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Diagnostic performance of next-generation sequencing and genetic profiling in thyroid nodules from a single center in China

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Abstract

Objective: The data regarding the mutation landscape in Chinese patients with thyroid cancer are limited. The diagnostic performance of thyroid nodules by fine-needle aspiration (FNA) cytology needs optimization, especially in indeterminate nodules.

Methods: A total of 1039 FNA and surgical resection samples tested using the targeted multigene next-generation sequencing (NGS) panel were retrospectively collected. The features of gene alterations in different thyroid tumors were analyzed, and the diagnostic efficacy was evaluated.

Results: Among 1039 samples, there were 822 FNA and 217 surgical FFPE samples. Among 207 malignant thyroid resections, a total of 181 out of 193 papillary thyroid carcinomas (PTCs) were NGS-positive (93.8%), with a high prevalence of *BRAF* mutations (81.9%, 158/193) and a low prevalence of *RAS* (1.0%, 2/193) and *TERT* promoter mutations (3.6%, 7/193). Gene fusions, involving the *RET* and *NTRK3* genes, were present in 20 PTCs (10.4%) and mutually exclusive with other driver mutations. Two of three follicular thyroid carcinomas harbored multiple mutations. *RET* gene point mutations were common in medullary thyroid carcinoma (8/11, 72.7%). The combination of cytology and DNA–RNA-based NGS analysis demonstrated superior diagnostic value (98.0%) in FNA samples. For indeterminate thyroid nodules, the diagnostic sensitivity and specificity of NGS testing were 79.2 (38/48) and 80.0% (8/10), respectively. Two mutation-positive benign cases harbored *NRAS* and *TSHR* mutations, respectively.

Conclusions: Our study revealed the distinct molecular profile of thyroid tumors in the Chinese population. The combination of NGS testing and FNA cytology could facilitate the accurate diagnosis of thyroid nodules, especially for indeterminate nodules.

Key Words

- ▶ thyroid nodule
- ▶ papillary thyroid carcinoma
- ▶ next-generation sequencing
- ▶ diagnostic performance
- ▶ genetic profiling

Introduction

With the increasingly available and progressively sensitive use of ultrasonography, thyroid cancer is one of the fastest growing types of malignancy worldwide and is especially prevalent among the young and adolescents, ranking ninth place for global incidence in 2020 (1). Mortality rates from the disease are low. However, a few patients do develop distant metastases and suffer significant morbidity secondarily to surgical procedures and radiation exposure. Therefore, a deep and comprehensive understanding of genetic alterations underlying their pathogenesis is crucial for accurate diagnosis and precise treatment. Previous studies revealed that the genetic alterations of thyroid cancer mainly involve the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, including point mutations in *BRAF*, *RAS*, *RET*, *TP53*, *PTEN*, and *PIK3CA* and chromosomal rearrangements in *RET*, *NTRK*, and *PPARG* (2). Large-scale studies have fully elucidated the genomic and pathological alterations of thyroid cancer, especially papillary thyroid cancer (PTC), in western populations; however, the data on the genetic landscape in Chinese patients, accounting for nearly one-third of global new cases, remain limited (3, 4, 5).

Fine-needle aspiration (FNA) followed by cytological examination is the standard diagnostic procedure for patients with suspicious thyroid ultrasound features. The diagnoses of most FNA samples are classified into malignant or benign categories, while approximately 20–30% of cases are cytologically indeterminate (6), presenting a dilemma for diagnosis and clinical management. In the updated Bethesda System, in addition to repeat FNA or diagnostic lobectomy, molecular testing has been recommended to further stratify the risk of cytologically indeterminate nodules. To date, various molecular testing techniques based on next-generation sequencing (NGS), such as ThyroSeq v1/v2/v3, have been widely applied in the clinical diagnosis of indeterminate thyroid nodules and personalized treatment. Published data regarding the value of the ThyroSeq v2 NGS assay for the diagnosis of indeterminate nodules show a high negative predictive value (NPV, 91–96%) and relatively low positive predictive value (PPV, 22–42%) (7, 8, 9, 10). The updated ThyroSeq v3 assay yields 112 genes with similar superior diagnostic NPV (94%) and favorable PPV (66%) (11). However, most of these molecular analyses are performed in the western populations, and ThyroSeq NGS assays are not available for Chinese patients. In the current study, we used a multigene NGS panel to detect targeted DNA mutations and RNA fusions in a large cohort of 1041 samples including FNA

materials and surgical resections. The purpose of this study was to depict the genetic landscape of different types of thyroid tumors in China and further assess the diagnostic performance of the NGS assay in FNA samples, especially in indeterminate nodules.

Materials and methods

Patients

All cases that were analyzed using the targeted multigene NGS panel were retrospectively and consecutively collected between January 2020 and May 2021 at the Fudan University Shanghai Cancer Center. Sample types for molecular testing included surgical formalin-fixed paraffin-embedded (FFPE) and FNA samples. Patients with suspicious thyroid ultrasound features underwent precisely ultrasound-guided FNA, and the FNA samples (liquid cytology) were analyzed by cytology and molecular testing at the same time. The diagnoses of surgical resections and FNA samples were confirmed by two experienced pathologists. Microscopically, the tumor size should not be smaller than 0.3 cm, the percentage of tumor should be more than 20% and the necrosis should be less than 50% in FFPE samples. Clinicopathological data, including age, gender, site, cytological and histological diagnoses, tumor diameter, and status of the lymph nodes at diagnosis were reviewed. The results of NGS testing were retrospectively analyzed. All patients gave their informed consent before FNA or surgery. This study protocol was reviewed and approved by the Institutional Ethics Committee at Fudan University Shanghai Cancer Center.

Library preparation and sequencing

Sequencing libraries were prepared using the thyroid cancer multigene panel (RigenBio) according to the manufacturer's instructions. The thyroid cancer NGS panel is a multiplex PCR-based NGS test for point mutations and insertions/deletions of 22 related genes (*AKT1*, *ALK*, *BRAF*, *CTNNB1*, *CHEK2*, *EIF1AX*, *EZH1*, *FGFR1*, *FLT3*, *GNAS*, *HRAS*, *KIT*, *KRAS*, *NRAS*, *ZNF148*, *PIK3CA*, *PTEN*, *RET*, *SPOP*, *TERT* promoter, *TP53*, and *TSHR*) and gene fusions occurring in thyroid cancer including *RET*, *NTRK1*, *NTRK3*, and *PPARG* (detailed description in Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). Briefly, genomic DNA and total RNA were isolated from FFPE samples, which were reviewed by a pathologist to ensure sufficient tumor content, and FNA samples using AllPrep DNA/RNA Kit (Qiagen). DNA and RNA were

quantified using a NanoDrop 2000 unit (Thermo Fisher Scientific). Total RNA was reverse-transcribed into cDNA. An aliquot of DNA or cDNA was subjected to multiplex PCR for the amplification of target regions. Subsequently, a unique index and a universal adapter were added to each amplified library using PCR amplification. The purified indexed libraries were quantified on a Qubit fluorometer (Thermo Fisher Scientific), and the size distributions of the libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies). Then, the qualified libraries were sequenced for 150bp paired-end reads on the NovaSeq 6000 platform (Illumina).

Bioinformatics analysis

Before alignment, residual adapter sequences were removed from the 3' and 5' ends of paired-end sequencing reads using Trimmomatic (v0.38). Then VarScan (v2.3.9) was used for SNV/InDel calling. The variants were annotated using ANNOVAR and Variant Effect Predictor (VEP) including Sorting Intolerant From Tolerant score, Polymorphism Phenotyping score, and Clinical Significance Functions). The output was reformatted using a custom script to ensure the description of sequence variants was compliant with the standards of HGVS nomenclature.

Statistical analysis

Fisher exact tests were performed to analyze the associations of gene alterations with different clinicopathological features using SPSS version 22.0 (SPSS, Inc.), and $P < 0.05$ (two-sided) was considered statistically significant.

Results

General characteristics

A total of 1039 samples from 1029 patients were reviewed, and 10 patients had two samples analyzed by NGS. Among the 1039 samples, there were 822 FNA and 217 surgical FFPE samples. Of 822 FNA samples, 780 samples originated from the thyroid and 42 from the cervical lymph node which were suspected of thyroid cancer metastasis. The age of patients ranged from 16 to 82 years (mean 45 years) and the ratio of female to male was 2.8:1. In terms of cytological diagnosis, 13 (1.6%) samples were classified as nondiagnostic, 202 (24.6%) as benign, 147 (17.9%) as indeterminate (142 atypia of indeterminate significance/follicular lesion of indeterminate significance AUS/FLUS, and 5 follicular neoplasm/suspicious for follicular neoplasm FN/SFN),

92 (11.2%) as suspicious for malignancy (SUSP), and 368 (44.8%) as malignant. Of 217 surgical resection samples, the site of 183 samples originated from the thyroid and 34 from the cervical lymph node. The age of patients ranged from 14 to 75 years (mean 41 years) and the ratio of female to male was 2.2:1. The histological diagnoses included 193 PTCs, 11 medullary thyroid carcinomas (MTCs), 3 follicular thyroid carcinomas (FTCs), 2 follicular tumors of uncertain malignant potential (FT-UMPs), 4 follicular adenomas (FAs), and 4 nodular goiters.

Genetic features of thyroid tumors

In the group of 217 surgical resection samples whose histopathological diagnoses were available for further analysis, there were 207 malignant samples (including PTC, MTC, and FTC), 8 benign (including FA and nodular goiter), and 2 FT-UMPs. For 207 malignancies, 123 cases presented with lymph node metastases at diagnosis (59.4%). Tumor diameter of 183 primary thyroid carcinomas ranged from 0.3 to 12 cm (mean 1.2 cm). Among the 217 thyroid lesions, the most commonly mutated gene is *BRAF* gene (73.3%), followed by *RET* fusions (7.8%), *TERT* promoter (4.1%), *RET* gene (4.1%), *NRAS* gene (2.3%), *TP53* (1.8%), and *NTRK3* fusions (1.4%). The detailed overall mutation profiling was shown in Fig. 1.

In the cohort of 193 PTCs, 181 samples (93.8%) had pathological/likely pathological mutations by NGS testing. *BRAF* was the most commonly mutated gene with a mutation prevalence of 81.9% (158/193) and T1799A (V600E) was the most common substitution (157/158). Alterations of *RAS* genes (including *KRAS*, *NRAS*, and *HRAS*) were present in 2 (1.0%) PTCs. *KRAS* (Gly12Val) and *NRAS* (Gln61Arg) mutations were detected in one PTC, and *NRAS* mutation (Gln61Lys) in another PTC. Mutations in the *TERT* promoter hotspot C228T were identified in seven cases (3.6%). Mutations in *TP53* and *PIK3CA* genes were relatively rare, with a positive rate of 1.6% (3/193) and 0.5% (1/193), respectively. All of mutations in *TERT* promoter, *TP53* and *PIK3CA* coexisted with the presence of *BRAF* V600E mutation. Furthermore, 20 cases (10.4%) harbored gene fusions, including 17 *RET* fusions (12 *CCDC6-RET* and 5 *NCOA4-RET*) and 3 *ETV6-NTRK3* fusions. Among three FTCs, two cases were NGS positive (one co-occurrence of *PTEN* and *TP53* mutations and one co-occurrence of *NRAS* and *TERT* promoter mutations). Eleven MTCs were analyzed using NGS testing, and ten tumors were positive in the analysis (90.9%). As expected, the most common mutation in MTC was *RET* missense mutations ($n=8$), followed by *BRAF* ($n=1$) and *HRAS* mutations ($n=1$).

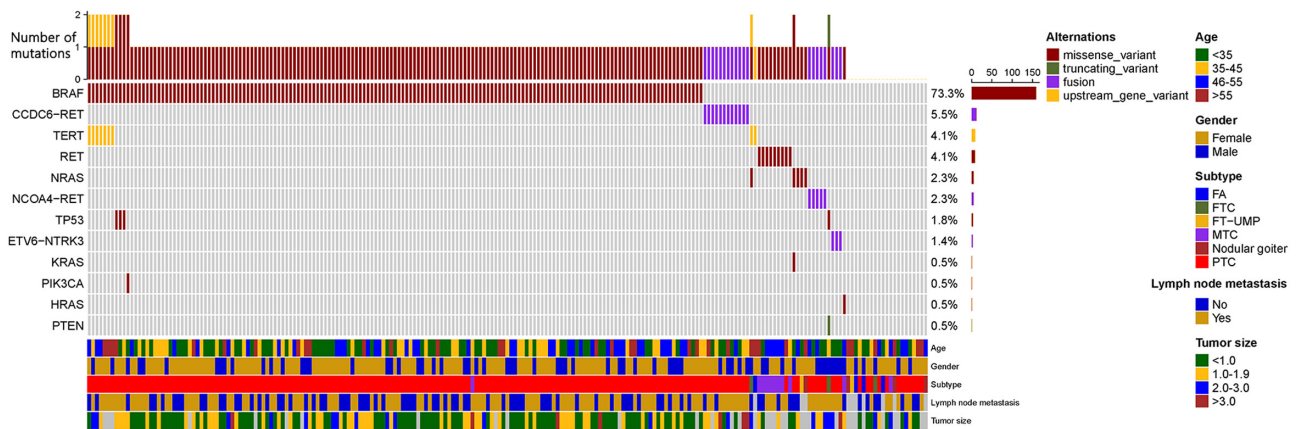


Figure 1

Mutation profiling of 217 surgical resection samples by multigene NGS testing. Mutated genes included *BRAF*, *RET*, *TERT* promoter, *NRAS*, *TP53*, *NTRK3*, *KRAS*, *PIK3CA*, *HRAS*, and *PTEN*. Clinicopathological features included age, gender, tumor subtype, lymph node metastasis, and tumor size. FA, follicular adenoma; FTC, follicular thyroid carcinoma; FT-UMP, follicular tumors of uncertain malignant potential; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma.

One of two FT-UMPs was NGS positive with a *NRAS* mutation. Among eight benign samples, a *NRAS* mutation was identified in one nodular goiter, and a *TERT* promoter (C228T) mutation in one substernal FA whose tumor growth rate was aggressive but prognosis was currently favorable after over 1 year follow-up.

The mutation profile of thyroid tumors showed that the majority of NGS-positive cases presented a single gene mutation, while a few harbored multiple genetic alterations. *TERT* promoter, *TP53*, and *PIK3CA* mutations were significantly associated with the presence of the *BRAF* mutation, while gene fusions involving *RET* and *NTRK3* genes were mutually exclusive with other driver mutations including *BRAF*, *RAS*, and *TERT*. In the cohort of 207 thyroid malignancies, statistical analyses showed that the presence of a *BRAF* mutation was associated with

a small tumor size ($P = 0.000$) and a *RAS* mutation was associated with an absence of lymph node metastasis ($P = 0.026$). A *TERT* promoter mutation was correlated with older age (>41 years, $P = 0.002$) and large tumor size (>1.2 cm, $P = 0.038$), while gene fusions were correlated with younger age (≤ 41 years, $P = 0.016$), relatively more male patients ($P = 0.020$), large tumor size (>1.2 cm, $P = 0.000$), and lymph node metastasis ($P = 0.003$) (Table 1).

Diagnostic performance of NGS testing in FNA samples

Among 822 FNA samples, the positive rates of NGS testing were 23.1% (3/13) in nondiagnostic cytology, 23.8% (48/202) in benign cytology, 49.7% (73/147) in indeterminate cytology, 83.7% (77/92) in SUSP cytology,

Table 1 Clinicopathological characteristics of different gene alterations in 207 thyroid malignancies.

	<i>BRAF</i>			<i>RAS</i>			<i>TERT</i> promoter			Fusions		
	Mut	Wild	<i>P</i>	Mut	Wild	<i>P</i>	Mut	Wild	<i>P</i>	Mut	Wild	<i>P</i>
Age												
Mean	41	41	0.946	50	41	0.332	57	40	0.002	35	42	0.016
≤ 41	87	26		1	112		0	113		16	97	
>41	72	22		3	91		8	86		4	90	
Gender												
Male	46	20	0.097	2	64	0.594	3	63	0.712	11	55	0.020
Female	113	28		2	139		5	136		9	132	
Tumor size												
Mean	1.1	1.7	0.000	1.3	1.2	0.595	1.7	1.1	0.038	2.2	1.1	0.000
≤ 1.2	109	15		2	122		1	123		3	121	
>1.2	38	21		2	57		4	55		12	47	
Lymph node metastasis												
Yes	92	31	0.406	0	123	0.026	6	117	0.477	18	105	0.003
No	67	17		4	80		2	82		2	82	

Table 2 Diagnostic performances of cytology, NGS analysis, and the combination of cytology and *BRAF* and NGS analysis for predicting thyroid malignancy in 313 cytologically negative (Bethesda I/II) and positive (Bethesda V/VI) cases.

	Histology		Sensitivity (%)	Specificity (%)	Accuracy (%)
	Malignant (303)	Benign (10)			
Cytology					
Positive	297	3	98.0	70.0	97.1
Negative	6	7			
NGS analysis (including DNA/RNA analysis)					
Positive	266	0	87.8	100.0	88.2
Negative	37	10			
Cytology + <i>BRAF</i> mutation					
Positive	299	3	98.7	70.0	97.8
Negative	4	7			
Cytology + NGS analysis					
Positive	300	3	99.0	70.0	98.0
Negative	3	7			

and 89.1% (328/368) in malignant cytology, increasing with advancing cytological malignancy risk. Furthermore, twenty-one *BRAF* V600E-mutated FNA samples with very low allelic frequency (AF, 1–2%) were verified as truly positive using amplification refractory mutation system (ARMS) testing. In the series of 313 cytologically negative (13 Bethesda I/II) and positive (300 Bethesda V/VI) cases who underwent surgical resections, 303 samples (4 Bethesda I/II and 299 Bethesda V/VI) were histologically confirmed as malignant (including PTC, MTC, FTC, and poorly differentiated carcinoma) and 10 samples were benign (including FA, nodular goiter, and hyperplastic nodules). FNA cytology showed a diagnostic performance sensitivity of 98.0% and specificity of 70.0% for thyroid nodules. By adding NGS testing to FNA cytology, the diagnostic sensitivity further increased to 99.0% and the overall accuracy reached 98.0%. It's worth noting that four Bethesda I/II nodules diagnosed as malignant papillary thyroid microcarcinomas were all NGS positive in FNA samples. Furthermore, compared to the combination of cytology and a single *BRAF* mutation analysis, the performance of the combination of cytology and DNA–RNA-based NGS analysis demonstrated superior diagnostic value (accuracy 98.0%, Table 2).

For 147 nodules with indeterminate cytology which cause a diagnostic dilemma in clinical management, surgical resections in 58 cases revealed 43 malignant nodules (41 PTCs and 2 FTCs), 5 FT-Umps and 10 benign nodules (1 FA, 7 nodular goiters, and 2 hyperplastic nodules). Among 40 FNA samples that were positive by NGS analysis, 38 samples were confirmed as histologically malignant (33 PTCs and 2 FTCs), 3 as FT-Umps, and 2 as benign (1 nodular goiter and 1 hyperplastic nodule). The most commonly affected gene was *BRAF* ($n=26$),

followed by *NRAS* ($n=10$) and *CCDC6-RET* gene fusions ($n=3$). The two NGS-positive benign samples harbored *NRAS* (Gln61Arg) and *TSHR* (Met453Thr) mutations, respectively. Among 18 NGS-negative FNA samples, there were 8 PTCs, 2 FT-Umps, 6 nodular goiters, 1 FA, and 1 hyperplastic nodule. Therefore, when considering FT-Ump as malignant, the diagnostic performance of NGS testing in indeterminate nodules revealed 79.2% sensitivity (38/48), 80.0% specificity (8/10), 44.4% NPV (8/18), 95.0% PPV (38/40), and 79.3% accuracy (46/58).

Due to the relatively high frequency of NGS-negative PTCs in our study, we further analyzed surgical resection tissues of the eight NGS-negative PTCs and found that five PTCs harbored pathological mutations (four *BRAF* V600E mutations with low allelic frequencies from 1.05 to 17.22%, and one *CCDC6-RET* fusion) and remaining three PTCs were mutation negative.

Discussion

In this study, we adopted an efficient molecular testing based on a comprehensive panel of DNA mutations and RNA gene fusions occurring in thyroid cancer that could provide both diagnostic and therapeutic information in a large series of 1039 samples. Compared with large-scale NGS assays containing several irrelevant genes that may reduce the amplicon coverage of clinically relevant indexes, our targeted panel could effectively detect DNA-based mutations and RNA-based gene fusions at a very low level of nucleic acid input for FNA and FFPE samples and is more cost-effective and time-saving in clinical practice. Additionally, given that *BRAF* V600E-mutated samples with relatively low AF (<2%) were verified to be

truly positive by ARMS testing, the present NGS testing was verified to be highly efficient in the detection of gene mutations. However, the cutoff level of AF was set as 5 or 10% in some previous studies (12, 13), which could probably miss a few malignant samples with compromised cellularity or a lower mutation frequency.

With the commercial availability of targeted therapies including *BRAF*, *RET*, and *NTRK* inhibitors (14, 15, 16) and numerous ongoing clinical trials revealing potential therapeutic targets in thyroid cancer (14), comprehensive analysis of genetic alterations using NGS, rather than single-gene testing, is considered to have high clinical value. Using this efficient approach, we comprehensively studied the genetic alterations of thyroid tumors in Chinese patients. PTC is the most common subtype, constituting 80–85% of all thyroid malignancies, mainly involving the MAPK and PI3K pathways (2, 13, 17). The frequency of *BRAF* mutations is about 51% in the Cosmic database based on western studies (18). However, similar to the results from Liang *et al.* in the Chinese population (72.4%) and Hong *et al.* in the Korean population (73.3%) (19), a high prevalence of *BRAF* mutation in PTC was also identified in our study (81.9%). However, the prevalence of *RAS* genes, including *NRAS*, *KRAS*, and *HRAS*, was only 1.0% in our PTC cases, which was lower than that in The Cancer Genome Atlas data (approximately 13%) (13, 17, 18). The genetic discrepancies in PTC between different populations may be related to different races and geographic regions. The most frequent type of *RET* gene alterations in PTC is gene fusion, whereas in MTC is a missense mutation. Consistent with the literature, the most common gene fusion was a *RET* fusion in our study (17/193, 8.8%), which frequently fused with the *CCDC6* and *NCOA4* genes (16, 17). Rearrangements of *RET* or *NTRK* genes are generally mutually exclusive with other driver mutations in the MAPK pathway including *BRAF* and *RAS* (3, 17, 20). However, Zou *et al.* also reported a 13% rate of concomitant mutations including concomitant *BRAF* V600E and *RET/PTC-1* fusion in 88 PTCs (21), indicating the importance of molecular analysis at both the DNA and RNA level to provide more potential therapeutic insights. Moreover, previous and present studies also revealed the association of kinase gene fusions with younger age, large tumor size, and lymph node metastasis (3, 17). In the literature, mutations of *TERT* promoter, *TP53*, and *PIK3CA* genes are considered to occur late in the progression of thyroid cancer and are associated with a more de-differentiated subtype and aggressive clinical course (16, 22, 23). In our study, mutations in the *TERT* promoter, *TP53*, and *PIK3CA* genes were associated with

the presence of a *BRAF* mutation. Furthermore, *TERT* promoter mutations were confirmed to be associated with aggressive features including older age and large tumor size, and only one histopathologically confirmed poorly differentiated thyroid carcinoma also harbored a *TP53* mutation in the FNA sample. Previous studies report a high prevalence of *RAS* mutations in FTC (30–40%) (13, 18), consistent with the result in our cohort of FTC (33.3%) and FT-UMP (50%). Compared with benign FA and low-grade FT-UMP, FTC harbored more high-risk aberrations including *PTEN*, *TP53*, and *TERT* promoter mutations, which could help the differential diagnosis in follicular lesions (24, 25, 26). Sporadic *RET* missense mutations were also the most common genetic alteration in MTC (72.7%) (3, 18).

Previous studies have verified that FNA cytology, as the standard preoperative tool for the diagnosis of thyroid nodules, is characterized by outstanding diagnostic sensitivity and specificity compared with surgical histology. However, approximately 20–30% of FNA samples reveal an indeterminate state (AUS/FLUS and FT/SFT), with only a 10–40% risk of malignancy (6). In recent years, several molecular tests were developed to improve the accuracy of preoperative diagnosis of cytologically indeterminate thyroid nodules and minimize false-positive and false-negative cytology (7, 12, 27, 28). The multigene NGS assay in the current study, targeting point mutations and gene fusions in thyroid cancer, showed superior diagnostic values. As the risk of malignancy increased in FNA cytology, so did the positive rates of NGS analysis. Compared with single FNA cytology or NGS analysis, the combination of cytology and NGS analysis showed better diagnostic performance in cytologically unequivocal nodules. Among cytologically negative (Bethesda I/II) and positive (Bethesda V/VI) nodules, although the diagnostic accuracy of the combination of cytology and a single *BRAF* mutation reached 97.8%, the DNA–RNA-based NGS testing could further improve the diagnostic value (98.0%) and provide more information about treatment and prognosis. Besides *BRAF* mutation, genetic alterations such as mutations of *NRAS*, *RET*, *PTEN*, *TERT* promoter, and gene fusions involving *RET*, *PPARG*, and *NTRK1* could also provide critical diagnostic, prognostic, and therapeutic clues for different types of thyroid cancer (29). Our results also highlighted that among 13 Bethesda I/II cases who underwent surgical resections, 4 cases (4/13, 30.8%) harbored driver mutations were confirmed as malignant by histopathology, indicating the high diagnostic value of NGS testing in thyroid nodules even in Bethesda I/II nodules. Therefore, for Bethesda I/II nodules

with mutations, we predict that there may be some missed malignant cases because of no surgical treatment. Close follow-up can enable patients with mutations to obtain timely diagnosis and treatment. Additionally, there are two mutation-positive benign cases presented with *NRAS* and *TSHR* point mutations, respectively. In the literature, Nikiforov *et al.* also reported that only one of three *TSHR* mutation-positive nodules was identified as cancer after surgery and two *NRAS*-mutated samples were benign on histology (30). Furthermore, *RAS* mutations are considered to be not specifically associated with malignant (75–88%) or potentially malignant outcomes (7, 27, 31, 32, 33) and are highly predictive of predominantly low-risk follicular-pattern carcinomas (34), which is further supported by our results of a significantly lower frequency of lymph node metastasis in *RAS*-mutated malignancies. Additional gene mutations including *SPOP*, *EZH1*, and *ZNF148* are also suggested to be benign markers (32). Therefore, additional features are needed for a more accurate malignancy prediction in thyroid nodules with *TSHR*, *NRAS*, or some 'low-risk' gene mutations.

Importantly, the diagnostic performance of molecular testing in indeterminate thyroid nodules is of great concern in clinical practice. The risk of malignancy (including FT-UMP) in indeterminate samples in our study (82.8%, 48/58) was higher than that in previous data (6), which may be attributable to experienced clinical surgeons in our cancer center. For indeterminate nodules, the diagnostic specificity and sensitivity of NGS testing in the FNA sample were 80.0 and 79.2%, respectively. In addition, among eight NGS-negative PTCs in FNA samples, we further detected pathological mutations in five PTCs using NGS testing in surgical resections. The discrepancies in molecular testing between FNA and surgical samples have been reported in previous reports (35) and may be due to the accuracy of aspiration varying depending on the experience of the ultrasound doctor, and the limited quantity of tumor cells in some FNA materials used for NGS testing, especially for the micro size of thyroid nodules. Moreover, it's worth noting that the histological diagnoses of ten NGS-negative malignancies in FNA samples were two FT-UMPs, five papillary thyroid microcarcinomas, and three classical PTCs (tumor diameter <2 cm) without lymph node metastasis, all of which were low-grade cancers with no histological features of aggressive behavior at presentation (12). Therefore, we suggest that patients with indeterminate cytology and no mutations detected by effective NGS testing should be monitored closely, and surgery should depend on follow-up and additional information. Compared with the widely accepted

ThyroSeq v2 NGS panel, which showed highly accurate diagnoses for nodules categorized as cytological FN/SFN (83% PPV, 96% NPV) (12), our targeted NGS assay featured with higher PPV (95.0%), but inferior sensitivity, specificity, and NPV, probably owing to the limited sample size of indeterminate nodules which underwent surgical resections and insufficient cytology accuracy in some FNA samples. Importantly, the superior PPV value suggested that the presence of a pathological mutation in our NGS panel was a strong indicator of cancer in indeterminate nodules. However, the limitation of the present study is that this is a retrospective study of clinical cases with complexity and variability, so the stated diagnostic value of NGS testing in the present study should be possibly skewed by the unresected nodules.

In summary, through targeted multigene NGS testing including DNA mutations and RNA fusions, our study further elucidated the distinct genetic profile of thyroid tumors in Chinese populations and showed a relatively higher frequency of *BRAF* mutation and lower frequency of *RAS* and *TERT* promoter mutations in PTC compared with that in western populations. For the diagnostic accuracy of cytologically unequivocal nodules (Bethesda I/II and V/VI), the combination of cytology and a targeted DNA–RNA-based NGS panel showed optimal clinical value. Besides, compared with single *BRAF* gene testing, the NGS testing could provide more diagnostic, prognostic and therapeutic information. In the diagnosis of indeterminate nodules, our NGS testing was characterized by superior PPV value. For indeterminate nodules with negative NGS results or single *TSHR* or *RAS* gene mutation, clinical management should be close surveillance, thereby avoiding immediate surgical resections. The clinical value of the present NGS panel, especially for indeterminate nodules, still needs further verification in prospective large-scale cohorts to facilitate the precise diagnosis and treatment of thyroid nodules.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ETJ-21-0124>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Min Ren: edited the manuscript and analyzed the data; Qianlan Yao: collected and analyzed the data; Longlong Bao: performed the experiments and collected original data; Zhiting Wang: performed the experiments; Ran Wei and Qianming Bai: analyzed the data; Bo Ping and Cai Chang: provided part of data; Yu Wang: provided pivotal opinions about the study; Xiaoli Zhu and Xiaoyan Zhou: conceived and designed the study and revised the paper.

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