RESEARCH

Molecular testing raises thyroid nodule fine needle aspiration diagnostic value

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Abstract

Thyroid fine needle aspiration biopsy (FNAB) remains indeterminate in 16–24% of the cases. Molecular testing could improve the diagnostic accuracy of FNAB. This study examined the gene mutation profile of patients with thyroid nodules and analyzed the diagnostic ability of molecular testing for thyroid nodules using a self-developed 18-gene test. Between January 2019 and August 2021, 513 samples (414 FNABs and 99 formalin-fixed paraffin-embedded (FFPE) specimens) underwent molecular testing at Ruijin Hospital. Sensitivity (Sen), specificity (Spe), positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated. There were 457 mutations in 428 samples. The rates of *BRAF*, *RAS*, *TERT* promoter, *RET/PTC,* and *NTRK3* fusion mutations were 73.3% (n = 335), 9.6% (n = 44), 2.8% (n = 13), 4.8% (n = 22), and 0.4% (n = 2), respectively. The diagnostic ability of cytology and molecular testing were evaluated in Bethesda II and V–VI samples. For cytology alone, Sen, Spe, PPV, NPV, and accuracy were 100%, 25.0%, 97.4%, 100%, and 97.4%; these numbers were 87.5%, 50.0%, 98.0%, 12.5%, and 86.2% when considering positive mutation, and 87.5%, 75.0%, 99.0%, 17.6%, and 87.1% when considering positive cytology or and positive mutation. In Bethesda III–IV nodules, when relying solely on the presence of pathogenic mutations for diagnosis, Sen, Spe, PPV, NPV, and AC were 76.2%, 66.7%, 94.1%, 26.8%, and 75.0%, respectively. It might be necessary to analyze the molecular mechanisms of disease development at the genetic level to predict patients with malignant nodules more accurately in different risk strata and develop rational treatment strategies and definite management plans.

Key Words

- thyroid nodule
- fine needle aspiration
- molecular test
- diagnosis
- gene mutation

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Introduction

Thyroid nodules are common in the general population and are detected in up to 50–65% of healthy individuals; about 95% are asymptomatic and discovered incidentally on physical examinations or imaging studies performed for reasons unrelated to thyroid disease ('incidentalomas') (1, 2). In a retrospective study investigating the prevalence of thyroid nodules in the healthy Chinese population, the overall prevalence was 36.9% (3). Most thyroid nodules (about 90%) are benign and require no treatment (1, 2). The goal of evaluation is to exclude malignancy, which occurs in 7–15% of cases depending on age, sex, radiation exposure history, family history, and other factors, and malignant nodules require surgery (1, 4). The current diagnostic modalities routinely used for thyroid disorders in China include palpation, ultrasonography, and serum testing of thyroid function. Ultrasonography based on the American Thyroid Association (ATA) risk stratification criteria is a common tool in evaluating and diagnosing thyroid nodules (5). A meta-analysis of 31 studies showed that ultrasonography alone does not accurately predict thyroid cancer (6). Ultrasonography can only determine the current growth status of the nodule and cannot predict its progression. Therefore, ultrasound alone has relatively poor value for diagnosing malignant thyroid nodules. Further definitive diagnosis can be made by fine needle aspiration biopsy (FNAB), which is a cost-effective approach, but 16–24% of FNABs cannot be diagnosed definitively (7). Still, cytology alone is only a snapshot of the nodule cells at a precise point in time and cannot predict the evolution of the nodule.

Next-generation sequencing (NGS) provides diagnostic assistance for FNAB with undetermined features in thyroid cancer. A multi-gene test panel allows the simultaneous detection of hundreds of genes, providing insight into the molecular mechanisms of disease formation while helping stratify management for patients according to the level of malignancy risk (8, 9). Several genes are involved in the pathogenesis of thyroid cancer (10, 11). Characteristic molecular markers identified in thyroid cancer include mutations in exons of *AKT1*, *BRAF*, *CTNNB1*, *EZH1*, *GNAS*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, *RET*, *SPOP*, *TP53*, *TSHR*, *ZNF148*, the promoter region of *TERT*, and fusions of *PAX8*/*PPARγ* and RET/PTC (10, 11). These molecular markers correlate with histological subtypes (12, 13, 14). Of note, the *RAS* mutations can also be found along the spectrum of adenomas to low-grade malignancies to high-grade malignancies (12, 13, 14). Pathogenic mutation in *TP53* has strong oncogenic potential and can be seen in almost all human cancers (15, 16). *TERT* encodes the telomerase protein, and telomerase overexpression confers the ability to split infinitely into cancer cells (17), including thyroid cancer cells (18). Medullary thyroid carcinoma (MTC) accounts for approximately 2–3% of all thyroid cancers and is predominantly sporadic (around 80%). Point mutations are the predominant type of *RET* in MTC (19), while normal thyroid development does not depend on RET protein expression (20). Specific *RET* germline mutations are associated with the clinical presentation, age of onset, and disease aggressiveness in hereditary MTC. In contrast, 1–7% of sporadic MTC can harbor germline *RET* mutations. Therefore, germline *RET* genetic testing is recommended for all sporadic MTCs (21).

Somatic molecular alterations, as described above, have been recognized as useful diagnostic and prognostic markers for thyroid cancer and have long been introduced into clinical practice (22). Three panels for genetic alterations in thyroid specimens are commercially available (Thyroseq V3, Afirma GSC, and ThyraMIR) (23), but these panels are not listed in China. Indeed, they were not developed based on Chinese population data, and available data suggest differences in thyroid cancer mutation patterns between Chinese and Western patients, with the Chinese patients showing higher frequencies of *BRAF* mutations and lower frequencies of *RAS* and *TERT* promoter mutations (24). Therefore, a thyroid cancer 18-gene detection kit was independently developed by us and is based on the method of multiplex amplification combined with high-throughput sequencing to detect single nucleotide variants (SNVs), insertions/deletions (Indels), and fusion genes in DNA and RNA. The product was confirmed by internal performance and can achieve 99.3% (SNVs/indels) and 100% (fusion genes) detection accuracy (25). Under the input of a 5-ng library construction, the limit of detection (LoD) of (DNA) *BRAF* p.V600E was variant allele frequency (VAF)-1.0%, the LoD of *TERT* C228T was VAF-2.0%, and the LoD of (RNA) *CCDC6*(E1)-RET(E12) fusion detection was 20 ng at a concentration of 20 copies/ng. The input amount for library construction is 400 total copy numbers/reaction. Hence, this assay requires only a small amount of genetic material, which is suitable for the small specimens obtained by FNAB, but validation is required.

Therefore, this study examined the NGS test results of 513 samples to provide a preliminary landscape to the gene mutation profile of patients with thyroid nodules in

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China and validate the use of this 18-gene detection kit to examine thyroid FNABs.

Materials and methods

Sample collection

This study pooled and analyzed the molecular results of 513 thyroid FNAB samples and surgical formalin-fixed paraffin-embedded (FFPE) specimens. The patients who underwent genetic testing between January 2019 and August 2021 and had comprehensive, relevant clinical information were included. The presence of coexisting diseases was not considered. The patients without genetic testing or with incomplete clinical information were excluded. All FNAB samples and surgical specimens tested with the 18-gene panel were included. All samples and specimens were collected from Ruijin Hospital, Shanghai Jiaotong University School of Medicine, and Shanghai Tenth People's Hospital. Basic patient information, cytopathology results, and surgical pathology results were collected from the electronic management system of the hospitals. The study was approved by Shanghai Tenth People's Hospital (no. 22K283). All genetic tests were performed with the patient's informed consent. Cytopathology classification criteria were performed according to the Bethesda criteria (26), and surgical pathology classification was performed according to the 2017 WHO classification of endocrine organ tumors (27).

18-gene panel testing

DNA and RNA were extracted simultaneously using a self-developed kit (25): 'Tissue DNA/RNA Extraction Kit (Centrifugation Method).' The nucleic acid elution volumes were 30 and 50 μL for FNAB samples and FFPE specimens, respectively. Nucleic acid quality control was performed using a NanoDrop One (Thermo Fisher Scientific). The library was constructed using a self-developed kit: 'Human Thyroid Cancer Gene Detection Kit (Amplification Sequencing), 18 Genes.' First-strand cDNA was synthesized from denatured RNA, and genomic DNA was removed. A cDNA library was obtained by twice multiplex polymerase chain reaction (PCR) amplifications, eventually used for fusion assay. The library input amount was not less than 40 and 80 ng for FNAB samples and FFPE specimens, respectively. The DNA library was obtained by twice multiplex PCR amplifications targeting gene amplification, and index sequences were added. The DNA library was eventually used for SNV/Indel assay with a minimum input of 20 (FNAB) or 40 (FFPE) ng. Sequencing was performed using a NOVA_S4-G-PE150 system (Illumina, Inc., San Diego, CA, USA).

Data analysis

For data pre-processing, the FastQ file package was downloaded from the sequencing system, and the Trimmomatic (v0.38) software was used to remove splice sequences and low-quality base fragments. The sequence alignment software BWA (BWA-MEM algorithm) and GATK were used to generate BAM files by aligning the sequences in FastQ files to the human reference genome hg19. Samtools were used to optimize the BAM files. A customized analysis procedure was used to perform data quality control such as Q30, Mean, Depth, and Mapping ratio for each sample. The VarScan (v2.3.9) software was used for SNV/Indel mutation analysis, and a customized analysis procedure was used for fusion analysis. Mutation annotation was performed using Annovar and VEP. The public databases ClinVar and COSMIC were used to interpret the variants' clinical significance. When a variant was recorded as 'Pathogenic' in ClinVar or COSMIC and reported in thyroid disease, it was recorded as a pathogenic mutation.

This kit can simultaneously detect SNVs/Indels of 15 genes (*AKT1*, *BRAF*, *CTNNB1*, *EZH1*, *GNAS*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, *RET*, *SPOP*, *TP53*, *TSHR*, and *ZNF148*), the *NTRK1/3*, *PAX8/PPARγ*, and RET/PTC fusion variants, and the *TERT* promoter region variants (c.-124C>T, C228T, c.-146C>T, and C250T).

Statistical analysis

Only descriptive statistics were used. Sensitivity (Sen), specificity (Spe), positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated. A subgroup analysis was performed in Bethesda III–IV nodules.

Results

Basic information on all samples

There were 414 FNAB samples and 99 FFPE specimens analyzed by gene sequencing in this study (Supplementary Fig. 1, see section on supplementary materials given at the end of this article), with 318 samples collected from

Table 1 Characteristics of the patients.

Category		Number of samples ($n = 513$)
Sex	Female	381
	Male	132
Age	≤14	2
	15–47	352
	48–63	132
	≥64	27

female patients and 132 samples from male patients. The age of the patients was stratified as childhood (<14 years), youth (15–47 years), middle-aged (48–63 years), and old (>64 years) (28). The age range was 6–89 years, with 68.6% ($n = 352$) of the patients in the age group of 15–47 years (Table 1). Surgical histopathological results were not obtained for 346 FNAB samples due to a decision to follow up without surgery, surgical scheduling, or ablative treatment (Table 2 and Supplementary Fig. 1). Nine specimens were surgically confirmed as benign tumors, and cytological results were not queried in two of them. In addition, 158 specimens were confirmed as malignant tumors. Samples classified as malignant lesions by cytopathology accounted for 63.9% (Bethesda V, $n = 258$; Bethesda VI, $n = 70$), while the benign sample accounted for 36.1% (Bethesda II, $n = 55$; Bethesda III, $n = 83$; Bethesda IV, $n = 20$) (Table 2). Cytopathology results were not obtained for 25 FFPE samples.

The landscape of molecular alterations in thyroid diseases

The 18-gene test panel was performed to examine the genetic alterations of the thyroid nodules. The molecular assays showed that 428 samples had genetic variants within the detection range, with a positive rate of 83.4% (428/513), 85 samples (FNAB, $n = 65$; FFPE, $n = 20$) had no mutation, and 27 samples had two or more mutations. The positivity rate of the FNAB samples was slightly higher than the FFPE samples, at 84.3% (349/414) and 79.8% (79/99), respectively (Supplementary Fig. 2). Therefore, 457 variants were detected in 428 samples (Figs. 1 and 2). *BRAF* mutations were detected in 73.3% of the samples (335/457), and 3 samples carried rare *BRAF* mutations: K601E (no surgical pathology results), V600_K601delinsE (confirmed as PTC), and K601_W604del (confirmed as PTC). *RAS* mutations were the second most common mutation, with a prevalence of 9.6% ($n = 44$). *NRAS* ($n = 24$) mutations were approximately twice as *KRAS* mutations ($n = 11$) and three times as *HRAS* mutations ($n = 9$). *RET/PTC* fusion was the third most common form of mutations, accounting for 4.8% ($n = 22$), and *RET/PTC1* (CCDC6-RET, $n = 18$) had a 4.5-fold higher incidence than *RET/PTC3* (NCOA4-RET, $n = 4$). No other forms of *RET/PTC* fusion mutation were detected (Figs. 1 and 2). Two samples carried *NTRK* fusions as *ETV6* (exon 4)-*NTRK3* (exon 14). Eleven of 13 samples carried *TERT* promoter region mutations in combination with other mutations. Three patients (two with *TP53/BRAF* V600E and one with *TP53/TERT* C228T/*NRAS* Q61R) were confirmed with malignant tumors by surgical pathology. All TP53 mutations in this study were not found alone. Unexpectedly, patient C-010 carried the *BRAF* V600E mutation but was confirmed to be a nodular goiter with adenomatous hyperplasia, and the surrounding thyroid tissue showed Hashimoto's thyroiditis. Seven FNAB samples carried *EZH1* mutations (two with *EZH1* Y642F, four with *EZH1* Q571R, and one with *EZH1* Q571R/TERT C228T). Five FNAB samples carried only *SPOP* P94R mutation. Two FNAB samples carried only *TSHR* mutation (*TSHR* M453T and *TSHR* D633Y, respectively). The cytopathology was Bethesda II–III, but surgical pathology was not obtained for all these samples (Figs. 1 and 2). Therefore, the 18-gene test panel could reveal the mutations present in many thyroid nodules.

Table 2 Cytology and surgical pathology results of samples.

Cytology		Surgical pathology		
Bethesda	Number of samples ($n = 513$)	Malignant ($n = 158$)	Benign ($n = 9$)	Unknown ($n = 346$)
II	55	0	1	54
III	83	11	1	71
IV	20	10	2	8
V	258	81	1	176
VI	70	31	2	37
No cytology	27	25	2	0

Comparison of the diagnostic effectiveness between cytological examination and molecular testing for thyroid nodules

Next, whether the 18-gene test panel could predict malignancy was examined. There were 140 samples that could be used for calculating the diagnostic efficiency, which was classified as Bethesda II–VI (Table 3).

The diagnostic efficacy values of cytology, molecular, and combinations of both were examined for cytologically determined lesions. Using surgical pathology results as the gold standard for diagnosis,

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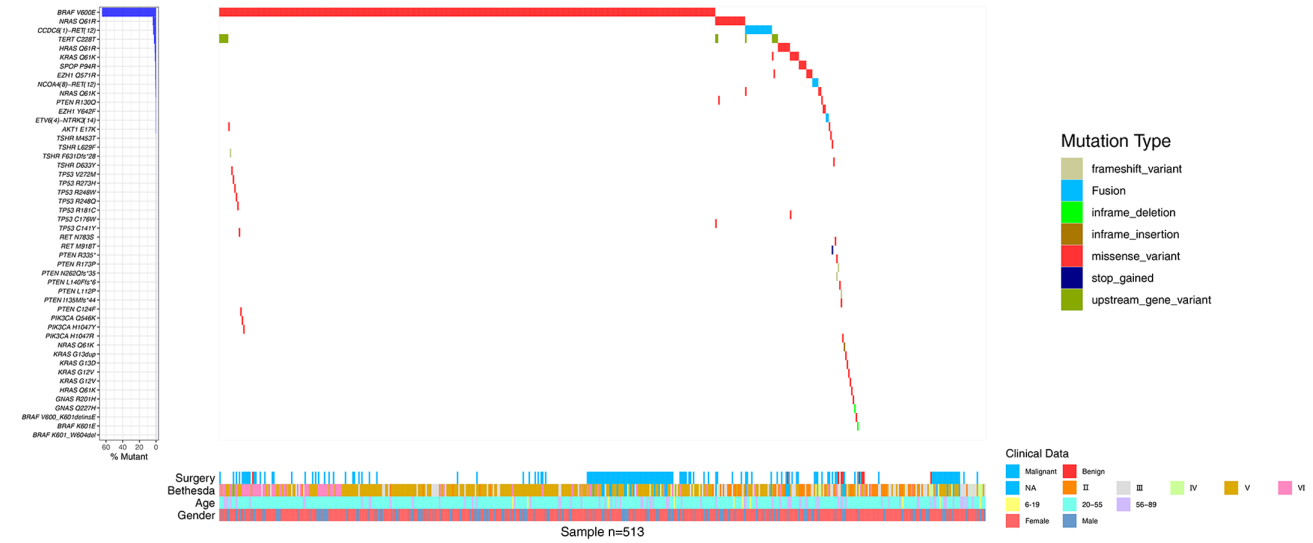

Figure 1
Waterfall plot of single nucleotide variants (SNVs) or fusion variants in all samples. Each square represents a mutation, and each column represents a sample. Mutation type, cytology, surgical pathology, age, and sex are shown by a different color in each sample.

when relying solely on the cytopathological results for diagnosis, Sen, Spe, PPV, NPV, and AC were 100%, 25.0%, 97.4%, 100%, and 97.4%, respectively. When using diagnosis by pathogenic mutation only, the Sen, Spe, PPV, NPV, and AC were 87.5%, 50.0%, 98.0%, 12.5%, and 86.2%, respectively. Combining the cytopathology with molecular assays led to a significant increase in specificity (to 75.0%) but without an actual increase in accuracy (to 87.1%) (Table 4).

In Bethesda III–IV nodules (i.e. of unclear cytological results), using the surgical specimen as the gold standard for diagnosis, when relying solely on the presence of pathogenic mutations for diagnosis, Sen, Spe, PPV, NPV, and AC were 76.2%, 66.7%, 94.1%, 26.8%, and 75.0%, respectively (Table 5).

Samples with two or more mutations

The clinical significance of carrying two or more mutations was examined. Twenty-seven samples carried at least two mutations. *TP53* ($n = 7$) or *TERT* ($n = 11$) were the most common (Table 6). Eleven of these samples were confirmed as malignant tumors, and one was benign (Table 6). This benign patient (C-006) carried two mutations in *PTEN* (the frameshift mutation I135Mfs*44 and the missense mutation C124F), and cytopathology showed Bethesda II. Surgical histopathology confirmed the follicular adenoma (FA) in the right-sided thyroid, and no tumorigenic component was observed in his right central group of lymph nodes or the lymph nodes behind the right laryngeal recurrent nerve.

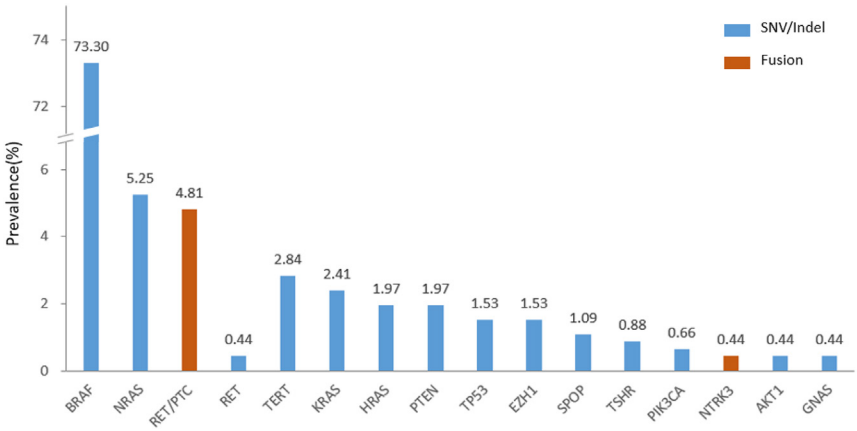

Figure 2
The detection rate of related genes in all variants.

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Table 3 Samples with both cytology results and surgical pathology results.

Alteration types		Total (n = 140)	Surgical pathology						
			II (n = 1)	III (n = 12)	IV (n= 12)	V (n = 82)	VI (n= 33)	Malignant (n = 133)	Benign (n = 7)
Single nucleotide/ insertion/deletion variation	*BRAF* V600E	89 (63.6%)	0	5	5	56	23	88	1
	BRAF V600_K601delinsE	1 (0.7%)	0	0	0	1	0	1	0
	BRAF K601_W604del	1 (0.7%)	0	0	0	1	0	1	0
	BRAF V600E/*PIK3CA* H1047R	1 (0.7%)	0	0	0	0	1	1	0
	BRAF V600E/*PIK3CA* H1047Y	1 (0.7%)	0	0	0	0	1	1	0
	BRAF V600E/*RET* N783S	1 (0.7%)	0	0	0	1	0	1	0
	BRAF V600E/*TERT* C228T	1 (0.7%)	0	0	0	0	1	1	0
	BRAF V600E/*TP53* R248Q	1 (0.7%)	0	0	0	0	1	1	0
	AKT1 E17K	1 (0.7%)	0	0	0	1	0	1	0
	HRAS Q61R	1 (0.7%)	0	0	0	1	0	1	0
	KRAS Q61K	3 (2.1%)	0	1	0	2	0	3	0
	NRAS Q61R	2 (1.4%)	0	1	0	1	0	2	0
	NRAS Q61R/*TERT* C228T	1 (0.7%)	0	0	1	0	0	1	0
	PTEN I135Mfs*44/*PTEN* C124F	1 (0.7%)	1	0	0	0	0	0	1
	PTEN L140Ffs*6/*PTEN* R173P	1 (0.7%)	0	0	0	0	1	1	0
	PTEN N262Qfs*35	1 (0.7%)	0	1	0	0	0	0	1
	PTEN R130Q	1 (0.7%)	0	0	0	1	0	1	0
	PTEN R335*/*TSHR* L629F	1 (0.7%)	0	1	0	0	0	1	0
	RET M918T	1 (0.7%)	0	0	0	1	0	1	0
	TERT C228T	1 (0.7%)	0	1	0	0	0	1	0
Gene fusion	*CCDC6*(1)-*RET*(12)	2 (1.4%)	0	0	0	1	1	2	0
	NCOA4(8)-*RET*(12)	3 (2.1%)	0	0	1	2	0	3	0
	ETV6(4)-*NTRK3*(14)	1 (0.7%)	0	0	0	0	1	1	0
Negative		23	0	2	5	13	3	19	4

No distinction in sample types (FNAB samples or FFPE samples).

The other patient (C-014) also carried mutations in *PTEN* (the frameshift mutation L140Ffs*6 and the missense mutation R173P), with cytopathology of Bethesda VI, and the surgical pathology confirmed as papillary thyroid microcarcinoma (PTMC), and the subtypes were classic papillary thyroid cancer (CPTC) and tall cell variant of papillary thyroid carcinoma (TCVPTC) (Table 5). Patient C-476 only had the frameshift mutation *PTEN* N262Qfs*35 and was confirmed as a nodular goiter by surgical histopathology. Patient C-415 only had the missense mutation *PTEN* R130Q and was confirmed as PTC with lymph node metastasis by

Table 4 Evaluation of diagnostic efficiency for different combination schemes in Bethesda II, V, and VI.

Identification scheme	Evaluation conditions	Surgical histopathology		Predictive value, %				
		Malignant (n = 112)	Benign (n = 4)	Sen	Spe	PPV	NPV	AC
Cytology	V–VI	112	3	100	25	97.4	100	97.4
	II	0	1					
Pathogenic mutation	NGS (+)	98	2	87.5	50	98	12.5	86.2
	NGS (–)	14	2					
Cytology with pathogenic mutation	V–VI and NGS (+)	98	1	87.5	75	99	17.6	87.1
	II or NGS (–)	14	3					

Cytology: Only cytology results were used to diagnose benign or malignant. 'Malignant'' was defined as Bethesda V–VI, and 'Benign/negative' was defined as Bethesda II (116 samples).
Pathogenic mutation: Only genetic test results were used to diagnose benign or malignant. 'Malignant' was defined as positive pathogenic gene mutations in the detection range, and 'Benign' was defined as negative pathogenic gene mutations in the detection range (116 samples).
Cytology with pathogenic mutation: 'Malignant' was defined as Bethesda V–VI with positive pathogenic gene mutations, and 'Benign' was defined as Bethesda II or negative gene mutations (116 samples).
AC, acuracy; NPV, negative predictive value; PPV, positive predictive value; Sen, sensitivity; Spe, specificity.

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Table 5 Evaluation of diagnostic efficiency of the presence of pathogenic mutations in Bethesda III–IV.

	Surgical histopathology		Predictive value (%)				
	Malignant (21)	Benign (3)	Sen	Spe	PPV	NPV	AC
Pathogenic mutation	16	1	76.2	66.7	94.1	26.8	75
	5	2					

AC, accuracy; NPV, negative predictive value; PPV, positive predictive value; Sen, sensitivity; Spe, specificity.

histopathology. Patient C-421 also carried *BRAF* V600E and *TP53* R273H and was confirmed as PTC with lymph node metastasis by surgical histopathology. Patient C-044 also carried *BRAF* V600E and *TP53* R248Q and was confirmed as PTC (subtypes CPTC and TCVPTC) by surgical pathology. Patient C-013 was confirmed as PTC (*NRAS* Q61R/*TERT* C228T). Patients C-455 (*NRAS* Q61R, *TP53* C141Ym, and *TERT* C228T) and C-456 (*NRAS* Q61K, *TERT* C228T, and *CCDC6* (exon1)-*RET* (exon 12)) were confirmed as metastatic thyroid cancer. Patient C-463 carried *PTEN* R335* and *TSHR* L629F mutations, developed recurrence within 6 months after ablative treatment, and was classified as a malignant tumor. Therefore, a higher mutation burden could indicate a higher likelihood of malignancy since 90.9% of the patients with two or more mutations had malignant thyroid nodules. Still, the sample size was too small to perform additional analyses and reach firmer conclusions.

Discussion

Results of thyroid FNAB remain indeterminate in 16–24%. Molecular testing could improve the diagnostic accuracy of FNAB. This study examined the gene mutation profile of patients with thyroid nodules and analyzed the diagnostic ability of molecular testing for thyroid nodules by a self-developed 18-gene test. The results suggested that the 18-gene test panel appears to increase the diagnostic ability of FNAB for malignant thyroid nodules. The three currently available major gene panels are unavailable in China, and their development and validation did not include Chinese patients. Therefore, the work presented here is original and important.

Association of mutations with thyroid nodule status

The *BRAF* K601E mutation is closely associated with FTC, especially with the FVPTC [19]. *BRAF* V600E is the most common variant in cytologically indeterminate thyroid nodules, followed by *RAS* mutations and *RET/PTC* fusions (29, 30), as observed in the present study. Afkhami *et al.* (19) reported that the K601E mutation accounted for 5.3% of all *BRAF* mutations in patients undergoing thyroidectomy. In the present study, there was only one patient with the *BRAF* K601E mutation, and the prevalence was lower than expected. The possible reason is that most patients had PTC or PTMC in this study. Interestingly, in a retrospective study (31), 17 and 4 samples with *BRAF* mutations did not exhibit malignant features in the first and second years of follow-up, and all 4 samples with *BRAF* mutations showed malignant features in the third year of follow-up. Therefore, cytology is only a snapshot, and the presence of a *BRAF* mutation suggests that malignant features will eventually develop. Therefore, a closer follow-up would be recommended. The present study suggests that *TP53* mutation does not occur alone in thyroid cancer, as supported by other studies (32, 33).

Table 6 Combined mutation carriers that have obtained surgical pathological results in this study.

Sample	Sex	Age	Bethesda	Disease manifestation	Molecular change
C-006	Female	58	II	Follicular adenoma	*PTEN* I135Mfs*44/*PTEN* C124F
C-007	Female	32	VI	Papillary thyroid carcinoma (CPTC+TVCPTC)	*BRAF* V600E/*PIK3CA* H1047Y
C-013	Female	53	IV	Papillary thyroid carcinoma	*NRAS* Q61R/*TERT* C228T
C-014	Female	37	VI	Papillary carcinoma (CPTC+TVCPTC)	*PTEN* L140Ffs*6/*PTEN* R173P
C-020	Male	48	VI	Papillary thyroid carcinoma	*BRAF* V600E/*PIK3CA* H1047R
C-026	Female	31	V	Papillary thyroid carcinoma	*BRAF* V600E/*RET* N783S
C-029	Female	30	VI	Papillary thyroid carcinoma	*BRAF* V600E/*TERT* C228T
C-044	Male	57	VI	Papillary thyroid carcinoma (CPTC+TVCPTC)	*BRAF* V600E/*TP53* R248Q
C-421	Female	44	NA	Papillary thyroid carcinoma	*BRAF* V600E/*TP53* R273H
C-455	Male	62	NA	Metastatic thyroid cancer	*NRAS* Q61R/*TP53* C141Y/*TERT* C228T
C-456	Female	59	NA	Metastatic thyroid cancer	*NRAS* Q61K/*TERT* C228T/*CCDC6*(1)-*RET*(12)
C-463	Female	22	III	Recurrent thyroid cancer	*PTEN* R335*/*TSHR* L629F

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Evidence suggests that *TERT* promoter mutation might be a late genetic event in tumorigenesis and is associated with tumor aggressiveness and poor prognosis. Compared with *BRAF* or *TERT* mutation alone, patients with coexisting mutations are diagnosed at an older age and have larger tumors and more clinically significant aggressiveness, suggesting a poorer prognosis (34). The overall incidence of *TERT* mutations in the present study was only 2.8% (13/457), which was lower than expected in both FNAB and FFPE samples, and seven of the patients also carried the *BRAF* V600E mutation.

RET is a proto-oncogene expressed in parafollicular C cells, and *RET* mutations lead to MTC (19). The risk of MTC in *RET* germline mutation carriers approaches 100%, and *RET* somatic mutations were identified in approximately half of the sporadic MTCs (35). Somatic RET mutations (e.g. M918T) are associated with more advanced pathological TNM staging, greater likelihood of disease recurrence, metastasis, and lower survival rates. It suggests somatic *RET* mutations can be prognostic biomarkers (36, 37, 38, 39). In this study, the *RET* M918T mutation was detected in an FFPE specimen with Bethesda V cytopathology, and histopathology confirmed the MTC. Considering that this is a clear pathogenic mutation, it should be recommended that this patient undergo germline testing to rule out a familial effect. In addition, one patient was detected with both *RET* N783S and *BRAF* V600E mutations, cytopathology and surgical pathology confirmed the PTC. A previous study reported the *RET* N783S mutation in MTC, but the investigators categorized this mutation as a variant of unknown significance (40). On the contrary, the present study suggests that the *RET* N783S variant could be pathogenic and be associated with thyroid cancer.

RET fusions are relatively common in PTC, with clonal *RET/PTC* fusions occurring in approximately 20% of PTCs, while non-clonal *RET/PTC* fusions occur in thyroid adenomas and some non-neoplastic lesions (41, 42, 43). Thirteen types of *RET/PTC* fusion protein have been identified, with *RET/PTC1* (~60%) and *RET/PTC3* (~30%) being the most common (41). There are different clinical features between *RET/PTC1* and *RET/PTC3,* and no consensus has been reached on their prognostic value. *RET/PTC3* is usually associated with a worse prognosis, such as a more aggressive phenotype, larger tumor size, and more advanced lesions at diagnosis. *RET/PTC1* is generally more common in younger patients (44). In this study, patients with *RET/PTC1* were 17–59 years old (>70% were <40 years old) and the male-to-female ratio was 1:5. The patients with RET/PTC3 were 25–56 years old and the male-to-female ratio was 1:3.

Ye *et al.* (45) found that three mutually exclusive mutations, *ZNF148*, *SPOP*, and *EZH1*, occur in 24.3% of adenomatoid nodules and have not been identified in PTCs. Therefore, any of the *EZH1*, *SPOP,* and *ZNF148* mutations alone may only occur in benign hyperplastic or adenomatous nodules and can be tentatively considered molecular markers of benign thyroid nodules. In this study, samples carrying the *EZH1* and *SPOP* P94R mutations have not been surgically pathologically confirmed, and cytopathology results are Bethesda II–III. Hence, these nodules are likely to be benign. Histopathology and additional protein function experiments are necessary to clarify the clinical significance of these genetic alterations.

The diagnostic ability of RAS and PTEN gene alterations in diagnosing thyroid nodules needs further evaluation

RAS mutations occur in approximately 23% of nodules and are the second most common mutation after *BRAF* V600E (29). A comprehensive analysis of several studies found that *RAS* mutations occur in multiple types of thyroid cancer (46): 40% in FTC, 26% in FA, 5% in Hurthle cell adenoma (HA), 11% in Hurthle cell carcinoma (HC), 11% in PTC, up to 53% in ATC, and 0–43.3% in sporadic MTC (mainly *HRAS* and *KRAS*) (47). *RAS* mutations have limited diagnostic value in preoperative thyroid nodules, especially follicular-like lesions (29). The weighted mean values of PPV and NPV in cytologically indeterminate thyroid nodules were 78.0% and 64.0%, respectively (48). It was found that only 28.6% (16 out of 56) of *RAS*-positive nodules were postoperatively confirmed to be thyroid cancer and were predominantly FVPTC. This study indicated that *RAS* mutations increased cancer risk when coexisting with other genetic mutations (*TP53*, *TERT* promoter region, *NTRK* fusion, etc.). The malignant risk was much higher in Bethesda V than in Bethesda III or IV for *RAS*-positive nodules (49). Gupta *et al.* (50) noted that most *RAS*-positive thyroid cancers have indeterminate cytologic features, few lymph node metastases, and distant metastases. It is also recommended that only total thyroidectomy should be performed in such patients; prophylactic lymph node dissection is not required unless preoperative ultrasound or intraoperative treatment reveals very distinct malignant features (50). In the present study, among the samples that carried

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only the *RAS* mutation, nine were malignant and two were benign, and the three samples was coexisting other mutations and were confirmed malignant.

PTEN is a relatively common tumor suppressor in cancer, dependent on its lipid phosphatase activity, which negatively regulates the PI3K-AKT-mTOR pathway, and loss-of-function mutations in *PTEN* have been identified in various cancers (51). Data from the COSMIC study show that PTEN mutations occur in 3% of benign adenomas and nodular goiters (25). This study also suggested that *PTEN* mutations can occur in benign and malignant lesions. Therefore, samples carrying *PTEN* mutations alone are difficult to diagnose directly, and combined with cytology or ultrasound results for further determination is necessary.

Combining cytology and multi-gene testing improves diagnostic accuracy

Cytology is only a snapshot of the thyroid nodule, which could have been performed before malignant changes appeared. In this way, using FNAB alone is generally associated with low sensitivity but relatively high specificity (1, 4, 52). Among them, cytology Bethesda III–IV cannot determine benign and malignant, and more clinical attention is needed. Therefore, combining cytology with genetic testing for mutations known to be pathogenic is an appealing strategy to identify malignant or future malignant nodules. In the present study, combining cytology and mutations increased the specificity from 25% to 75%. This increase in accuracy is supported by Ren *et al.* (25). Nevertheless, 23 samples had no gene mutations. Sixteen showed malignancy (Bethesda types V or VI) by cytology, and the operation was performed. The other seven samples were types III–IV, and their exact nature could not be determined; combined with their medical history, the patients request surgery due to anxiety. A larger molecular assay panel might be needed to explore more types of molecular alterations. The commercial Thyroseq V3, Afirma GSC, and ThyraMIR panels are available for thyroid specimens (23), but they are not available in China because they did not include Chinese patients, and thyroid cancer in Chinese patients display different mutation patterns than in Western patients (24). The 18 genes were selected based on the data from the Chinese population. Whether the panel can be applied to other populations remains to be investigated.

Multi-gene mutations are more associated with malignant tumors, which could help in diagnosis

The diagnostic role of *BRAF* V600E mutation-based assays in thyroid nodules has been fully recognized due to high incidence, but NGS panels based on multiple genes and multiple detection types (such as Thyroseq V3) provide higher-precision diagnostic performance for all common types of thyroid cancer and parathyroid disease and can simultaneously consider higher sensitivity, specificity, and accuracy (9). Tumorigenesis is associated with the activation of various proto-oncogenes (e.g. *BRAF* and *KRAS*), inactivation of tumor suppressors (e.g. *TP53* and *PTEN*), and abnormalities in value-added apoptosis regulatory genes and repair genes (53). This study indicated that the malignant risk was higher in the presence of multiple mutations, but the number of patients with multiple mutations ($n = 11$) was too small for more detailed analyses.

Subgroup analysis in Bethesda III and IV nodules

The performance of cytology alone in Bethesda II and VI nodules is well-documented (26, 54). The panel's efficacy was assessed on cytologically indeterminate nodules (Bethesda III and IV) as that is the result that will most likely trigger testing for molecular markers to guide further management. The subgroup analysis specifically for Bethesda III and IV suggests that the molecular gene panel improves accuracy for indeterminate cytology nodules. These results have the most immediate translational potential for patient care. Still, the numbers of patients were small in the subgroups, and the results need to be confirmed in future studies.

Limitations

The number of benign samples in this study was small, and only seven samples could be used for statistical analysis. Limited to the number of benign samples, the different biomarker combinations in this study did not yield promising results in terms of specificity. Indeed, the NPV of the test reported here is lower than the NPV of the three major approved tests in Western countries (23). It could be attributed to the overall low proportion of benign nodules in the present study. Additional studies in larger numbers of patients with more various lesions are necessary to complete the validation process of the

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test. The present study aimed to examine the cytology and genetics of the thyroid specimens. Because several sonographers performed the ultrasound examinations and sonography is notoriously operator dependent, the ultrasound features were not analyzed in the present study. Finally, the present did not consider the health economics of the 18-gene panel. Whether using the 18-gene panel is cost-effective by allowing the early treatment of thyroid cancer remains to be examined.

Conclusion

The 18-gene test panel appears to increase the diagnostic ability of FNAB for malignant thyroid nodules. Combining cytology and mutations increased the diagnostic accuracy compared with cytology alone. It might be necessary to analyze the molecular mechanisms of disease development at the genetic level to predict patients with malignant nodules more accurately in different risk strata and develop rational treatment strategies and definite management plans.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-23-0135.

Declaration of interest
All authors declare that they have no competing interests.

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Author contribution statement
Lei Dong, Zhiqiang Yin, and Jian Fei conceived and supervised the study; Min Ding and Rongli Xie designed experiments; Dongyan Han, Min Ding, and Rongli Xie performed experiments; Dongyan Han provided new tools and reagents; Zhengshi Wang, Guohui Xiao, and Xiaohong Wang analyzed data; Dongyan Han, Min Ding, and Rongli Xie wrote the manuscript; Lei Dong, Zhiqiang Yin, and Jian Fei made manuscript revisions. All authors reviewed the results and approved the final version of the manuscript.

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