ORIGINAL RESEARCH

BRAF\textsuperscript{V600E} mutation analysis in fine‐needle aspiration cytology specimens for diagnosis of thyroid nodules: The influence of false‐positive and false‐negative results

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Abstract

**Background:** The accurate evaluation of BRAF\textsuperscript{V600E} mutation in preoperative fine‐needle aspiration cytology (FNAC) specimens is important for making management decisions in thyroid nodules (TNs). The aim of this study was to assess the false‐positive and false‐negative BRAF\textsuperscript{V600E} mutations in thyroid FNAC specimens and their influence on diagnosis of TN.

**Methods:** This prospective study enrolled 292 nodules in 269 patients who underwent BRAF\textsuperscript{V600E} mutation analysis using amplification refractory mutation system‐quantitative real‐time polymerase chain reaction (ARMS‐qPCR) both in FNAC specimens and formalin‐fixed, paraffin‐embedded (FFPE) tissue samples after surgery. The false‐positive and false‐negative mutations for BRAF\textsuperscript{V600E} analysis using ARMS‐qPCR in FNAC specimens were recorded, with reference to the results of BRAF V600E mutation analysis using ARMS‐qPCR in FFPE tissue sample. Diagnostic performances of FNAC, BRAF\textsuperscript{V600E} mutation analysis in FNAC specimens, BRAF V600E mutation analysis in FFPE tissue sample, and the combination of FNAC and BRAF\textsuperscript{V600E} mutation analysis for predicting thyroid malignancy were assessed.

**Results:** The false‐positive and false‐negative mutations for BRAF\textsuperscript{V600E} analysis using ARMS‐qPCR in FNAC specimens were 10.1% (19/189) and 7.1% (7/98), respectively. FNAC combined with preoperative BRAF\textsuperscript{V600E} mutation analysis significantly increased the diagnostic sensitivity from 75.7% to 92.3%, and accuracy from 78.7% to 90.6% in comparison with FNAC alone (both \( P < .001 \)). No significant differences were found between the combination of FNAC and BRAF\textsuperscript{V600E} mutation analysis in FNAC specimens and the combination of FNAC and BRAF\textsuperscript{V600E} mutation analysis in FFPE tissue sample (sensitivity: 92.3% vs 91.9%; accuracy: 90.6% vs 91.3%; both \( P > .05 \)).

**Conclusions:** FNAC combined with preoperative BRAF\textsuperscript{V600E} mutation analysis can significantly increase the diagnostic performance in comparison with FNAC alone. False‐positive and false‐negative BRAF\textsuperscript{V600E} mutation results are found in preoperative FNAC specimens, whereas it does not affect the overall auxiliary diagnosis of TNs.
Ultrasound (US)-guided fine-needle aspiration cytology (FNAC) is a standard diagnostic method for thyroid nodules (TNs), but some cytological results such as nondiagnostic, indeterminate, and false-negative results might cause confusion in the management of TNs. To overcome these limitations, several molecular markers have been applied in combination with FNAC results. Among various molecular markers related to thyroid cancer, BRAFV600E mutation is the most common molecular marker found in papillary thyroid carcinoma (PTC). Because the BRAFV600E test has a high positive predictive value (PPV), it can be used to improve the diagnostic accuracy of FNAC and to overcome the limitations of FNAC as mentioned above. In addition, many studies have found that the presence of a BRAFV600E mutation is associated with more aggressive tumor characteristics, such as extrathyroidal extension, lymph node involvement, resistance to radioactive iodide, PTC recurrence, and PTC-related mortality.

Some authors advocated preoperative BRAFV600E mutation analysis as an aid for improving risk stratification of PTC patients in order to define a more individualized treatment plan. Therefore, it is very important to evaluate the accuracy of BRAFV600E mutation analysis in preoperative FNAC specimens.

Conventional Sanger sequencing is a standard method to detect the BRAFV600E mutation because of its high reliability. However, this molecular technique is not sensitive enough for the detection of low frequency mutations (less than 20%) in the sample, which leads to a higher false-negative result. Moreover, this methodology is not suitable for batch testing, which limits its clinical application. Of note, the BRAFV600E mutation is present only in the malignant cells in thyroid FNAC specimens which are admixed with other cell types and blood. Therefore, a highly sensitive assay is required to detect the BRAFV600E mutations in FNAC specimens. Efforts to increase sensitivity have produced methods that can detect BRAFV600E with a limit of detection (LOD) in as few as 0.1%-2% of the total cell population. However, false-positive mutations for the BRAFV600E mutation are present in 0.08%-5.4% of the cases when highly sensitive analytic methods such as pyrosequencing, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), dual priming oligonucleotide (DPO)-based multiplex PCR, mutant enrichment with 3’-modified oligonucleotide (MEMO) sequencing, real-time PCR, or droplet digital PCR are used.

Recently, the amplification refractory mutation system-quantitative real-time PCR (ARMS-qPCR) method is developed to specifically detect the BRAFV600E mutation with high sensitivity (0.1%-1% of total cells), which can provide quantitative and rapid (<4 hour) result. And, it has been commercially used at a low cost of $87/test in clinical practice. However, there has been no report evaluating the false-positive and false-negative mutation results of BRAFV600E analysis in thyroid FNAC specimens before surgery using ARMS-qPCR until now, which may cause uncertainty in interpreting the BRAFV600E analysis and the following management. Thus, the purpose of this study was to assess the false-positive and false-negative BRAFV600E mutation results using ARMS-qPCR in preoperative thyroid FNAC specimens, with reference to the BRAFV600E mutation results using ARMS-qPCR in formalin-fixed, paraffin-embedded (FFPE) tissue samples after surgery. In addition, we also investigated whether those false results would affect the added diagnostic value of BRAFV600E mutation analysis to FNAC for evaluation of TNs.
analysis did not match those subject to histopathologic examination for BRAF^{V600E} mutation analysis. Additionally, two nodules in two patients were excluded because of PCR failures. Finally, the study group consisted of 264 patients with 287 TNs (Figure 1). Of the 264 patients, one nodule per patient was analyzed in 241 patients, and two nodules per patient were analyzed in 23 patients.

2.2 | US-guided FNAC procedure

All FNAC procedures were performed under US guidance by one of the three radiologists who had more than 3 years experience in thyroid FNAC and more than 5 years experience in thyroid US. Using a high-frequency linear array transducer with the US instrument, the FNAC specimens were obtained from the nodule with a 5-mL syringe. Local anesthesia was performed routinely with 1% lidocaine. The needle was inserted into the nodule and moved rapidly back and forth in different directions within the nodule when suction was applied. For BRAF^{V600E} mutation analysis, after acquisition of the optimal amount for cytological diagnosis, additional aspiration was performed to isolate genomic DNA. The FNAC aspirates were expressed onto frosted-end glass slides and immediately fixed in 95% alcohol for hematoxylin and eosin (H&E) staining.

One of the three cytopathologists experienced in thyroid cytology reviewed all the FNAC specimens in the Department of Pathology of the University Hospital. According to the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) criterion, cytological diagnoses consist of six categories: (I) Nondiagnostic; (II) Benign; (III) Atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS); (IV) Follicular neoplasm or suspicious for follicular neoplasm (FN/SFN); (V) Suspicious for malignancy (SUSP); (VI) Malignant. The criterion for an adequate smear was the presence of six groups of cells with more than 10 cells per group.

2.3 | Detection of BRAF^{V600E} mutation by ARMS-qPCR in FNAC specimens

BRAF^{V600E} mutation analysis was also performed at the Department of Pathology in the University Hospital by a single PCR operator who had more than 3 years experience in BRAF^{V600E} mutation analysis of thyroid specimens.

2.3.1 | Manual macrodissection of the FNAC slide

The “manual macrodissection” methodology chosen for the molecular study has been previously described and is routinely used in the laboratory. Firstly, the tumor cells or suspicious cells from H&E staining FNAC smears were identified and selected by cytopathologists using microscopy to assure adequate thyroid cell representation. After choosing the most representative slide or marking the regions of the slides containing numerous lesional thyrocytes, the slides were deparaffinized using xylene and ethanol. Once the slide was air dried, lysis solution without proteinase K was poured on the slide to scrape material from the entire smear or marked area of the slide using a single-edged razor blade. Finally, the scraped tissues were collected to a microcentrifuge tube including lysis solution with proteinase K for the DNA extraction and analysis.

2.3.2 | DNA extraction, quantification, and dilution

DNA extraction was successfully completed in all samples following the manufacturer’s instructions with a DNA

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**FIGURE 1** Flowchart of patient enrollment and exclusion
extraction kit (ADx-FF01). After DNA isolation, its concentration was assessed using a NanoDrop 2000 spectrophotometer (Thermo). For identification of DNA purity and quality, the spectral absorbance (OD value) and the 260/280 and 260/230 ratios were calculated. The purity of DNA was considered high if the OD 260/280 ratio was close to 1.8, whereas an OD 260/280 ratio of >2.0 is suggestive for RNA contamination, and an OD 260/280 ratio of <1.6 is indicative of organic solvent contamination. DNA with an OD 260/230 >2.0 was diluted to ~1 ng/μL with elution buffer ATE (QIAGEN). The extracted DNA was stored in a −20°C refrigerator (Haier Pharmaceutical Co., Ltd.) until used.

2.3.3 | BRAF V600E mutation detection

A validated, China Food and Drug Administration (CFDA)-approved fluorescent PCR detection Kit (ADx-ARMS, Amoy Diagnostics Co. Ltd) based on ARMS technique was used for the detection of the BRAF V600E mutation in the samples. The ARMS-qPCR is a highly sensitive method employed for specifically detecting the BRAF V600E (c.T1799A in exon 15). Using specific primers, the ARMS-qPCR amplifies the target sequence and the amplified products are analyzed by amplicon-specific fluorescent probes (double loop probes). Briefly, each PCR reaction system included 5 μL of extracted DNA, 0.4-μL TaqDNA polymerase, and 35-μL reaction mixture in a kit containing oligonucleotide primers, dNTPs, double loop probes, MgCl2, ammonium sulfate, and potassium chloride. The PCR reaction was carried out on ABI 7900 Fast real-time fluorescence-PCR machine (Applied Biosystems) with the following conditions: cycle 1 with an initial denaturation step at 95°C for 5 minutes; cycle 2:15 annealing cycles at 95°C for 25 seconds, 64°C for 20 seconds, and 72°C for 20 seconds, followed by cycle 3:31 extension cycles at 93°C for 25 seconds, 60°C for 35 seconds, 72°C for 20 seconds. The FAM (mutation type) and VIC (wild-type) signals were captured at 60°C during cycle 3. Fluorescence increases geometrically corresponding to the exponential increase of the PCR products, which is used to determine the threshold cycle (Ct value). The Ct value was calculated automatically by this system at the end of the reaction. Each run contained a negative control and a positive control.

If the collected signals met the following three criteria, the testing was regarded as success and the results trustworthy: Firstly, the VIC signal and FAM signal of the positive control rose and the Ct value of the FAM signal was less than 20. Secondly, the VIC signal and FAM signal of the negative control did not rise. Thirdly, the VIC signals of the sample and the positive control were supposed to rise and the VIC signal Ct value of the sample ranged from 13 to 21. If the Ct value of the FAM signal in the sample was less than 28, it was considered to indicate the presence of a BRAF V600E mutation, otherwise the sample was deemed to be negative for BRAF V600E.

2.4 | Detection of BRAF V600E mutation by ARMS-qPCR in FFPE tissue samples

The ARMS-qPCR analysis for BRAF V600E mutation was performed on the same nodule as the preoperative FNAC by macrodissection on FFPE slides after surgery. Four sections (5-μm thick) were cut from FFPE tumor tissue blocks and were mounted onto microscopic slides. One section slide was stained with H&E for histopathological examination. The H&E staining section was used as a reference, and tumor-rich regions of the sections were chosen from the three slides based on H&E staining patterns. Three 5-μm thick FFPE slides were deparaffinized using xylene and ethanol. The tumor-rich regions of the three sections slides were scraped using a single-edged razor blade and were collected into a microcentrifuge tube. DNA extraction, quantification and dilution, and BRAF V600E mutation detection procedure in FFPE tissue samples were similar as those in FNAC specimens except for DNA was diluted to ~2 ng/μL with elution buffer ATE (QIAGEN).

2.5 | Statistical analysis

BRAF V600E mutation analysis in the histological diagnosis after surgery was used as the reference standard. The following four entities were considered: True-positive mutation = BRAF V600E mutations detected both in FNAC specimens and FFPE tissue samples; True-negative mutation = no BRAF V600E mutations detected both in FNAC specimens and FFPE tissue samples; False-positive mutation = BRAF V600E mutations detected in FNAC specimens but not in FFPE tissue samples; False-negative mutation = BRAF V600E mutations negative in FNAC specimens, whereas positive in FFPE tissue samples. The rates of true-positive mutation, true-negative mutation, false-positive mutation, and false-negative mutation were calculated.

Statistical analyses were performed with SPSS version 19.0 software (IBM Corporation, Armonk, NY). With the histopathological analysis after surgery as the reference standard, the sensitivity, specificity, PPV, negative predictive value (NPV), and accuracy were calculated by the diagnostic test 2 × 2 contingency table. With regard to FNAC results for predicting thyroid malignancy, FNAC results of SUSP and malignant cytology were considered as malignant, whereas the others were considered as benign. With regard to BRAF V600E analysis in FNAC specimens or FFPE tissue samples for predicting thyroid malignancy,
BRAFV600E-positive mutation was considered as malignancy, whereas BRAFV600E-negative mutation was considered as benign. For the combination of FNAC results and BRAFV600E analysis in FNAC specimens or FFPE tissue samples for predicting thyroid malignancy, either of two methods diagnosed the nodule as malignant was regarded as malignant, while only both two methods diagnosed the nodule as benign was regarded as benign. The McNemar test was used to compare the differences in sensitivity, specificity, and accuracy, whereas Chi-squared test or Fisher exact test was used in PPV and NPV. A P-value of .05 or less was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | Final diagnosis

The 264 patients included 62 men and 202 women with a mean age ± standard deviation of 46.4 ± 13.6 years (range, 19-75 years). The mean size of nodules at the longest diameter was 10.8 ± 6.7 mm (range, 5-48 mm) (Table 1).

Sixty-five nodules were histologically confirmed as benign, and 222 nodules confirmed by pathological specimens were malignant. The benign nodules included 32 nodular hyperplasias, 14 Hashimoto's nodules, 13 adenomatous hyperplasias,

### Table 1 Basic characteristics of patients and nodules

| Characteristic                      | Patients, n 264 |
|-----------------------------------|----------------|
| Patient sex                       | Men, n 62      |
|                                   | Women, n 202    |
| Age, yr 46.4 ± 13.6 (19-75)       | Nodules, n 287 |
| Nodule size, mm 10.8 ± 6.7 (5-48) | Malignant nodules, n 222 |
| Papillary thyroid Carcinoma, n (%)| Follicular thyroid carcinoma, n (%) 3 (1.4) |
| Anaplastic thyroid carcinoma, n (%)| 1 (0.4) |
| Benign nodules, n 65              | Nodular hyperplasia, n (%) 32 (49.2) |
| Hashimoto's nodule, n (%) 14 (21.6) | Adenomatous hyperplasia, n (%) 13 (20) |
| Follicular adenoma, n (%) 4 (6.2) | Subacute thyroiditis nodule, n (%) 1 (1.5) |
| Parathyroid nodule, n (%) 1 (1.5) | |

Note: Data are presented as mean ± SD (range) where applicable.

Abbreviation: n, number.

### Table 2 The results of preoperative BRAFV600E mutation analysis using ARMS-qPCR in relation to FNAC results and final histology after surgery

| Cytology | Nondiagnostic (n = 12) | Benign (n = 36) | AUS/FLUS (n = 57) | FN/SFN (n = 5) | SUSP (n = 46) | Malignant (n = 222) | Total (n = 287) |
|----------|------------------------|----------------|-------------------|---------------|--------------|-------------------|-----------------|
| BRAFV600E | (+)                    | (+)            | (+)               | (+)           | (+)          | (+)               | (+)            |
|          | (−)                    | (−)            | (−)               | (−)           | (−)          | (−)               | (−)            |
|          | (±)                    | (±)            | (±)               | (±)           | (±)          | (±)               | (±)            |

Abbreviations: ARMS-qPCR, amplification refractory mutation system-quantitative real-time polymerase chain reaction; FNAC, fine-needle aspiration cytology; SUSP: suspicious for malignancy; follicular neoplasm or suspicious for follicular neoplasm; FNAC, fine-needle aspiration cytology; SUSP, suspicious for malignancy.
four follicular adenomas, one subacute thyroiditis nodule, and one parathyroid nodule. Thyroid malignancy consisted of 218 PTCs, three follicular thyroid carcinomas (FTCs), and one anaplastic thyroid carcinoma (ATC) (Table 1).

3.2 | FNAC results

Of 287 FNAC analyses performed (Table 2), 12 (4.2%) nodules were reported to be nondiagnostic (Bethesda I), 38 (13.6%) nodules benign cytology (Bethesda II), 108 (37.6%) indeterminate cytologies (57 [19.9%] AUS/FLUS [Bethesda III], 5 [1.7%] FN/SFN [Bethesda IV], and 46 [16.0%] SUSP [Bethesda V]), and 129 (45.6%) malignant cytology (Bethesda VI).

3.3 | BRAFV600E mutation analysis in FNAC specimens

The BRAFV600E mutation rates were 25% (3/12) in the nondiagnostic cytology, 26% (10/38) in benign cytology, 46% (26/57) in AUS/FLUS cytology, 20% (1/5) in FN/SFN cytology, 74% (115/129) in SUSP cytology, and 89% in malignant cytology, respectively (Table 2).

3.4 | BRAFV600E mutation analysis in FFPE tissue samples

Among the 222 malignant nodules, 177 (79.7%) showed positive results for the BRAFV600E mutation analysis in FFPE tissue samples. The prevalence of PTC in thyroid malignancy was 98.1% (218/222) and the positive mutation rate of BRAFV600E in PTC was 81.2% (177 of 218). No positive BRAFV600E mutation was found in the three FTCs, one ATC, and four nodular hyperplasias which showed BRAFV600E mutation in FNAC specimens.

3.5 | Consistency of BRAFV600E mutation analysis between FNAC specimens and FFPE tissue samples

Consistency of BRAFV600E mutation analysis between FNAC specimens and FFPE tissue samples occurred in 90.9% (261/287) of TNs while inconsistency in 9.1% (26/287) of nodules. The true-positive mutation rate was 89.9% (170/189) and true-negative mutation rate was 92.9% (91/98).

### Table 3: Characteristics of patients who showed false-positive mutation by ARMS-qPCR for BRAFV600E analysis in FNAC specimens

| No. | Age (yr) | Sex | US size (mm) | FNAC results | BRAFV600E mutation analysis |
|-----|----------|-----|--------------|--------------|-----------------------------|
|     |          |     |              |              | FNAC Specimens ARMS-qPCR | FFPE Tissue Samples ARMS-qPCR | Histology          |
| 1   | 53       | F   | 12           | II           | +                           | −                           | Nodular Hyperplasia |
| 2   | 41       | F   | 26           | II           | +                           | −                           | Nodular Hyperplasia |
| 3   | 50       | F   | 6            | III          | +                           | −                           | Nodular Hyperplasia |
| 4   | 32       | F   | 5            | V            | +                           | −                           | Nodular Hyperplasia |
| 5   | 25       | F   | 5            | VI           | +                           | −                           | Classic PTC        |
| 6   | 75       | F   | 6            | V            | +                           | −                           | Classic PTC        |
| 7   | 63       | F   | 9            | VI           | +                           | −                           | Classic PTC        |
| 8   | 38       | F   | 7            | VI           | +                           | −                           | Classic PTC        |
| 9   | 58       | F   | 5            | III          | +                           | −                           | Classic PTC        |
| 10  | 28       | F   | 6            | VI           | +                           | −                           | Classic PTC        |
| 11  | 33       | F   | 5            | VI           | +                           | −                           | Classic PTC        |
| 12  | 29       | F   | 6            | VI           | +                           | −                           | Classic PTC        |
| 13  | 42       | F   | 9            | VI           | +                           | −                           | Classic PTC        |
| 14  | 35       | F   | 8            | II           | +                           | −                           | Classic PTC        |
| 15  | 19       | F   | 5            | V            | +                           | −                           | Classic PTC        |
| 16  | 56       | F   | 5            | VI           | +                           | −                           | Classic PTC        |
| 17  | 46       | F   | 9            | VI           | +                           | −                           | Classic PTC        |
| 18  | 57       | F   | 10           | VI           | +                           | −                           | Classic PTC        |
| 19  | 23       | M   | 15           | IV           | +                           | −                           | Minimally Invasive FTC |

**Abbreviations**: ARMS-qPCR, amplification refractory mutation system-quantitative real-time polymerase chain reaction; FNAC, fine-needle aspiration cytology; FTC, follicular thyroid carcinomas; PTC, papillary thyroid carcinoma; US, ultrasound.
The inconsistency group consisted of 26 nodules in 26 patients (24 women and 2 men; mean age ± standard deviation, 34 ± 14 years; range, 19-75 years). In 73.1% (19/26) of the inconsistent nodules, BRAFV600E mutations were detected in FNAC specimens, whereas not in FFPE tissue samples. These results were considered as false-positive mutation. Thus, the false-positive mutation rate was 10.1% (19/189). Of 19 false-positive mutation nodules, four were nodular hyperplasias, one was minimally invasive FTC, and 14 were classic PTCs (Table 3) (Figures 2, 3).
In the remaining 26.9% (7/26) nodules, BRAFV600E mutation was negative in FNAC specimens, whereas positive in FFPE tissue samples, indicating false-negative mutation. Thus, the false-negative mutation rate was 7.1% (7/98). Of the seven false-negative mutation nodules, six were classic PTCs and one was mixture of follicular variant of PTC and classic PTC (Table 4) (Figure 4).

FIGURE 3 The false-positive mutation for BRAFV600E analysis using ARMS-qPCR in preoperative FNAC specimen from a 28-year-old woman with classic PTC. A, Transverse US scan of left thyroid reveals a 6-mm solid marked hypoechoic nodule (white arrow), with taller-than-wide shape and microcalcifications. B, The cytologic diagnosis from US-guided FNAC is malignancy (H&E staining, ×400). C, BRAFV600E analysis using ARMS-qPCR in FNAC specimens shows positive mutation result (The light green amplification plot of the sample shows that the VIC signal and FAM signal [red arrow] rise, and the Ct value of the FAM signal is 21.75). D, Histology of surgical specimens shows classic PTC (H&E staining, ×100). E, BRAFV600E analysis using ARMS-qPCR in FFPE tissue samples shows negative mutation result (The light green amplification plot of the sample shows that the VIC signal rises but FAM signal [red arrow] does not rise).
### 3.6 Diagnostic performances of FNAC, BRAFV600E analysis in FNAC specimens, BRAFV600E analysis in FFPE tissue samples, and combination of FNAC and BRAFV600E analysis

Concerning the diagnostic performance for TNs, FNAC showed a sensitivity of 75.7%, specificity of 89.2%, PPV of 95%, NPV of 51.7%, and accuracy of 78.7% in diagnosing thyroid malignancy, with the histopathological analysis after surgery as the reference standard (Table 5).

Adding the molecular test of BRAF V600E mutation analysis to FNAC significantly improved the diagnostic performance, with sensitivity increasing from 75.7% to 92.3% ($P < .001$), and accuracy from 78.7% to 90.6% ($P < .001$) (Table 5). Forty nodules with negative cytology (nondiagnostic, benign, AUS/FLUS, and FN/SFN) presented positive results for BRAFV600E by ARMS‐qPCR analysis. These patients underwent thyroidectomy. Three showed benign (all nodular hyperplasias) and 37 showed malignancy in the final histology (three false‐positive mutation cases including one minimally invasive FTC and two classic PTCs).

To evaluate whether the inconsistency of BRAFV600E mutation analysis before and after surgery would affect the diagnostic performance of combination of FNAC and BRAFV600E mutation analysis, we performed the following comparison study and found that no significant differences were found between the combination of FNAC and BRAFV600E mutation analysis in FNAC specimens and the combination of FNAC and BRAFV600E mutation analysis in FFPE tissue samples (sensitivity: 92.3% vs 91.9%; accuracy: 90.6% vs 91.3%; both $P > .05$) (Table 5).

### 4 DISCUSSION

In the present study, 98.1% of thyroid cancers were PTCs and the prevalence of BRAFV600E in PTC patients was 81.2%, which were consistent with other reports from China.\(^{20,22,25}\) Similarly, 95% or more of thyroid cancers are PTCs and 80% or more of PTCs harboring the BRAFV600E mutation in another Eastern country of South Korea.\(^{6,17}\) The prevalence of PTC in thyroid cancer (80%-90%) and the prevalence of BRAFV600E mutation in PTC (30%-50%) in Western countries, however, are much lower than that in Eastern Asian countries.\(^{12,17}\) These differences may originate from the detection methods used and the variable prevalence of genetic alterations in thyroid malignancy according to ethnicity, geographic area, and iodine consumption.

Comparing with previous studies,\(^{5,6,12,17}\) we found a higher false-positive mutation rate using ARMS‐qPCR (10.1%) than other PCR‐based methods (0.08%-5.4%). To the best of our knowledge, those studies evaluated BRAFV600E mutation only in FNAC specimens, but not in both FNAC specimens and surgical specimens. Surgically proven benign cases of false‐positive BRAFV600E mutation have been documented in the literature. Using preoperative conventional Sanger sequencing for BRAFV600E mutation testing, Chung et al\(^ {17}\) found one false‐positive mutation case that was proven to be an adenomatoid nodule with indeterminate FNAC result. Kim et al\(^ {26}\) reported two false‐positive mutation cases among 17 nodules treated by thyroidectomy, which were confirmed to be an adenomatoid hyperplasia with underlying lymphocytic thyroiditis and a fibrotic nodule with dense calcification. Using the preoperative DPO‐based multiplex PCR analysis, Direnzo et al\(^ {27}\) reported one false‐positive mutation case that was proven to be an adenomatoid nodule with indeterminate FNAC result. Kim et al\(^ {5}\) reported five false‐positive mutation cases that surgery revealed to be benign (one follicular adenoma and four nodular hyperplasias). In our study, we also observed four false‐positive mutation cases, which
were confirmed to be benign (all nodular hyperplasias). The false-positive mutation results for benign nodules may cause unnecessary thyroid surgery. In additional, we found 15 false-positive mutation cases, which were confirmed to be malignant but no BRAF \textsuperscript{V600E} mutations were detected in FFPE tissue samples (one minimally invasive FTC and 14 classic PTCs). Thus, the real false-positive mutation rate of BRAF \textsuperscript{V600E} testing methods in FNAC specimens may be underestimated. Additionally, this might be attributed to the difference in detection mechanisms of the difference assays. The ARMS-qPCR, similar to DPO-based multiplex PCR, is based on the ARMS method which is known to
have a relatively high false-positive rate. The false-positive mutation is a result of the overly sensitive assay. On the other hand, it is noteworthy that of the 19 false-positive mutation nodules, 15 (79%, 15/19) were malignant, including one minimally invasive FTC and 14 classic PTCs. Therefore, the false-positive mutation results seem not affect the following management strategy.

Seven false-negative mutation cases were found, which were confirmed to be malignant and BRAF 

V600E mutations were detected in FFPE tissue samples (six were classic PTCs and one was a mixture of follicular variant of PTC and classic PTC). One of the possible reasons might be that the FNAC specimen was admixed with normal thyroid cells, other type cells, and blood. And, the LOD for mutant alleles was as low as 1% for the ARMS-qPCR method used in the present study.28,29 Thus, to reduce the possibility of false-negative results due to low numbers of tumor cells, it is necessary to select more regions of representative slides for assuring adequate lesional thyrocytes in “manual macrodissection” method.

The added value of BRAF 

V600E test is its ability to detect PTCs that might have been missed by FNAC. Examples may include tumors with indeterminate (AUS/FLUS and FN/SFN), benign, and nondiagnostic cytology. In our study, the molecular test increased the sensitivity of FNAC from 75.7% to 92.3%. Diagnostic accuracy also increased from 78.7% to 90.6%. These findings were consistent with those of Kim et al.,5 who reported gains in sensitivity (from 67.5% to 89.6%) and accuracy (from 90.9% to 96.6%). The greatest increase in sensitivity came from the detection of PTC in the patients with indeterminate (AUS/FLUS and FN/SFN) cytology of FNAC results. About 10%–75% nodules with indeterminate (AUS/FLUS and FN/SFN) cytology are follicular neoplasms which cannot be reliably differentiated from malignancy by FNAC and intraoperative frozen section analysis.30 This creates a difficult clinical dilemma concerning the extent of surgery (total thyroidectomy or hemithyroidectomy). The molecular test for indeterminate FNAC result patients may be beneficial in planning the extent of surgery. It is interesting that no significant difference in sensitivity and accuracy were found between the combination of FNAC and the BRAF 

V600E mutation analysis in FNAC specimens and the combination of FNAC and the BRAF 

V600E mutation analysis in FFPE tissue samples. The reason is that the majority of (17/26) inconsistent cases show malignant or suspicious malignant cytology in FNAC results, in which molecular test did not change the cytological judgment for TNs. These results suggested that those false-positive and false-negative mutation cases using ARMS-qPCR for the BRAF 

V600E analysis in preoperative FNAC specimen would not affect the main outcomes of molecular test as a complementary diagnostic tool to FNAC in the diagnosis of TNs. Meanwhile, considering the final assessment of TNs, the value of molecular test should be evaluated in combination with FNAC result.

At last, several limitations are existed in this study. First, surgery-proven benign TNs were not tested for BRAF 

V600E mutation analysis except for those showing BRAF 

V600E mutation in FNAC specimens. As we know, no nodules reported as benign tested positive for the BRAF 

V600E mutation. Second, allelic fraction was able to be analyzed by conventional Sanger sequencing, whereas not applicable for ARMS-qPCR, and the comparison between ARMS-qPCR and other molecular techniques, such as ddPCR which is a novel and ultrasensitive method for BRAF 

V600E mutation analysis in FNAC specimens19, was not carried out in the current study. Thus, it should be evaluated in further studies. Third, the “gold standard” for determining detection accuracy of ARMS-qPCR for BRAF 

V600E mutation analysis in FNAC specimens is ARMS-qPCR from FFPE specimen in this study. Conventional Sanger sequencing is a highly reliable and widely used method to detect the BRAF 

V600E mutations in FFPE specimen,6,14 which might be used as the “gold standard” in this study to explicitly reflect detection accuracy of ARMS-qPCR for BRAF 

V600E mutation analysis from FNAC specimens. In addition, this work was a single institution study. Therefore, a larger multicenter study for evaluation the false-positive

### Table 5: Diagnostic performances of FNAC, BRAF 

V600E analysis in FNAC specimens, BRAF 

V600E analysis in FFPE tissue samples, and the combination of FNAC and BRAF 

V600E analysis for predicting thyroid malignancy

| Diagnostic modality | Sensitivity | Specificity | PPV | NPV  | Accuracy |
|---------------------|-------------|-------------|-----|------|----------|
| FNAC                | 75.7% (168/222) | 89.2% (58/65) | 96% (168/175) | 51.7% (58/112) | 78.7% (226/287) |
| BRAF 

V600E analysis in FNAC specimens | 83.3% (185/222) | 93.8% (61/65) | 97.9% (185/189) | 62.2% (61/98) | 85.7% (246/287) |
| FNAC + BRAF 

V600E analysis in FNAC specimens | 92.3% (205/222) | 84.6% (55/65) | 95.3% (205/215) | 76.4% (55/72) | 90.6% (260/287) |
| BRAF 

V600E analysis in FFPE tissue samples | 79.3% (176/222) | 100% (65/65) | 100% (176/176) | 58.6% (65/111) | 84.0% (241/287) |
| FNAC + BRAF 

V600E analysis in FFPE tissue samples | 91.9% (204/222) | 89.2% (58/65) | 96.7% (204/211) | 76.3% (58/76) | 91.3% (262/287) |

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; FNAC, fine-needle aspiration cytology; NPV, negative predictive value; PPV, positive predictive value.
and false-negative mutation rates of the BRAF^{V600E} analysis using ARMS-qPCR in preoperative FNAC specimen is mandatory in the near future.

In conclusion, FNAC combined with preoperative BRAF^{V600E} mutation analysis can significantly increase the diagnostic performance in comparison with FNAC alone. False-positive and false-negative BRAF^{V600E} mutation results are present in FNAC specimens using ARMS-qPCR, whereas it does not affect the added diagnostic value of BRAF^{V600E} mutation analysis to FNAC for evaluation of TNs.

CONFLICT OF INTEREST
The authors do not have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS
Concept and design: H-X X. BRAF V600E mutation analysis: Hui‐Xiong Xu. Collection and assembly of data: C-KZ, J-YZ, and QW. Data analysis: C-KZ, J-YZ, and L-PS. Manuscript written: C-KZ. Reviewed the manuscript: All authors.

DATA AVAILABILITY STATEMENT
The data are available from the corresponding author when upon reasonable requests.

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