

Diagnostic value and lymph node metastasis prediction of a custom-made panel (thyroline) in thyroid cancer

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Abstract. Differentiation of benign and malignant thyroid nodules is crucial for clinical management. Here, we explored the efficacy of next-generation sequencing (NGS) in predicting the classification of benign and malignant thyroid nodules and lymph node metastasis status, and simultaneously compared the results with ultrasound (US). Thyroline was designed to detect 15 target gene mutations and 2 fusions in 98 formalin-fixed, paraffin-embedded (FFPE) tissues, including those from 82 thyroid cancer (TC) patients and 16 patients with benign nodules. BRAF mutations were found in 57.69% of the papillary thyroid cancer (PTC) cases, while RET mutations were detected among all the medullary thyroid cancer (MTC) cases. Multiple mutations were positive but none showed dominance in anaplastic thyroid cancer (ATC) and follicular thyroid cancer (FTC). The sensitivity and specificity of NGS prediction in differentiation of benign and malignant thyroid nodules were 79.27 and 93.75%, respectively, and the positive predictive value (PPV) and negative predictive value (NPV) were 98.48 and 46.88%, respectively. The sensitivity and

specificity of US were 76.83 and 6.25%, respectively, and the PPV and NPV were 80.77 and 5.00%, respectively. The area under curve (AUC) of NGS and US were 0.865 and 0.415, respectively. A total of 27 patients had ≥ 1 metastases to lymph nodes, 19 of which carried mutations, including BRAF, RET, NRAS, PIK3CA, TP53, CTNNB1 and PTEN. However, there was no correlation between the variant allele frequency of specific gene mutations and the number of metastatic lymph nodes. In conclusion, the prediction value of NGS was higher than the US-based Thyroid Imaging Reporting and Data System (TI-RADS). NGS is valuable for the accurate differentiation of benign and malignant thyroid nodules, and pathological subtypes in FFPE samples. The findings of the present study may pave the way for the application of NGS in analyzing fine-needle aspiration (FNA) biopsy samples.

Introduction

Thyroid cancer (TC) is the most common endocrine tumor and its incidence has continually increased over recent decades (1-5). Thyroid nodules are found in 50-60% of the population in adulthood, while only 5% of thyroid nodules are malignant (6). A previous study found that preoperative image examination and operation process missed 33% of lymph node metastases in TC patients (7). As a result, the main challenge in the management of thyroid nodules is to rule out malignancy from massive thyroid nodules and predict lymph node metastasis.

The diagnosis of TC is typically obtained through ultrasound (US) examination and fine-needle aspiration (FNA) biopsy (8). However, current US has an accuracy of only 72.6% (9). Between 20 and 30% of FNA biopsy samples may draw incorrect or indeterminate conclusions based on simple cytology (10,11). The current predictive value of US and FNA cannot fully meet the requirements of accurate diagnosis of TC. Accurate identification of the pathological subtype and lymph node metastasis status of TC preoperatively is important in developing individualized therapeutic strategies.

Genetic examination, as a newly-developed technique for cancer diagnosis, has been demonstrated to have advantages in the early diagnosis of cancer and in accelerating the examination process (12,13). As the most common gene mutation found

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Abbreviations: TC, thyroid cancer; US, ultrasound; FNA, fine-needle aspiration; NGS, next-generation sequencing; FFPE, formalin-fixed, paraffin-embedded; TI-RADS, Thyroid Imaging Reporting and Data System; PPV, positive predictive value; NPV, negative predictive value; PTC, papillary thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; FTC, follicular thyroid cancer; ROC, receiver operator characteristic; AUC, area under curve

Key words: thyroid cancer, next-generation sequencing, Thyroid Imaging Reporting and Data System, accurate diagnosis, metastasis

in TC, BRAF plays an important role in TC diagnosis and risk stratification (12). With the introduction of next-generation sequencing (NGS), high throughput sequencing is able to detect target mutations using only a small amount of DNA. The detection of mutations in TC is useful for providing a specific diagnosis in cytologically indeterminate cases (12,14).

In this study, we focused on the application of NGS for a panel of mutations in TC and evaluated its predictive efficacy in classifying benign and malignant nodules and their metastasis status, compared with conventional US examination and histological diagnosis.

Materials and methods

Thyroid samples. A total of 98 formalin-fixed, paraffin-embedded (FFPE) tissue specimens from surgically removed thyroid samples were collected at the Department of Pathology of the First Affiliated Hospital of Sun Yat-sen University between 2011 and 2016. Subsequent analyses were performed on 16 thyroid benign nodules and 82 TC specimens. Based on histological results, all tumors were classified by two independent pathologists. All patients provided informed consent before enrollment in the study, which was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

Tissue DNA extraction. DNA was extracted using QIAamp DNA FFPE tissue kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. DNA concentration was measured using a Qubit dsDNA assay.

NGS library preparation. DNA shearing was performed using Covaris M220, followed by end repair, phosphorylation and adaptor ligation. Fragments of 200–400 bp in size were selected by beads (Agencourt AMPure XP kit; Beckman Coulter, Inc., Brea, CA, USA), followed by hybridization with capture probe baits, hybrid selection with magnetic beads and PCR amplification. A bioanalyzer high-sensitivity DNA assay was performed to assess the quality and size of the fragments. Indexed samples were sequenced on a NextSeq 500 sequencer (Illumina, Inc., San Diego, CA, USA) with pair-end reads.

Capture-based targeted DNA sequencing. Genetic profiles of all tissue samples were assessed by capture-based targeted deep sequencing, using a lymphoma panel consisting of critical exons and introns of 31 genes (Burning Rock Biotech, Ltd., Guangzhou, China), covering 209 kb of human genomic regions. DNA quality and size were assessed by high sensitivity DNA assay using a bioanalyzer. All indexed samples were sequenced on a NextSeq 500 (Illumina, Inc.) with pair-end reads. When the NGS analysis was performed in thyroid-derived tumor samples, the white blood cells (WBCs) from the same patient were used as their own inner controls to ensure that the detected mutations were definitely somatic.

Sequence data analysis. After a successful sequencing reaction, the raw sequence data were mapped to the human genome (hg19) using BWA Aligner 0.7.10. Local alignment optimization, variant calling and annotation were performed using GATK 3.2, MuTect and VarScan. Variants were filtered

Table I. Demographic and nodule characteristics.

	Histological diagnosis	
	Benign group (n=16)	Malignant group (n=82)
Patient information		
Age (years)	48.00±6.07	45.95±3.20
Sex ratio (M/F)	6/10	25/57
Nodule characteristic		
Unique/multinodular	16/0	73/9
Maximum size (mm; mean ± SD)	7.3±6.1	13.7±11.6
Echo genicity		
Hypoechoic	8	39
Isoechoic	3	12
Hyperechoic	2	10
Unknown	3	21
Blood flow		
Rich	9	35
Normal	1	0
Low	4	22
Unknown	2	25
Microcalcification		
Positive	9	38
Negative	5	15
Unknown	2	29
Aspect ratio		
<1	11	46
>1	4	5
Unknown	1	24
Boundary		
Clear	7	33
Unclear	6	18
Unknown	3	31
TI-RADS category 4	15/16	63/82
Pathological subtype		
PTC	-	52
ATC	-	13
MTC	-	10
FTC	-	7
Lymph node metastasis		
Positive	-	27
Negative	-	55

SD, standard deviation; TI-RADS, Thyroid Imaging Reporting and Data System; PTC, papillary thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; FTC, follicular thyroid cancer.

using the VarScan fpfilter pipeline. At least 5 supporting reads were required for INDELs, while 8 supporting reads were required for SNVs to be defined. According to the ExAC, 1000 Genomes, dbSNP and ESP6500SI-V2 databases,

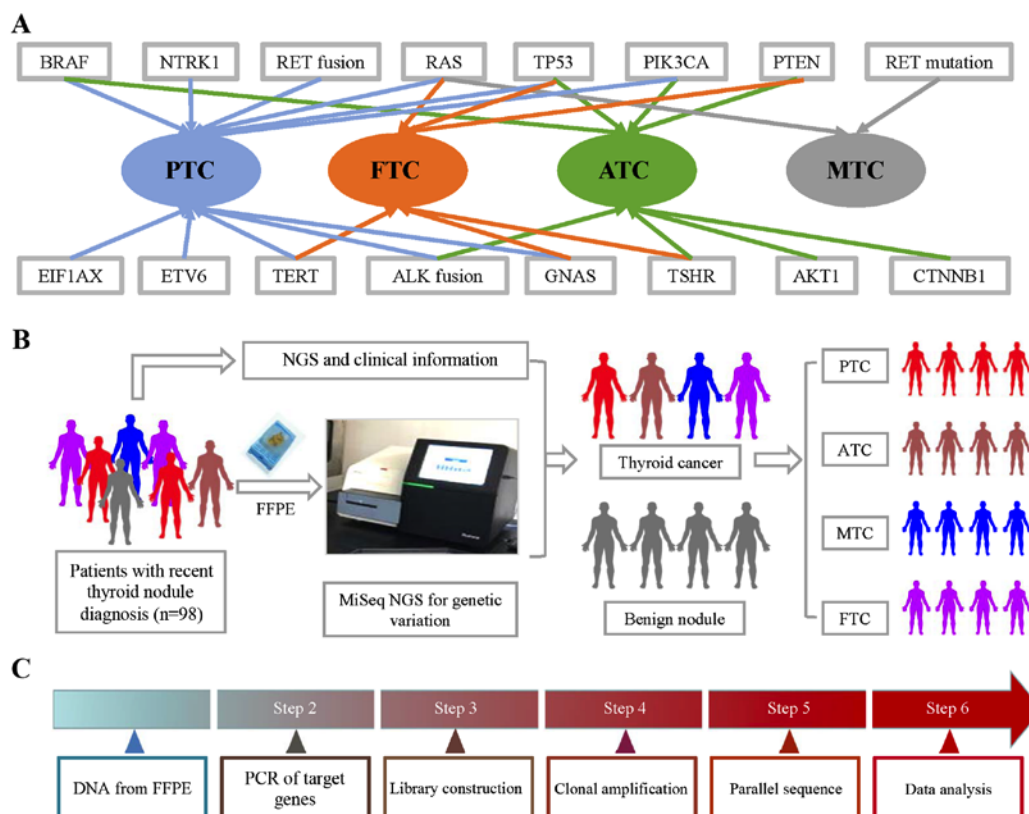


Figure 1. Gene mutations and fusions in subtypes of TC and workflow of NGS. (A) BRAF, RAS, TERT, ETV6, EIF1AX, GNAS, PIK3CA, TP53 and NTRK1 mutations, as well as RET and ALK fusions, were found in PTC. BRAF, TERT, ALK fusion, GNAS, AKT1, PIK3CA, TP53 and PTEN were found in ATC. RAS, TERT, TSHR, GNAS, PENT and TP53 were found in FTC, while only RET and RAS mutations were found in MTC. (B) FFPE samples were obtained from 98 thyroid nodule patients, which was followed by CTC enumeration on NanoVelcro Chips. After collecting clinical information, we analyzed the correlation between pathological information and NGS results. (C) DNA from FFPE tissue was amplified for enrichment of target regions in a multiplex PCR reaction. Then, the library was prepared by ligating the PCR amplicons into platform-specific adapters and adding bar codes for specimen multiplexing. Finally, the library was enriched by clonal amplification (emPCR) and sequenced by massively parallel sequencing on the Ion Torrent PGM. The data analysis and variant calling were performed using bioinformatic pipelines followed by a custom SeqReporter algorithm for filtering and annotation of genetic variants. TC, thyroid cancer; NGS, next-generation sequencing; PTC, papillary thyroid cancer; ATC, anaplastic thyroid cancer; FTC, follicular thyroid cancer; MTC, medullary thyroid cancer; FFPE, formalin-fixed, paraffin-embedded.

variants with population frequency >0.1% were grouped as SNPs and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using both TopHat2 and Factera 1.4.3.

Thyroid Imaging Reporting and Data System (TI-RADS) category. According to TI-RADS (15), thyroid nodules classified as category 4 with at least one suspicious US feature are believed to have a high probability of malignancy. The following US features showed a significant association with malignancy: solid component, hypoechoogenicity, marked hypoechoogenicity, microlobulated or irregular margin, microcalcification and taller-than-wide shape. Thyroid nodules without the above features were classified as category <4 (low risk).

Statistical analysis. Calculations of specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and receiver operator characteristic (ROC) were conducted using IBM SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Correlations were studied using Spearman's rank correlation coefficients. Significance level was set as $\alpha=0.05$.

Results

Thyroidine NGS panel design. To design a NGS sequencing panel for thyroid nodule patients, we conducted a review-based search in PubMed to collect TC-related genetic information. These gene mutations and fusions were found in particular subtypes of TC (Fig. 1). Papillary thyroid cancer (PTC) and anaplastic thyroid cancer (ATC) had the most related gene mutations. The literature reported that BRAF, RAS, TERT, ETV6, EIF1AX, GNAS, PIK3CA, TP53 and NTRK1 mutations, as well as RET and ALK fusions, were found in PTC. BRAF, TERT, ALK fusion, GNAS, AKT1, PIK3CA, TP53 and PTEN were found in ATC. Fewer gene mutations were found in medullary thyroid cancer (MTC) and follicular thyroid cancer (FTC). RAS, TERT, TSHR, GNAS, PENT and TP53 were found in FTC, while only RET and RAS mutations were found in MTC. The workflow of the NGS mutation analysis is shown in Fig. 1.

Demographic and nodule characteristics. A total of 98 patients with thyroid nodules who had undergone US and pathological examination were included (Table I). DNA samples were taken from FFPE tissue and amplified for sequencing. Based on the

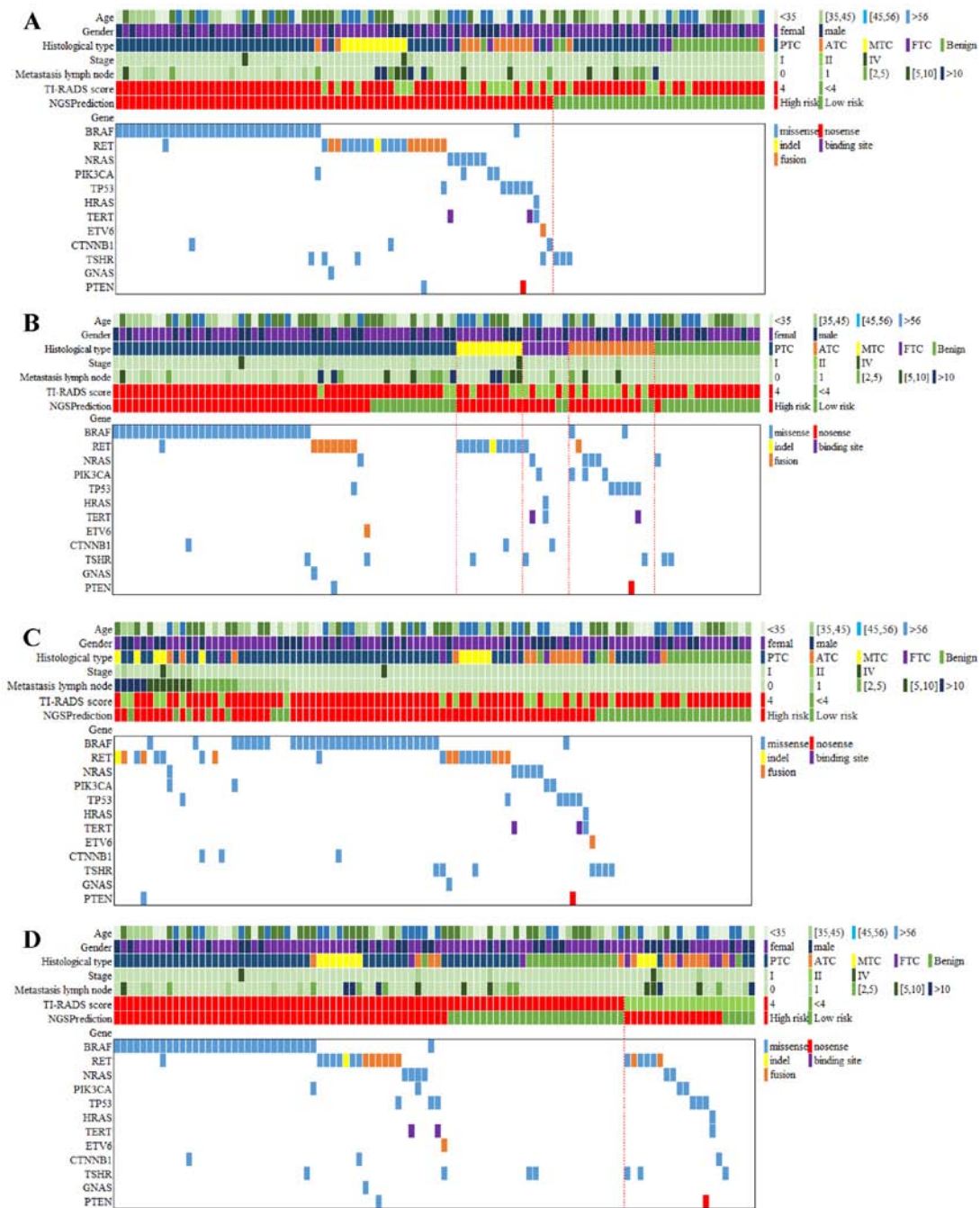


Figure 2. Gene spectrum grouped by benign and malignant thyroid nodules, pathological subtypes, metastatic lymph nodes and TI-RADS. (A) The gene spectrum of the 98 patients contained age, sex, histological diagnosis of cancer type, tumor stage, metastasis lymph node number, TI-RADS category, NGS prediction result and detailed genetic information. (B) The gene spectra of the four TC subtypes varied. For PTC, BRAF mutations were predominant, while RET mutations were detected among all the MTC. A wide range of mutations were positive but none of them showed dominance in ATC and FTC. (C) A total of 27 patients had 1 or more metastasis lymph nodes, 19 of whom carried 23 mutations, including BRAF (n=8), RET (n=8), NRAS (n=1), PIK3CA (n=2), TP53 (n=1), CTNNB1 (n=2) and PTEN (n=1). (D) Among the 78 US-predicted high-risk patients, 63 cases were diagnosed with TC by histological examination. Out of the 20 US-predicted low-risk patients, only 1 case was diagnosed with benign thyroid nodule by histological examination. TI-RADS, Thyroid Imaging Reporting and Data System; NGS, next-generation sequencing; TC, thyroid cancer; PTC, papillary thyroid cancer; MTC, medullary thyroid cancer; ATC, anaplastic thyroid cancer; FTC, follicular thyroid cancer; US, ultrasound.

histological diagnosis, the demographic data of these patients were separately presented in two groups: benign (16 patients) and malignant (82 patients). Age and sex ratio (M/F) in the two groups were 48.00 ± 6.07 years and 6/10, and 45.95 ± 3.20 years and 25/57, respectively. Characteristics of the nodules, including number, size and various US features (echogenicity, blood flow, microcalcification, aspect ratio and boundary) were collected. Fifteen patients in the benign group and 63 patients

in the malignant group were assigned to TI-RADS category 4. The 82 TC patients included 52 PTC, 13 FTC, 10 MTC and 7 ATC cases. Of these, 27 patients were also pathologically diagnosed with lymph node metastasis.

Gene spectrum between benign and malignant thyroid nodules. The gene spectrum of the 98 patients is presented in Fig. 2. In general, 86 mutations of all types of target genes were detected

in 69 patients (70.4% of 98 patients). Thyroline reported 66 cases (67.3%) in high risk, slightly lower than the number of cases with gene mutation. A total of 16 patients (16.3%) simultaneously carried two gene mutations and no one had >3 different gene mutations.

The majority of mutations were missense (73 cases, 84.8%), while other mutation types included nonsense (1 case, 1.0%), indel (1 case, 1.0%), binding site (2 cases, 2.0%) and fusion (9 cases, 9.2%). In addition to those cases confirmed to be malignant, 16 patients were diagnosed with benign thyroid nodules through pathological examination. Of these, 3 patients (18.75%) were positive for gene mutations, including NRAS (n=1) and TSHR (n=2). None of the 16 patients carried ≥ 2 mutations. Missense mutations were the only mutation type for both NRAS and TSHR.

Gene spectrum in different pathological subtypes of TC. The gene spectra of the four TC subtypes varied. For PTC, BRAF mutations were predominant, while RET mutations were detected among all the MTC cases. A wide range of mutations were positive but none of them showed dominance in ATC or FTC (Fig. 2). Among the 52 PTC patients, 39 (75%) patients were identified with mutations, an indication for high risk in NGS prediction. Specifically, BRAF mutations occurred in 30 patients (57.69%), followed by RET (n=8), TSHR (n=2), NRAS (n=1), TP53 (n=1), ETV6 (n=1), CTNNB1 (n=1), GNAS (n=1) and PTEN (n=1). Seven patients (13.46%) had multiple mutations, including BRAF/CTNNB1, BRAF/RET, BRAF/TSHR, RET/GNAS, RET/PTEN, RET/TP53 and ETV6/TSHR (Fig. 2). Missense mutations were the most common type of mutation (38 of 46 mutations), detected in all BRAF, NRAS, TP53, CTNNB1, TSHR, GNAS and PTEN genes, while fusion was only observed in RET and ETV6 (8 of 46 mutations). Ten patients were confirmed to be MTC by histological test and all of them were positive for gene mutations, including only RET (n=10), CTNNB1 (n=1) and TSHR (n=1). Other mutations were negative among MTC patients. Two patients (20%) exhibited multiple mutations, RET/CTNNB1 (n=1) and RET/TSHR (n=1). Missense mutations still accounted for the most mutations (11 of 12 overall mutations) and just 1 case was detected to be an indel mutation. FTC was diagnosed in 7 patients, among whom 5 (71.4%) were detected with gene mutations. A wide range of mutations were positive, including RET (n=1), NRAS (n=1), PIK3CA (n=1), HRAS (n=1), TERT (n=2), CTNNB1 (n=1) and TSHR (n=1). Three patients (23.1%) had multiple mutations, NRAS/TERT, RET/TSHR and HRAS/TERT. Missense mutation was also the most common (7 of 8 overall mutations) mutation pattern, and only 1 TERT mutation was reported to occur in the DNA-binding site. A total of 13 patients were diagnosed with ATC, 12 of whom (92.31%) had gene mutations in BRAF (n=2), RET (n=1), NRAS (n=3), PIK3CA (n=3), TP53 (n=5), TERT (n=1), TSHR (n=1) and PTEN (n=1). Five patients had multiple mutations, such as BRAF/PIK3CA, NRAS/PIK3CA, BRAF/TP53, TP53/PTEN and TP53/TERT. The greatest diversity in mutation patterns occurred in ATC patients. Missense mutations were again predominant (14 of 17 overall mutations). There was also 1 case of RET fusion mutation, 1 case of TERT binding site mutation, and 1 PTEN nonsense mutation.

Table II. Comparison of the prediction value of NGS and US for clinical TC diagnosis.

A, NGS				
Pathological diagnosis	High risk	Low risk	Sensitivity (%)	Specificity (%)
Malignant	65	17	79.27	
Benign	1	15		93.75
PPV	98.48%			
NPV		46.88%		
Histological type	Count	Malignant	Benign	Sensitivity (%)
PTC	53	39	13	75.00
ATC	13	11	2	84.62
MTC	10	10	0	100.00
FTC	7	5	2	71.43
B, US				
Pathological diagnosis	4	<4	Sensitivity (%)	Specificity (%)
Malignant	63	19	76.83	
Benign	15	1		6.25
PPV	80.77%			
NPV		5.00%		
Histological type	Count	4	<4	Sensitivity (%)
PTC	52	49	3	94.23
ATC	13	5	8	38.46
MTC	10	7	3	70.00
FTC	7	2	5	28.57

(A) Among the NGS high-risk patients, 65 were confirmed to be malignant, with only 1 benign sample. In the low-risk patients, 15 were benign and 17 were malignant. The sensitivity and specificity of NGS were 79.27 and 93.75%, respectively. The PPV and NPV were 98.48 and 46.88%, respectively. The sensitivity of NGS was >70% in all subtypes, particularly in MTC, when it was up to 100%. (B) Among the high-risk patients by US examination, 63 were confirmed to be malignant and 15 were benign. In the low-risk patients, 1 was benign and 19 were malignant. The sensitivity and specificity of US were 76.83 and 6.25%, respectively. The PPV and NPV were 80.77 and 5.00%, respectively. As analyzed by pathological subtypes, US had the highest sensitivity in PTC, up to 94.23%. However, the sensitivity in ATC and FTC was only 38.46 and 28.57%, respectively. NGS, next-generation sequencing; US, ultrasound; TC, thyroid cancer; PPV, positive predictive value; NPV, negative predictive value; PTC, papillary thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; FTC, follicular thyroid cancer.

Greater prediction value for NGS compared to US. The overall predictive abilities for thyroid nodule malignancy

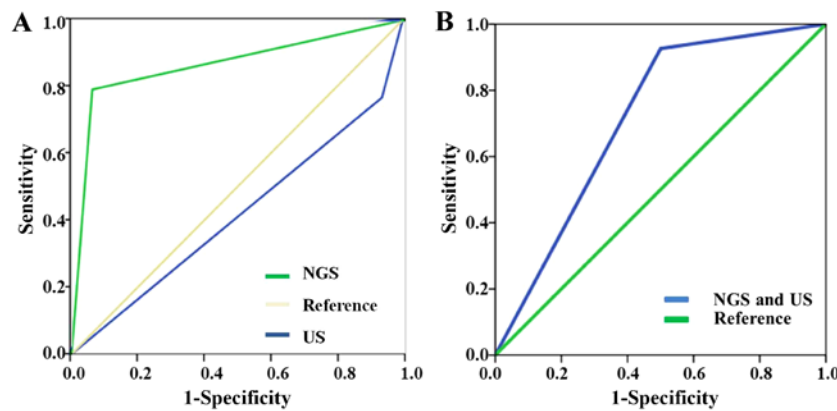


Figure 3. ROCs of NGS and US. (A) The AUC of NGS was 0.865, while the AUC of US was 0.415. (B) In situations where NGS and US drew the same conclusion, the sensitivity of combined NGS and US was 92.6% and the specificity was 50%. The AUC of combined NGS and US was 0.712. ROC, receiver operator characteristic; NGS, next-generation sequencing; US, ultrasound; AUC, area under curve.

by NGS and US are presented in Table II. Among the NGS high-risk patients, 65 were confirmed to be malignant, with only 1 benign sample. All patients with multiple mutations were malignant and reported with high risk. In the low-risk patients, 15 were benign and 17 were malignant. Thus, the sensitivity and specificity of NGS prediction were 79.27 and 93.75%, respectively. The PPV and NPV for NGS were 98.48 and 46.88%, respectively. Among the high-risk patients identified by US examination, 63 were confirmed to be malignant and 15 were benign. In the low-risk patients, 1 was benign and 19 were malignant. Thus, the sensitivity and specificity of US examination were 76.83 and 6.25%, respectively. The PPV and NPV were 80.77 and 5.00%, respectively.

In terms of different pathological subtypes, the sensitivity of NGS was consistently >70%, particularly in MTC, when it was up to 100%. US had the highest sensitivity in PTC, up to 94.23%. The panel reported all the cases (100%) to be at high risk, while only 7 cases (70%) were defined as high risk based on US. However, the sensitivity in ATC and FTC was only 38.46 and 28.57%, respectively. A total of 11 ATC patients (84.62%) were reported as having high risk through NGS test, slightly lower than the number of patients with mutations. However, US only found 5 ATC cases to be at high risk. The panel reported 5 in 7 FTC cases (71.43%) to be high risk, and only 2 cases (28.57%) were defined as high risk through US. The ROCs of NGS and US are presented in Fig. 3. The area under curve (AUC) of NGS test was 0.865, while the AUC of US test was 0.415. The prediction value of NGS was higher than that of US. In situations where NGS and US drew the same conclusion, the sensitivity of combined NGS and US was 92.6% and the specificity was 50%. The AUC of combined NGS and US was 0.712.

Differential profile among lymph node metastasis degrees of thyroid nodules. By examining the lymph nodes of all patients with thyroid nodules, 27 patients had ≥ 1 metastasis lymph nodes, including PTC (n=18), MTC (n=5), FTC (n=1) and ATC (n=3) patients. Specifically, 5 (18.52%) patients had >10 metastasis lymph nodes, 7 (25.93%) had 5-10 nodes, 7 (25.93%) had 2-5 nodes and 8 (29.63%) had 1 node. Patients with >1 metastatic lymph node tended to show a greater diversity in cancer subtype, while all patients with only 1 metastasis lymph node

were diagnosed with PTC. A total of 8 patients (29.63%) with metastasis lymph nodes were detected with no gene mutation by NGS and reported to be at low risk as well. The remaining patients had a variety of mutations that differed in both gene and mutation pattern. Nineteen patients carried 23 mutations, including BRAF (n=8), RET (n=8), NRAS (n=1), PIK3CA (n=2), TP53 (n=1), CTNNB1 (n=2) and PTEN (n=1). However, no correlation was found between the variant allele frequency of BRAF, NRAS, RET mutation, RET fusion and number of metastatic lymph nodes (Fig. 4; $P>0.05$).

Prediction difference for the high-risk thyroid nodules between NGS and US. There was inconsistency in high-risk identification between NGS and US (Fig. 2). Among the 78 US-predicted high-risk patients, 51 (65.38%) were reported as high risk by NGS. Among 20 US-predicted low-risk patients, only 5 (25%) were reported as low risk by NGS. We picked out two patients (case 1 and 2) diagnosed with benign thyroid nodule by ultrasonic diagnosis but assessed as malignant by pathological results (Fig. 5). US showed no typical features such as hypoechoogenicity, unclear margins, rich blood flow, microcalcification or taller-than-wide shape. However, both cases were PTC with BRAF mutation. Simultaneously, we also selected two other patients (case 3 and 4) with consistent results among NGS, US and pathological diagnosis. For these cases, US demonstrated typically malignant features, including microcalcification and hypoechoogenicity (Fig. 5). Consistently, NGS also detected TC-related driver mutation, such as NRAS and TERT in case 3, and TP53 and TERT in case 4.

Discussion

A complete understanding of the molecular mechanisms of tumor formation is essential for providing accurate diagnoses and personalized treatments. In the past, single gene assays have been commonly used for finding molecular alterations in TC. TC harbors characteristic genetic alterations, including point mutations for proto-oncogenes (BRAF and RAS) and chromosomal rearrangements (RET), which vary with histological subtypes (12,16). Presently, NGS technology can simultaneously analyze hundreds of genes of interest using targeted sequencing panels, indicating

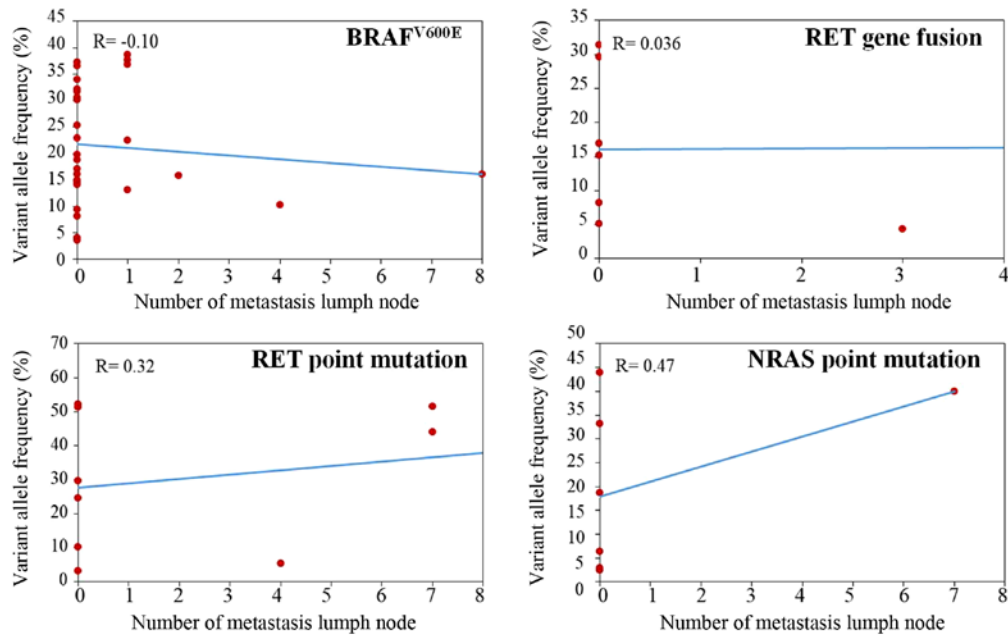


Figure 4. Correlation between variant allele frequency and number of metastatic lymph nodes. Spearman's analysis was performed for the variant allele frequency of BRAF, NRAS and RET mutation, RET fusion and number of metastatic lymph nodes. As a result, no correlation was found between the variant allele frequency of BRAF, NRAS and RET mutation, RET fusion and number of metastatic lymph nodes. $P > 0.05$.

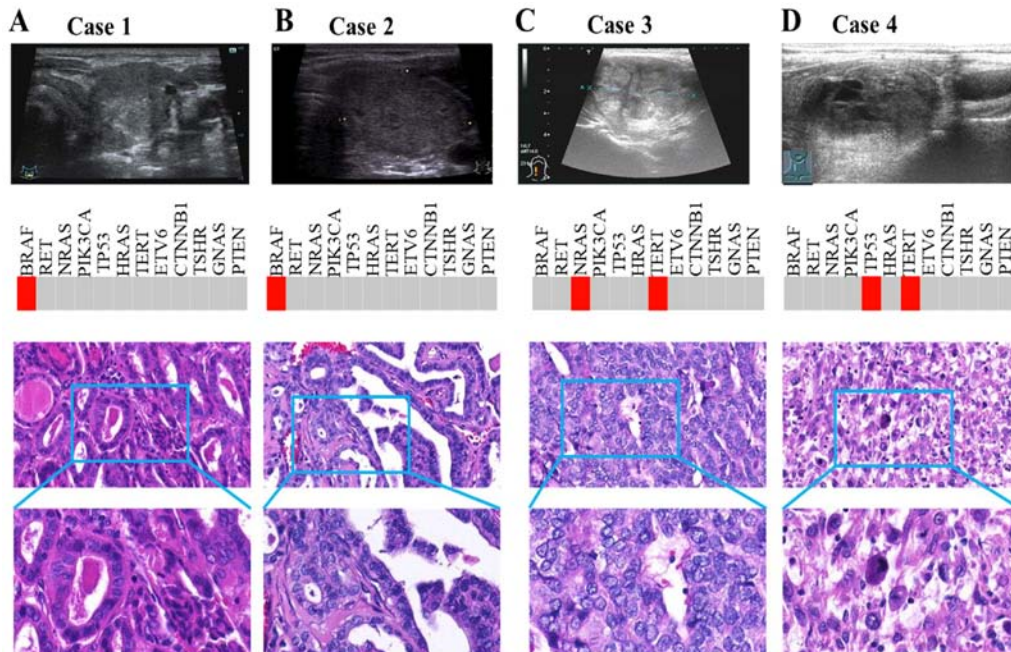


Figure 5. Prediction difference between NGS and US for high-risk thyroid nodules. (A and B) Case 1 and 2 were diagnosed via US with benign thyroid nodule, but pathological results revealed malignancy. US showed no typical features. However, both cases were PTC with BRAF mutation. (C and D) Case 3 and 4 had consistent results among NGS, US and pathological diagnosis. US demonstrated typically malignant features, including microcalcification and hypoechogenicity. Consistently, NGS also detected TC-related driver mutations, such as NRAS and TERT in case 3, and TP53 and TERT in case 4. NGS, next-generation sequencing; US, ultrasound; PTC, papillary thyroid cancer; TC, thyroid cancer.

that NGS-based molecular tests for oncology research and clinical practice are rapidly evolving (17). NGS allows for simultaneous high-throughput sequencing analysis of variable genetic alterations and provides a comprehensive understanding of tumor biology, which can improve diagnostic accuracy and is useful for providing personalized treatments for TC patients.

Published literature increasingly indicates that gene mutations can be specific biomarkers for clinical TC diagnosis (13,16,18). As the most common gene mutation of TC, BRAF mutations were found in ~45-60% of PTC cases (12,19). RAS mutations (HRAS, KRAS and NRAS) were found in 40-53% of FTC cases (20,21). RET was commonly found to be mutated in both familial and sporadic MTC (22). In our

study, analysis of patients with malignant nodules showed great heterogeneity in gene and mutation patterns between different TCs. BRAF mutations were detected in 57.69% of PTC, while RAS mutations were only found in 28.57% (2/7) of FTC cases. A previous study including 35 FTCs found that 16 patients (45.7%) harbored RAS mutations (23). Compared with this, the low positive rate of RAS in the current study may be attributed to the recruited sample size (7 FTC patients). Interestingly, in the FTC subgroup, two patients had either NRAS/TERT or HRAS/TERT multiple mutation patterns. TERT promoter mutations are more prevalent in advanced TCs, particularly those harboring BRAF and RAS mutations (24,25). In addition, RET mutations were detected in all MTC patients. Specifically, RET mutations may suggest a strong genotype-phenotype correlation with MTC (26), indicating that it may be a reliable biomarker for MTC diagnosis.

In addition to well-known BRAF, RAS and RET mutations, NGS technology facilitated detection of new somatic alterations in TC, the significance of which has not yet been explored in detail. We found many gene mutations in particular tumor subtypes that are seldom reported, such as CTNNB1 and TSHR in PTC; NRAS and TSHR in ATC; CTNNB1 in MTC; and PIK3CA and CTNNB1 in FTC. Interestingly, TSHR was traditionally reported in FTC (27), but found in all subtypes by our gene spectrum. Thus, we hypothesize that TSHR may be a common driver gene for TC. Due to sample size limitations, our gene spectrum results may vary from previous studies (27-29), and some mutations in our panel were negative among TC patients.

US is the preferred non-invasive diagnostic method recommended by current American Thyroid Association guidelines (8). Nonetheless, the diagnosis value of TI-RADS is controversial. A prospective study including >2,000 patients reported that the diagnostic accuracy of US of ≥ 1 malignant features was only 72.6% (9). In our study, we focused on the application of NGS for predicting biological characteristics of thyroid nodules, including a novel comparison of the predictive efficacy between NGS and US. Both NGS and US demonstrated a high sensitivity in diagnosis of TC, but both showed inconsistency in high-risk identification. TC patients with BRAF were all classified as high risk by US. However, for those without BRAF mutations, prediction accuracy dropped markedly. BRAF mutations were mainly detected in PTC (12,19), and the US features varied between subtypes (30,31). The clinical application of US-based TI-RADS may be confined to PTC, which may lead to missed and delayed diagnosis of other subtypes. The results suggest that NGS technology in differentiation of benign and malignancy possess more advantages in terms of sensitivity, specificity, PPV and NPV compared to US. The sensitivity and specificity of combined NGS and US were superior to US alone. Therefore, NGS in combination with US may have better diagnostic and/or prognostic value for TC patients.

Another purpose of the panel was to investigate its ability to reflect lymph node metastasis status. Previous studies found that 19.4-84% of TC patients have lymph node metastases (32,33), 33% of which are missed by preoperative US examination (7,34). Patients with lymph node metastasis often need more aggressive therapy, including radiative iodine treatment and more frequent follow-up (8). Based on histological

examination in the current study, 27 patients had ≥ 1 metastatic lymph nodes. Mutations in genes including BRAF, RET, NRAS, PIK3CA, TP53, CTNNB1 and PTEN were found in 19 patients. These mutations may be a key driving factor for lymph node metastasis. For deeper insight into the relationship between the abundance of mutant allele and metastasis status, we selected BRAF, RET and NRAS mutations as well as RET fusion for correlation analysis. However, we did not find a significant correlation between the variant allele frequency of aforementioned gene mutations and the number of metastatic lymph nodes. This finding may have been due to the small sample size. Therefore, the impact of genetics on lymph node metastasis has yet to be determined.

The association of BRAF mutation with more aggressive clinicopathological features and poorer outcomes has been under debate for some time. Certain studies have demonstrated a role of BRAF in tumor aggressiveness in PTC (35,36), but others have failed to do so (37,38). A new genetic alteration, the point mutation in the TERT promoter, was recently described in TC and has been shown to be associated with increased aggressiveness and poorer clinical prognosis (39,40). Notably, Xing *et al* found that PTC patients with coexisting BRAF and TERT mutations had the worst clinicopathological outcomes (41). TERT mutations were found in 12.9% (21/163) of Portuguese PTC patients (42). However, we did not find TERT mutations in PTC patients, or coexisting BRAF and TERT mutations. TERT mutation was found in 4.4% (20/455) and 4.1% (27/653) PTC patients in another two Chinese studies, respectively (43,44). Additionally, the prevalence (14.3%, 1/7) of TERT promoter mutation in ATC patients from our study was significantly <46.3% (25/54) and 38.7% (41/106) from Liu *et al* and Shi *et al* respectively (39,45). TERT promoter mutations may play an important role in distant metastases of TC (42). However, no ATC patients in our cohort presented distant metastases. Thus, the reason why the mutation rate of TERT promoters was low in our detection platform may be attributed to the small sample size of ATC. On the other hand, it may be related with the heterogeneity of ATC biological behavior.

In conclusion, NGS exhibits advantages in discriminating benign and malignant thyroid nodules compared with traditional US-based TI-RADS, simultaneously providing insight into the pathological subtypes of TC. The novel use of NGS-based Thyroline in conjunction with US may pave the way for increasingly accurate and timely diagnoses of TC compared to conventional methods. However, the current findings may assist the application of NGS in FNA samples. A limitation of our analysis was that it was performed in FFPE tissues, not in FNA samples. In the future, the combined evaluation of target-medicine-related gene status included in the Thyroline panel may be used to assist precision treatment for TC patients, leading to improved clinical outcomes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZK, YL, YZ and HX conceived and designed the study. ZK, LC and CS performed the histological examination. JLi was a contributor to the US examination. SP and BL were contributors in the data statistics. ZK and YL wrote the report. JLi, MK, LS, SY, JC and WL reviewed and edited the manuscript and were also involved in the conception of the study. All authors read and approved the final manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study had got the ethics approval of the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University and the reference number was 2017224.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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