BRAF mutation					
	Wild-type, n (%)	Mutation, n (%)	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
Sanger sequencing	44 (23.8)	141 (76.2)	97.2	95.2	98.6	90.9
Cobas 4800 BRAF V600	39 (21.1)	146 (78.9)	99.3	90.5	97.3	97.4
Immunohistochemistry	43 (23.2)	142 (76.8)	98.6	97.6	99.3	95.3

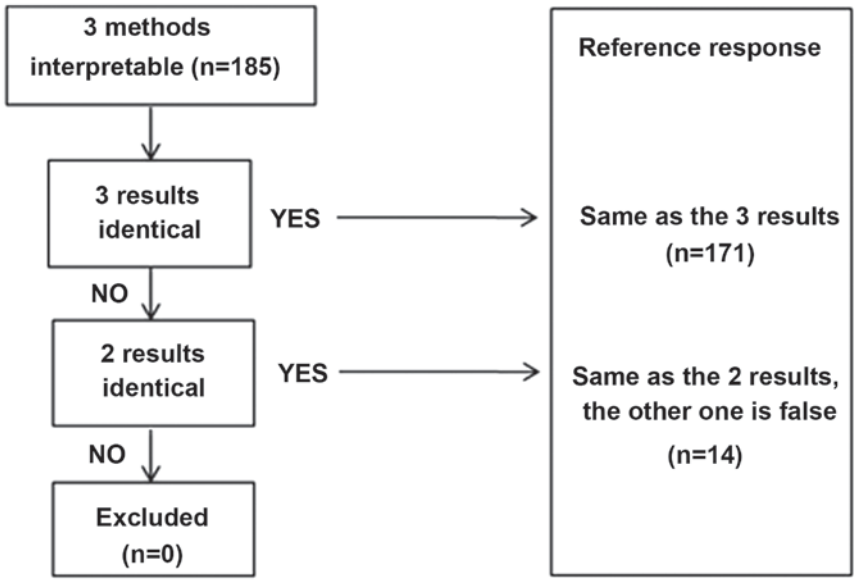

Figure 1. Determination of the reference responses by the results of the three diagnostic methods.

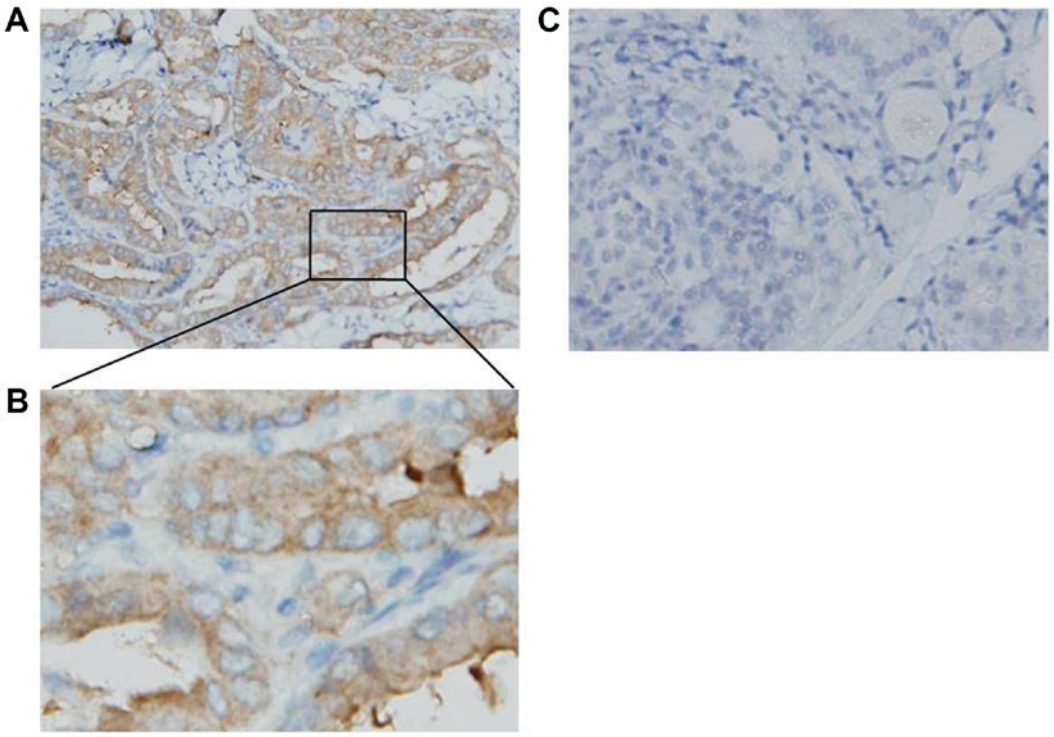

Figure 2. BRAF V600E expression in papillary thyroid carcinoma tissues using VE1 antibody. Representative images of samples positive (A) at x100 magnification, (B) at x200 magnification and (C) negative (x100 magnification) for BRAF expression.

Table II. Characteristics of the three diagnostic methods.

	Method		
Characteristic	Cobas 4800 BRAF V600	Immunohistochemistry	Sanger sequencing
CE-IVD mark	Yes	No	No
Specificity, %	90.5	97.6	95.2
Sensitivity, %	99.3	98.6	97.2
Detection of rare mutations	No	No	Yes
Turnaround time, days	1	1	2-3
Costs	Medium	Low	Medium

Table III. Association between BRAF mutation status and clinicopathological features in patients with papillary thyroid cancer.

	BRAF mutation status			
Feature	Negative, n (%)	Positive, n (%)	χ^2	P-value
Age, years			0.015	0.902
<55	35 (22.9)	118 (77.1)		
≥55	7 (17.6)	25 (82.4)		
Sex			0.400	0.527
Female	30 (21.6)	109 (78.4)		
Male	12 (26.1)	34 (73.9)		
Lymph node metastasis			1.650	0.199
Yes	25 (26.6)	69 (73.4)		
No	17 (18.7)	74 (81.3)		
Central node metastasis			0.550	0.458
Yes	23 (25.0)	69 (75.0)		
No	19 (20.4)	74 (79.6)		
Lateral node metastasis[a]			0.404	0.525
Yes	11 (30.6)	25 (69.4)		
No	3 (42.9)	4 (57.1)		
Extrathyroid extension			4.040	0.044
Yes	4 (10.5)	34 (89.5)		
No	38 (25.9)	109 (74.1)		
Multifocality			5.226	0.022
Yes	5 (10.6)	42 (89.4)		
No	37 (26.8)	101 (73.2)		
Stage			0.072	0.789
I and II	38 (23.3)	125 (76.7)		
III and IV	4 (18.2)	18 (81.8)		
Tumor size, cm			0.384	0.535
≤1	10 (19.6)	41 (80.4)		
>1	32 (23.9)	102 (76.1)		

[a]Lateral neck dissection was performed in 43 patients.

time-consuming. However, rare mutations in codon 600 could be detected using Sanger sequencing, but not with the other two methods.

In the present study, the results of 14 samples were discordant between the three methods. The analysis of the results revealed that there was complete agreement in

171/185 cases (92.4%). Using the results from two methods only, the highest concordance rate was observed with the combination of the Cobas 4800 BRAF V600 testing and IHC (177/185 samples) (95.7%).

Association between BRAF mutations and clinicopathological features. The association of BRAF mutation and clinicopathological features was analyzed using a χ^2 test. As presented in Table III, the presence of the BRAF mutation was associated with extrathyroid extension and multifocality ($P<0.05$), but not associated with age, sex, lymph node metastasis, central node metastasis, lateral node metastasis, Tumor-Node-Metastasis stage or tumor size ($P>0.05$).

Discussion

The effect of BRAF mutation on the diagnosis and prognosis of PTC has been extensively investigated. Previous studies have assessed the BRAF V600E mutation as a prognostic factor for PTC (20-22), of which a number have revealed an association of the BRAF V600E mutation with aggressive clinicopathological features of PTC (6,23-25). Similarly, in the present study, the BRAF mutation was associated with extrathyroid extension and multifocality of PTC. To date, the evidence supporting routine preoperative BRAF testing to determine the clinical treatment remains insufficient. The clinical use of BRAF testing must be clarified through prospective and randomized trials, and its molecular mechanism investigated. The BRAF mutation, as a promising marker, may improve diagnosis and risk stratification of patients with PTC.

To identify the optimal method in routine clinical diagnostics, three methods for the detection of BRAF mutations in PTC were compared. For a number of years, Sanger sequencing has been considered to be the most reliable method to detect BRAF mutations in diagnostic laboratories. However, if there is a low percentage of tumor cells in the sample, it may not be a feasible method (26,27). In the present study, four samples in which a V600E mutation was detected by the two other methods yielded a negative result when tested with Sanger sequencing. The sensitivity and specificity of Sanger sequencing were 97.2 and 95.2%, respectively. The results revealed that the sensitivity of Sanger sequencing was lower compared with that of the Cobas 4800 BRAF V600 test and IHC.

The results of the present study indicated that immunostaining with the anti-BRAF V600E antibody is highly sensitive in detecting the BRAF V600E mutation in PTC tissue. Of the 185 samples, two were negative for the mutation according to IHC, but were positive according to the other two methods. The sensitivity and specificity of IHC were 98.6 and 97.6%, respectively. IHC is cheaper and widely available in most diagnostic laboratories, so this method may be used as the first analysis. Although the IHC antibody has cross-reactivity with other BRAF mutations, including V600K (28) and V600R (18), it is designed to detect the V600E mutation, and therefore may not reliably detect other mutations.

The Cobas 4800 BRAF V600 test, unlike the other two included in the present study, meets the requirements regarding safety, health and environmental protection that are essential for the European Conformity-*In Vitro* Diagnostics CE-IVD mark.

As with the IHC test, a major limitation of the Cobas 4800 BRAF V600 test is that it is designed to detect the BRAF V600E mutation, and therefore does not detect other mutations. The sensitivity of the Cobas 4800 BRAF V600 test was 99.3%, which was more sensitive compared with that of IHC and Sanger sequencing. Although the Cobas 4800 BRAF V600 test requires a small amount of DNA, it may be not able to detect the mutation if <10% of a sample consists of tumor cells (27). However, Lopez-Rios *et al* (29) demonstrated a lower limit of detection of 5% mutant alleles for the V600E mutation.

Although the Cobas 4800 BRAF V600 test relies on molecular genetic techniques that are standard diagnostic methods to detect BRAF mutations, it requires high tumor content and special equipment. IHC is a relatively cheap, accurate and reliable screening method, and can be used routinely for BRAF mutation analysis in clinical diagnostics.

The present study has a number of limitations. First, no gold standard method to detect the BRAF V600E mutation was included. However, improved concordance was observed with the combination of two methods, particularly the Cobas 4800 BRAF V600 test and IHC. Secondly, the present study was performed at a single center and included a small patient population.

In conclusion, the results of the present study demonstrated that IHC is a simple, highly sensitive and reliable screening method for detection of the V600E mutation, which can be used as a first-line method in routine clinical diagnostics. Furthermore, at least one molecular method should be performed in negative or doubtful cases. Therefore, a combination of IHC and the Cobas 4800 BRAF V600 test may be the most efficient and reliable method in routine practice.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JZZ, PPL and JTZ performed the experiments and drafted the manuscript. JPY, YY and MG participated in the design of the study. XQZ performed the statistical analysis and helped to draft the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (Tianjin), and informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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