Received: 29 May 2021 | Revised: 8 July 2021 | Accepted: 15 July 2021

DOI: 10.1002/jcla.23920

RESEARCH ARTICLE

WILEY

A five-gene panel refines differential diagnosis of thyroid nodules

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Funding information
This work was supported in part by the National Natural Science Foundation of China (81800749), Two-hundred Talent (20161318) and Clinical Research Program of 9th People's Hospital, Shanghai Jiao Tong University School of Medicine (JYLJ202019)

Abstract

Background: Molecular testing for oncogenic mutations in fine-needle aspiration has showed high predictive value in identifying malignant lesions from thyroid nodules with indeterminate cytology.

Methods: To figure out an efficient and economical gene panel for most medical institutions in China, we designed a five-gene panel including *BRAF*/*NRAS*/*KRAS*/*HRAS*/*TERT* genes and conducted a retrospective study to evaluate the role of this five-gene diagnostic panel in differential diagnosis of thyroid nodules.

Results: A total of 665 patients with 695 thyroid nodules were investigated in the current study. The fine-needle aspiration biopsy and surgically separated thyroid tissue specimens were harvested to test *BRAF*, *TERT*, *NRAS*, *KRAS*, and *HRAS* mutations. We identified 261 mutations in 665 patients, including 177 V600E mutations in *BRAF*. Three hundred and sixty-nine patients who underwent thyroid surgery after completion of the initial clinical and cytological evaluation were enrolled in the final analysis. The diagnostic sensitivity, specificity, and accuracy of the combination of FNAB cytology and five-gene detection were 74.7%, 93.8%, and 84.8%, respectively. *BRAF* V600E and five-gene panel could recognize 46.4% and 53.6% of papillary thyroid carcinoma in the patients with cytologically indeterminate nodules.

Conclusion: The five-gene panel can effectively improve the sensitivity, negative predictive value, and accuracy of fine-needle aspiration biopsy cytology, especially in the patients with cytologically indeterminate nodules.

Sang-Yu Lu, Ying-Chao Chen, Chen-Fang Zhu and Jing Chen contributed equally to this work.

KEYWORDS
BRAF, cytology, differential diagnosis, *RAS*, thyroid nodules

1 | INTRODUCTION

Fine-needle aspiration biopsy (FNAB) has dramatically improved the diagnostic accuracy of thyroid nodules since it was introduced into clinical practice because of its high positive predictive value (PPV) and high negative predictive value (NPV).[1,2] It has been wildly used all over the world and recommended as the preoperative gold standard for distinguishing malignant and benign lesions by many guidelines.[3] Its unequivocal value in the personal treatment of thyroid nodules has been demonstrated by innumerable researches. However, to our knowledge, the clinical application of this technique encountered some troubles in China. Some hospitals use FNAB results as an indication for surgery, while some surgeons choose to send patients with ultrasound (US) suspected nodules to the operating table directly, because of their confusion about the evaluation of FNAB. Researches showed that about 30% of FNAB cytology represents indeterminate results, which may cause overtreatment (e.g., surgery for benign nodules) or inappropriate treatment (e.g., lobectomy of malignant lesions).[4] Besides, the successful application of FNAB requires not only mastering technical skills, including a comprehensive understanding of the limitations and the factors affecting the acquisition of adequate specimens, but also owning extensive experience in FNAB cytological evaluation, which puts high demands on the endocrinologists and pathologists.[5] These aspects limit the diagnostic value of FNAB in guiding clinical decisions on thyroid nodules in most regions in China.[6] Therefore, additional methods are needed to improve FNAB cytological diagnosis, especially for those with cytologically indeterminate nodules.

The breakthrough, aided by genome-scale technologies, came in 2003 when the oncogenic *BRAF* V600E was initially descripted about its association with thyroid cancer.[7] Subsequently, a large number of biomarkers were unearthed and demonstrated the potential of molecular diagnostic test. In spite of this, *BRAF* V600E remains the most frequent genetic marker in PTC (papillary thyroid carcinoma) with a mutation rate of 53.0%–80.6%, followed by *RAS* (15%).[8,9] In addition, *TERT*, which was reported mutated in less than 10% of PTC, has been a hotspot recent years owing to its association with aggressive clinicopathological features and poor outcomes, especially increased risk for distant metastasis.[8,10–12] Different kinds of panels involving these biomarkers sprung up and developed rapidly recent ten years. From Galectin-3 to GSC (Genomic Sequencing Classifier) consisting of 10,196 genes,[13,14] the general trend of molecular diagnosis panels is containing more gene mutations and more expensive. The management of thyroid nodules seems to be shifting from "surgical over-diagnosis" to "molecular over-diagnosis." Herein, based on the current research results and our previous exploration, we developed a five-gene panel including *BRAF*/*NRAS*/*KRAS*/*HRAS*/*TERT* genes and conducted a retrospective study to evaluate the role of this five-gene diagnostic panel in differential diagnosis of thyroid nodules in a cohort of FNAB and surgically separated thyroid tissue specimens.

2 | PATIENTS AND METHODS

2.1 | Patients and samples of FNAB/paraffin-embedded thyroid tissue

Altogether, 665 patients with 695 thyroid nodules were enrolled into the study at the Department of Endocrinology, Shanghai Ninth People's Hospital. All patients provided informed consent, and the study was approved by the ethics committee of the Shanghai Ninth People's Hospital (CRC/IRB-C-BD-16-V3.1, Ethic No. SH9H-2020-T346-1). All these patients underwent surgery removing thyroid nodules and the removed nodules were pathologically evaluated. Among 665 patients, 394 thyroid nodules from 369 patients were aspirated and yielded FNAB samples. US-guided FNAB was performed under a standardized protocol by an experienced endocrinologist. Material from the needle pass through the nodule was used to prepare a direct smear for cytological evaluation, and the remaining material plus the needle washing was collected for molecular testing. Totally, two or three needle passes were taken in each thyroid nodule. The harvest of material for molecular testing was conducted in a way to ensure the routine cytological evaluation. The remaining 296 patients without FNAB examination accepted surgery directly after ultrasound examination. The surgically separated thyroid tissues were paraffin-embedded and collected for molecular testing.

2.2 | Detecting the point mutations of *BRAF*/*NRAS*/*KRAS*/*HRAS*/*TERT* in tissues of thyroid nodules

DNA was isolated using QIAamp DNA Micro Kit for FNAB samples and GeneRead DNA FFPE Kit for paraffin-embedded samples (Qiagen). The quantity of isolated DNA was assessed by NanoDrop 8,000 spectrophotometer (Thermo Scientific). Mutations including *BRAF* V600E and K601E, *TERT* C228T and C250T, *NRAS* codon 61, *HRAS* codon 61, and *KRAS* codons 12 and 13 were detected by Sanger sequencing using Universal sequencing reaction kit (Anjia). Droplet digital PCR (ddPCR) was performed with the QX200 Droplet Digital PCR system (Bio-Rad Technologies) to confirm *BRAF* V600E mutation in 92 patients who were diagnosed as malignancy and with no *BRAF* V600E mutations by Sanger sequencing. The QX200 ddPCR System was used per the manufacturer's protocol using assays for *BRAF* V600E mutation (Cat. No. 1863026, Bio-Rad Technologies). Amplification was performed as follows: 95°C for 10 min (1 cycle),

94°C for 30 s and 55°C for 1 min (40 cycles), and 98°C for 10 min (1 cycle) with a ramp rate of 1°C/s, and the reaction was then held at 4°C with a ramp rate of 1°C/s. The absolute quantification of mutant alleles and wild-type alleles was estimated using QuantaSoft v1.7.4 analysis software (Bio-Rad Technologies). The threshold was defined as described in the "Droplet Digital Application Guide." The sample is interpreted as *BRAF*-positive when the number of positive droplets exceeds 5.

2.3 | Cytology review of FNAB samples

Our routine cytological evaluation of all FNAB samples is classified into five categories: benign (benign lesions such as nodular goiter, Hashimoto thyroiditis, and adenoma; predicted risk of PTC is less than 20%), suspicious for PTC (atypia of individual cellular structure and nucleus; predicted risk of PTC is 30%–40%), atypical for PTC (atypia of some cellular structure and nucleus; predicted risk of PTC is 50%–60%), tendentious for PTC (one or two cancer cells were observed; predicted risk of PTC is 90%–94%), and confirmed for PTC (prominent cancer cells were observed; predicted risk of PTC is 95%–99%).

2.4 | Statistical analysis

Calculations of specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and Cochran-Armitage trend test were performed using SAS (version 8.2).

3 | RESULTS

Six hundred and sixty-five patients received surgery and constituted the "Surgery Group," of which surgically removed thyroid nodules were pathologically determined whether PTC or benign. Among them, 369 patients underwent FNAB cytological evaluation constituted the "FNAB + Surgery Group" and the FNAB specimens were collected for further molecular testing (Figure 1). Other 296 patients without FNAB examination accepted thyroid surgery directly after ultrasound examination, including those met the surgical indications (i.e., symptoms of oppression) or requested surgery voluntarily due to suspicious malignancy by US detection. The surgically separated thyroid tissues from these 296 patients were paraffin-embedded and collected for molecular testing.

We designed a five-gene diagnostic panel consisting of point mutations on *BRAF* V600E/K601E, *TERT* C228T/C250T, *NRAS* codon 61, *HRAS* codon 61, and *KRAS* codons 12/13. The mutations of *BRAF*/*NRAS*/*KRAS*/*HRAS*/*TERT* were detected in 695 samples including FNAB specimens and paraffin-embedded thyroid tissues.

In the surgery group, we identified 261 mutations from 665 patients in Surgery Group, including 177 *BRAF* V600E (*BRAF*), 3 *BRAF* K601E, 45 *NRAS* codon 61 (*NRAS-61*), 1 *HRAS* codon 61 (*HRAS-61*), 1 *KRAS* codon 12 (*KRAS-12*), 1 *KRAS* codon 13 (*KRAS-13*), 2 *TERT* C228T (*TERT*), 1 *TERT* C250T, 1 both *BRAF* V600E and *NRAS* codon 61 (*BRAF* + *NRAS*), and 14 both *BRAF* V600E and *TERT* C228T (*BRAF* + *TERT*).

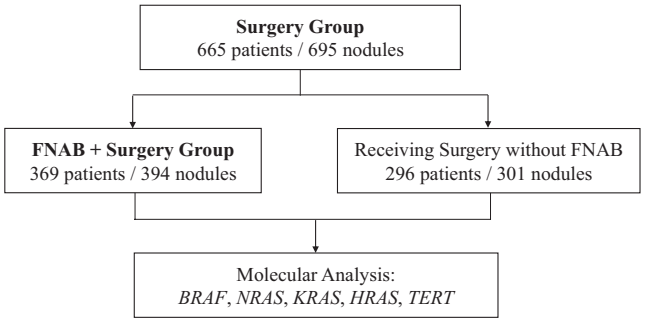

FIGURE 1 Schematic representation of the study design

To determine the efficiency of molecular testing for differential diagnosis of thyroid nodules, we analyzed the performance of molecular testing according to the pathological examination result. The sensitivity and specificity of the *BRAF*/five-gene molecular testing in differential diagnosis of thyroid nodules are 59.9%/63.8% and 99.4%/95.3%, respectively (Tables S1 and S2). There are 2 benign nodules detected with *BRAF* mutation and 17 with five-gene mutation.

According to the cytological pathological evaluation of FNAB by pathologists of our hospital, thyroid nodules are classified into five categories: benign, suspicious, atypical, tendentious, and confirmed. The thyroid nodules evaluated as suspicious or atypical in the FNAB examination were considered as cytologically indeterminate nodules and tendentious or confirmed nodules were interpreted malignant.

Among 369 patients in FNAB + Surgery Group, 86 (23.3%) patients were considered with malignant nodules (including confirmed and tendentious) in the cytological pathology of FNAB, 116 (31.4%) patients were considered as indeterminate (including atypical and suspicious), and 167 (45.3%) patients were benign (Figure 2; Table 1). The rates of PTC of these three categories were 97.7%, 48.3%, and 20.4%, respectively. In the view of a high false-negative rate of 20.4% (34/167) for benign cytology, an effective diagnostic method should be introduced to improve the quality of cytological analysis to avoid inappropriate treatment. As for molecular testing of these FNAB samples, all 104 (28.2%) *BRAF*-positive patients were diagnosed as papillary carcinoma by pathological examination after surgery. However, 126 (34.1%) patients were detected with five-gene mutations, of whom 116 (92.1%) were diagnosed as papillary carcinoma after surgery (Table S3).

BRAF V600E was the most frequent mutation as expected. Among the 104 *BRAF*-positive patients in this Group, 63 patients were firstly determined malignant nodules, 26 were indeterminate, and 15 were benign by the cytological pathology of FNAB (Figure 2). The ranking second mutation was *RAS* mutations (20 patients). After surgery, 12 patients with *RAS* mutations (including 2 patients with *BRAF* + RAS mutations) were found to be with papillary carcinomas, 1 with follicular carcinoma, 4 with follicular adenomas, and the other

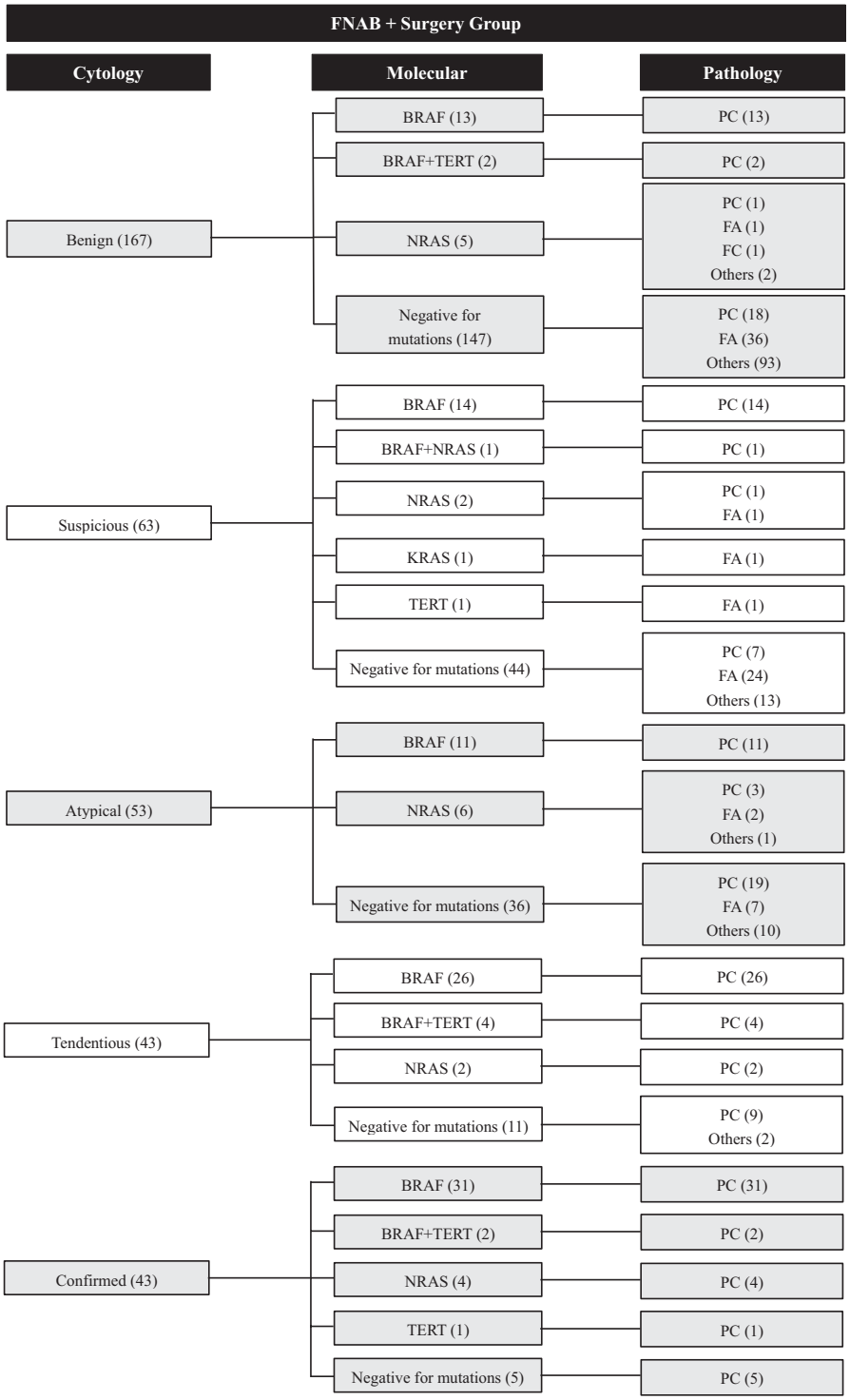

FIGURE 2 Correlation between cytology, molecular findings, and histological diagnosis in the FNAB + Surgery Group. PC, papillary carcinoma; FA, follicular adenoma; FC, follicular carcinoma; and Others, include subacute thyroiditis, nodular goiter, Hashimoto thyroiditis, thyroid cyst, metastatic clear cell renal cell carcinoma, and medullary carcinoma

3 with benign lesions. In addition, 8 of 10 patients carrying *TERT* mutation were coexistent with *BRAF* mutation. After surgery, 9 of 10 patients with *TERT* mutations were diagnosed as papillary carcinomas on pathological examination and 1 was considered with follicular adenoma, which was loss of follow-up.

To confirm the role of *BRAF* V600E and five-gene panel in differential diagnosis of thyroid nodules using FNAB specimens, we compared the performance of molecular testing with FNAB cytological analysis in FNAB + Surgery Group. Despite a high specificity for distinguishing the malignancy (100.0%/94.9%), the sensitivity of the *BRAF*/five-gene molecular testing alone was only 59.8%/66.7% (Table 2), which was similar to the corresponding results of Surgery Group. The sensitivity and accuracy of *BRAF*/five-gene molecular testing combining with FNAB cytology increased to 71.8%/74.7% and 86.2%/84.8%. For all this, a combination of FNAB cytology and the five-gene panel significantly improved the sensitivity, negative predictive value (NPV), and accuracy of preoperative diagnosis of thyroid nodules, while kept the specificity and positive predictive

TABLE 1 Consistency between FNAB cytology and surgical pathology of 369 patients in FNAB + Surgery Group

	Surgery pathology					
		PTC ($n = 174$)		Benign ($n = 195$)		PTC rate†(%)
FNAB	*n*	*n*	%	*n*	%	
Malignant	86	84	48.3	2	1.0	97.7
Confirmed	43	43	24.7	0	0.0	100.0
Tendentious	43	41	23.6	2	1.0	95.3
Indeterminate	116	56	32.2	60	30.8	48.3
Atypical	53	33	19.0	20	10.3	62.3
Suspicious	63	23	13.2	40	20.5	36.5
Benign	167	34	19.5	133	68.2	20.4

Note: PTC rate† means the proportion of PTC patients confirmed by surgical pathology to the total number of patients in the corresponding FNAB cytological classification.

TABLE 2 Performance characteristics of *BRAF* V600E, five-gene, FNAB cytology, FNAB cytology + BRAF V600E, and FNAB cytology + five-gene in FNAB + Surgery Group

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
BRAF V600E	59.8	100.0	100.0	73.6	81.0
Five-Gene	66.7	94.9	92.1	76.1	81.6
FNAB cytology	48.3	99.0	97.7	68.2	75.1
FNAB cytology + BRAF V600E	71.8	99.0	98.4	79.8	86.2
FNAB cytology + Five-Gene	74.7	93.8	91.5	80.6	84.8

Note: Patients with malignant FNAB cytology were defined as positive; patients with indeterminate and benign FNAB cytology were defined as negative.

value (PPV) (Table 2). Besides, the detection of the *BRAF*/five-gene contributed to better predicting the probability of malignancy in nodules with indeterminate FNAB cytology. The vast majority of patients with confirmed and tendentious malignant nodules in the FNAB examination were eventually confirmed to be malignant by surgical pathology. Patients with confirmed and tendentious malignant nodules showed the highest proportion carrying *BRAF*/five-gene mutations (*BRAF*-positive 75.0%; five-gene positive 83.3%) (Table 3). Out of 56 patients with indeterminate FNAB cytology while confirmed malignant, 26 (46.4%) carried *BRAF* mutations and 30 (53.6%) carried five-gene mutations (Table 3), indicating molecular testing could identify about 50% PTC patients with indeterminate cytology. It is worth noting that 20.4% (34/167) patients with cytological benign nodules were diagnosed as PTC, of whom 44.1% (15/34) carried *BRAF* mutations and 47.1% (16/34) carried five-gene mutations, revealing a well performance of molecular testing in either cytological indetermined or benign nodules. The sensitivity, specificity, and accuracy of *BRAF* mutation in the 116 patients with indeterminate cytology were 46.4%, 100.0%, and 74.1% and that of five-gene mutations were 53.6%, 90.0%, and 72.4%, respectively (Tables S4 and S5). Obviously, the patients with worse cytology tended to have a greater chance of carrying *BRAF*/five-gene mutation (*BRAF* V600E, $Z = 0.0016$; five-gene, $Z < 0.001$). It was significant that molecular testing played a crucial role in distinguishing malignant nodules especially in those with indeterminate FNAB cytology. The ROC curves demonstrated that combing molecular testing with FNAB was an effective method to evaluate thyroid nodules (Figure S1). Moreover, a few five-gene mutations especially in *RAS* genes were found in the patients with benign nodules determined by surgical pathology, which was reasonable since *RAS* mutations occurred in both thyroid carcinoma and benign lesions.

4 | DISCUSSION

The current study focuses on the feasibility and utility of a five-gene panel containing *BRAF*/*RAS*/*TERT* mutation detection in the improvement of FNAB cytology in differential diagnosis of thyroid nodules in China. Our results demonstrated that *BRAF*/five-gene mutations detected in the remaining material of FNAB samples, without another invasive manipulation, could significantly refine FNAB cytology diagnosis, for example, improving the sensitivity and accuracy, particularly in patients with indeterminate cytology, accounting for 31.4% in the cohort. Notably, 20.4% patients with benign nodules determined by FNAB cytology were diagnosed as thyroid carcinoma by surgical pathology, of which molecular testing of *BRAF*/five-gene reduced about 44.1% (15/34) or 47.1% (16/34) false-negative rate.

Given the fact that the application of FNAB was limited in China since skilled endocrinologists and pathologists were required, the introduction of molecular testing seems much more necessary, especially for medical institutions newly introduced FNAB. To satisfactorily evaluate a thyroid FNA specimen, more than six groups of

TABLE 3 Performance of molecular testing in different categories of cytology in FNAB samples from thyroid nodules

	Surgery pathology									
	PTC(n=174)					Benign(n = 195)				
		MUT		WT			MUT		WT	
FNAB	*n*	*n*	%‡	*n*	%‡	*n*	*n*	%‡	*n*	%‡
BRAF V600E										
Malignant	84	63	75.0	21	25.0	2	0	0.0	2	100.0
Confirmed	43	33	76.7	10	23.3	0	0	N.A	0	N.A
Tendentious	41	30	73.2	11	26.8	2	0	0.0	2	100.0
Indeterminate	56	26	46.4	30	53.6	60	0	0.0	60	100.0
Atypical	33	11	33.3	22	66.7	20	0	0.0	20	100.0
Suspicious	23	15	65.2	8	34.8	40	0	0.0	40	100.0
Benign	34	15	44.1	19	55.9	133	0	0.0	133	100.0
Five-Gene										
Malignant	84	70	83.3	14	16.7	2	0	0.0	2	100.0
Confirmed	43	38	88.4	5	11.6	0	0	N.A	0	N.A
Tendentious	41	32	78.0	9	22.0	2	0	0.0	2	100.0
Indeterminate	56	30	53.6	26	46.4	60	6	10.0	54	90.0
Atypical	33	14	42.4	19	57.6	20	3	15.0	17	85.0
Suspicious	23	16	69.6	7	30.4	40	3	7.5	37	92.5
Benign	34	16	47.1	18	52.9	133	4	3.0	129	97.0

Note: n (%)‡ indicates number (the percentage of the molecular testing MUT/WT patients in the corresponding FNAB cytology and surgical pathology classification).

follicular cells are required, and each group composed of at least 10 cells.[15] In contrast, molecular testing is a relatively objective test, thereby reducing the requirements for the rich experience of endocrinologists and pathologists. In view of this, the ability to evaluate thyroid nodules for a FNAB cytology novice could be effectively improved by combining cytology and molecular detection result, which is one of the profound meanings of this study, probably contributing to change the current situation that few FNAB was performed in China.

BRAF mutation is the most common gene mutation as expected and plays a crucial role in molecular diagnostics. The high specificity makes *BRAF* V600E mutation the best indicator for PTC up to now. However, 2 benign nodules were detected with *BRAF* V600E mutation in Surgery Group and indicated that *BRAF* V600E mutation does not entirely equal to PTC. That's what corresponds to the results of previous researches and endocrinologists should pay attention to. Here, we found no obvious relationship between *BRAF* K601E and thyroid carcinoma. There are 3 patients with *BRAF* K601E mutations determined follicular adenomas, revealing *BRAF* K601E a probable marker of benign lesions. Besides, *RAS* mutation is the second most common mutation in both malignant (papillary and follicular carcinoma) and benign nodules (follicular adenoma and other benign lesions). It seems the specificity and accuracy of *RAS* mutation are not as good as *BRAF* mutations in malignancy diagnosis, but *RAS* mutation is more frequent in patients with follicular adenoma and carcinoma than other mutations. Considering that FNAB is currently difficult to distinguish between follicular carcinoma and adenoma, and adenoma may be the precursor lesion of follicular carcinoma, *RAS* mutations in follicular adenomas and carcinomas may have potential special significance; thus, it should be included in molecular testing and fully considered in refining thyroid nodules risk assessment. The function of *TERT* mutation in differential diagnosis is limited by its low prevalence in PTC. But its strong association with PTC and correlation with greater chance of distant metastases make it a non-negligible gene mutation in clinic. That's why we kept it in our panel.

Molecular testing for thyroid nodules evolved from the earliest immunohistochemical evaluation with humble Galectin-3 to costly GSC.[13,14] The general trend of molecular diagnosis development is that more gene mutations and rearrangements are included in the detection panel and more money and resource are needed. A suitable instead of costly gene combination seems much more important and meaningful for molecular testing of cytologically indeterminate nodules. The five-gene panel demonstrating to improve thyroid nodules diagnosis efficiently and economically corresponds with our original intention to figure out a suitable panel for most medical institutions in China.[16]

ACKNOWLEDGMENTS

We thank all subjects for participating in this study.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

X.P.Y. and Y.C.C. conceived and designed the project. X.P.Y., Y.C.C. and S.Y.L. contribute to the project management. Y.C.C., J.C. and C.F.Z. took part in the of samples collection. Q.Y.Z., M.L., L.Y. and J.W. extracted DNA and sample quality control. S.Y.L., M.M.Z., S.X.Z. and Q.Y.Z. conducted the PCR experiments. S.Y.L., H.D.S. and X.P.Y. wrote the article.

CONSENT TO PARTICIPATE

All patients provided informed consent and agreed to participate this study.

CONSENT FOR PUBLICATION

All patients provided informed consent and agreed with the publication of this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Lu S-Y, Chen Y-C, Zhu C-F, et al. A five-gene panel refines differential diagnosis of thyroid nodules. *J Clin Lab Anal*. 2021;35:e23920. https://doi.org/10.1002/jcla.23920