Original Article

Clinical Utility of Next-Generation Sequencing-Based Molecular Panel in Thyroid Nodules

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Abstract

Introduction: The molecular testing of indeterminate thyroid nodules (ITNs) empowers clinicians to make informed treatment decisions. Despite being recommended by the ATA 2015 guidelines, the utility of molecular testing in India is hindered by challenges related to availability and cost-effectiveness, thereby limiting its widespread adoption. We aimed to evaluate the clinical utility of next-generation sequencing (NGS)-based molecular testing in Indian patients with ITNs. **Methods:** The study included patients with Bethesda III and IV and selected Bethesda II nodules who underwent Thyrotrack NGS-based assay on fine needle aspirate (FNA) material. Surgery was recommended for patients with clinically significant mutations, while others were followed sonographically. Post-surgical histopathology results were compared with mutation variants to calculate the sensitivity, specificity, and negative predictive value of the NGS assay. **Results:** Among 35 patients (mean age 37.7 ± 12.4 years, 80% female), 20 (57%) had clinically significant mutations. Surgery was performed on 11 patients. The most common mutation was RAS (detected in 15 patients), followed by BRAF, TSH-R, ETV6/NTRK3, and PAX8/PPARG. Post-surgical outcomes showed an overall sensitivity of 86% and a specificity of 74%, with a negative predictive value of 94%. Among the mutation-negative group, only one patient had a malignancy, and the rest were benign showing a high negative predictive value of the NGS-based testing. **Conclusion:** NGS-based assays provide a reliable and cost-effective option for ruling out malignancy in ITNs in India, offering a high negative predictive value and complementing ACR-TIRADS and Bethesda cytology classifications.

Keywords: Bethesda, FNAC, indeterminate cytology, next-generation sequencing, thyroid nodule

Introduction

The prevalence of thyroid nodules is high, affecting about 60% of individuals by their sixth to seventh decade of life.[1] Fine-needle aspiration cytology (FNAC) classified according to The Bethesda System for Reporting Thyroid Cytopathology provides excellent histopathological correlation, especially in Bethesda II, V, and VI categories. However, diagnosing and treating indeterminate thyroid nodules (ITNs) in Bethesda III (Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance) and IV (Follicular Neoplasm) categories remains challenging with a variable rate of malignancy ranging from 10 to 40%.[2,3]

The ATA 2015 guidelines recommend molecular testing for ITNs. Other options include diagnostic hemithyroidectomy.[1] Molecular testing is crucial for identifying mutations that drive malignancy and provide valuable insights into tumor biology. Among all the available commercial platforms for molecular testing in thyroid nodules, the next-generation sequencing (NGS) using ThyroSeq test has recently shown good accuracy for diagnosing thyroid cancer in FNA samples with indeterminate cytology.[4,5]

In a study with 247 FNA samples diagnosed as AUS/FLUS, 88% mutation-positive nodules were malignant, giving a negative predictive value of 94% and a specificity of 99%.[6]

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In a study by Nikiforov *et al*.,[5] ThyroSeq v2.1 accurately classified 20 out of 22 cancers identified by histologic analysis, yielding a sensitivity of 90.9% and a specificity of 92.1%.

Data on the molecular profiling of ITNs in Indian patients are limited. This study is one of the first in India to explore the clinical utility of molecular profiling in thyroid nodules.

Materials and Methods

This is a retrospective observational study using electronic medical records at a tertiary centre cancer hospital in southern India in which patients with ITN on cytology examination (Bethesda III or IV) or sonographically suspicious Bethesda II nodules (TIRADS 3, 4, or 5) who underwent NGS-based molecular testing were included. Patients with Bethesda V and VI or Bethesda II which were either TIRADS 1 or 2 were excluded. Following the protocols as per standards of care given by 2015 ATA guidelines for management of thyroid nodules, in patients who underwent fine needle aspiration for cytological examination, some material was collected in RNAlater solution [ThermoFisher Scientific] and was transported to Medgenome Labs at 2–4°C for molecular profiling through ThyroTrack test. The Thyrotrak test is a laboratory-developed CAP-accredited NGS-based mutation analysis test, and gene content is curated as per ATA guidelines[1] and published literature.[7] The ThyroTrack test covers 57 genes related to thyroid cancer prognostication and therapeutics. It has been designed to detect single-nucleotide variations (SNVs), small insertions/deletions (InDels) in multiple genes including important genes like *BRAF, RAS,* and *P13K* pathway genes, *TERT* promoter, and fusions in genes like *RET*, *NTRK, ALK,* and others to predict the risk of malignancy [see Table 1 for the complete gene list].

NGS assay

The FNA fluid obtained in RNAlater solution was spun down to pellet the cells. Total DNA and RNA were extracted and subjected to quality check for concentration using a spectrophotometer. Approximately 100 ng of DNA and RNA were used for library preparation using a hybrid-capture custom kit. The quality-passed libraries were subjected to sequencing on the Illumina NovaSeq6000 platform at a minimum depth of 250X. The sequenced data were processed using a validated bioinformatics pipeline for variant and fusion calling and prioritisation. NGS analysis quality metrics checks were performed as per our in-house pipeline validation criteria.

Analysis and interpretation

Only non-synonymous and splice site variants in coding regions were used for clinical interpretation. Variants were annotated using an in-house pipeline with the VEP program against Ensembl release 91, and clinically relevant mutations were cross-referenced with databases like ClinVar, OMIM, and HGMD. Common variants were filtered based on minor allele frequencies from 1000Genome Phase 3, ExAC, gnomAD, and an internal Indian database. The biological effect of variants was assessed using prediction algorithms such as PolyPhen, SIFT, Mutation Taster2, and LRT. Annotated variants were uploaded into the proprietary Oncominer platform and visualised on IGV. Variants with a minimum variant allele frequency (VAF) of 3–5% and fusions with >10 junction reads were prioritised and classified according to AMP/ASCO/CAP and ATA guidelines. Positive mutations led to surgical recommendations, while mutation-negative cases either underwent surgery or were monitored sonographically after multi-disciplinary discussion. Final histopathology classified nodules as true positive (malignant) or true negative (benign) based on mutation status and follow-up.

Table 1: Gene list included in the ThyroTrack NGS test

SNVs and InDels				
AKT1	***EZH1***	***MET***	***PPARG***	***TERT****
ALK	*FARSB*	*NF2*	*PTEN*	*TG*
APC	*FGFR2*	*NRAS*	*PTH*	*TP53*
BRAF	*GNAS*	*NTRK1*	*RAF1*	*TSC2*
CHEK2	*HRAS*	*NTRK2*	*RET*	*TSHR*
CTNNB1	*IDH1*	*NTRK3*	*RNF213*	*VHL*
DICER1	*IDH2*	*PI3K*	*ROS1*	
EIF1AX	*KDM6A*	*PICALM*	*SLC5A5*	
EP300	*KRAS*	*PIK3CA*	*STK11*	
ERBB4	*MEN1*	*PIK3R2*	*SYN2*	
Fusions				
ALK	*FGFR2*	*PAX8*	*ROS1*	
BRAF	*KIF5B*	*PICALM*	*SS18*	
CLIP1	*MET*	*PPARG*	*SYN2*	
EML4	*NTRK1*	*RAF1*	*THADA*	
ERBB4	*NTRK2*	*RET*	*UACA*	
FARSB	*NTRK3*	*RNF213*		

*TERT promoter region is covered

Ethical aspect

The current study involves use of a commercially available NGS panel and data was retrospectively collected from patient records. The manuscript was approved by the institutional ethics committee (NHH/AEC-CL-2024-1199), and patient's consent was waived off in view of the retrospective nature of the study using data from medical records.

Results

Of the patients who presented to the clinic with thyroid nodules, 35 patients (36 nodules, 1 patient AT036 had 2 nodules) underwent molecular testing using ThyroTrack assay by NGS. The mean age of the patients was 37.7 ± 12.4 years with 80% (n = 28) females. Of these 36 nodules, 10 (28%) were classified as Bethesda II (TIRADS 3/4/5) category. Another 18 patients (48%) were found to be Bethesda III, and the remaining 8 (24%) as Bethesda IV nodules. The mean size of the nodule was 39.8 ± 10 mm in Bethesda II, 36.3 ± 13.7 mm in Bethesda III, and 26.8 ± 11.2 mm in Bethesda IV category.

The molecular testing on FNA fluid collected from 36 nodules showed that 21 (58%) of the patients tested positive for

one or more clinically significant gene mutations. Among seven patients aged <30 years, four had nodules with RAS mutations (PV003, RO035, ST016, SL026), while the remaining three had no mutations. PV003 underwent surgery, which revealed follicular nodular disease. Two mutation-negative patients (DM008 and AN001), who underwent surgery due to sonographic suspicion, had FVPTC and colloid goiter, respectively. In the 30–60-year age group, 27 nodules were analysed. Of these, 15 had tested mutation positive, with RAS mutations being the most common (10 patients). The remaining mutations included BRAF, TSHR, TP53, ETV6/NTRK3, and PAX8/PPARG. Eight mutation-positive patients who underwent surgery had the following diagnoses: FVPTC (NRAS), classic PTC (BRAF), follicular thyroid cancer (PAX8/PPARG), and FVPTC (ETV6/NTRK3). Among the four mutation-negative patients who had surgery, three (CP018, SM031, UM011) had follicular nodular disease, and one with a TP53 mutation (TP005) had multi-nodular goiter. Two patients aged >60 years had BRAF and HRAS mutations with histology consistent with Classic PTC and Tall Cell PTC, respectively.

ACR-TIRADS scoring system was used to decide regarding FNA sampling of thyroid nodules. Among 36 nodules that were aspirated, there were 14 TIRADS-3 nodules, 20 TIRADS-4 nodules, and 2 TIRADS-5 nodules. 57% (n = 8) of TIRADS-3, 63% (n = 12) of TIRADS-4, and 1 TIRADS-5 nodule tested positive for mutation. The spectrum of different mutations in each category of ACR-TIRADS is given in Table 2. While *RAS* mutations were found with equal frequency in TIRADS-3 and 4 categories (75% of nodules in each), *PAX-8/PPARG* and *ETV6/NTRK3* gene fusions were found in TIRADS-4 only. One TIRADS-5 nodule (TP005) was reported to have *TP53* mutation, and it had benign multi-nodular goiter on post-operative histopathology. Characteristics of all the nodules in different Bethesda categories and their genomic profiles were studied in detail as mentioned below.

Table 2: Molecular testing results of thyroid nodules in ACR-TIRADS categories

Mutations Identified	TIRADS-3 (*n*=14)	TIRADS-4 (*n*=20)	TIRADS-5 (*n*=2)
NRAS	6	3	-
HRAS	-	6	-
BRAF	1	1	-
TSHR	1	-	-
PAX-8/PPARG	-	1	-
ETV6/NTRK3	-	1	-
TP53	-	-	1
Total mutation-positive nodules	8 (57%)	12 (63%)	1

In Bethesda II category, five out of nine patients were positive for *RAS* gene mutations. Four of them carried *NRAS* p.Gln61Arg mutation (CH009, SM031, ST016, AT036), and one had *NRAS* p.Gln61Lys mutation (RB007). The AT036 patient was found to have *HRAS* p.Gln61Lys additionally. The patient SM031, who carried *NRAS* gene mutation, underwent hemithyroidectomy and showed follicular nodular disease on histology. The other four mutation-positive patients are awaiting hemithyroidectomy. Among the patients who were found to be negative on molecular testing, DE010, GL014, PA012, and RI020 have been suggested follow-up and are undergoing sonographic surveillance [Table 3].

Out of 18 nodules with Bethesda III cytology, there were 12 with nuclear atypia and six with architectural atypia. Six out of 12 patients with nuclear atypia tested positive for mutation (MK002, RG022, RO035, SL026, TS033, and TP005). Three out of six patients with architectural atypia tested positive for mutation (CP018, PV003, and SN029). Among 18 patients with Bethesda III nodules, nine carried clinically significant mutations [Figure 1]. Three patients carried hotspot oncogenic mutation in the *NRAS* gene (MK002 and RO035 with p.Gln61Lys and PV003 with p.Gln61Arg mutations). Patient MK002 underwent hemithyroidectomy and was found to be a follicular variant of papillary thyroid carcinoma (FVPTC). Patient PV003 underwent hemithyroidectomy and was found to have benign pathology, while RO035 is awaiting surgery. Patients CP018 and SL026 were found to have *HRAS* gene mutations, p.Gln61Lys and p.Gln61Arg, respectively [Figure 1]. CP018 underwent hemithyroidectomy and was found to have follicular nodular disease, while SL026

Table 3: Molecular testing results and clinical follow-up summary of Bethesda II nodules (*n*=10)

Subject ID	Bethesda Category	Genomic Results	Gene	Variant	VAF (%)	TERT	Final nodule Histopathology after surgery
AT036	II	Positive	*NRAS*	p.Gln61Arg	11.36	Negative	Awaiting surgery
AT036	II	Positive	*HRAS*	p.Gln61Lys	14.8	Negative	Awaiting surgery
CH009	II	Positive	*NRAS*	p.Gln61Arg	43.0	NA	Awaiting surgery
DE010	II	Negative	NA	NA	NA	NA	Managed conservatively
GL014	II	Negative	NA	NA	NA	NA	Managed conservatively
RB007	II	Positive	*NRAS*	p.Gln61Lys	1.7	Negative	Awaiting surgery
SM031	II	Positive	*NRAS*	p.Gln61Arg	2.9	Negative	Benign
ST016	II	Positive	*NRAS*	p.Gln61Arg	29.6	NA	Awaiting surgery
PA012	II	VUS	*TP53*	p.Val216Ala	49.0	Negative	Managed conservatively
RI020	II	Negative	NA	NA	NA	Negative	Managed conservatively

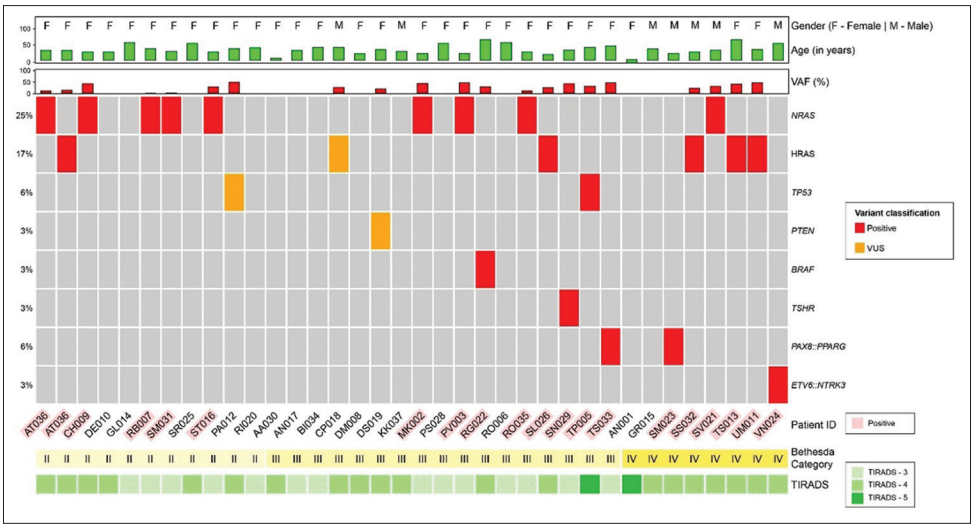

Figure 1: Variants reported in FNA samples (n = 36). Each column represents a patient's FNA sample. Each row highlights mutations in the indicated genes. Red indicates clinically significant variants, and the orange color indicates tier 3 (variants of unknown significance) variants in the indicated gene, for each patient. On the top, age and gender are depicted for each sample. The VAF (%) of each variant detected is shown below age and gender. The Bethesda classification and TIRADS category are indicated below the heatmap

is awaiting surgery. Patients RG022 and SN029 were detected with *BRAF* oncogenic variants, p.Val640Glu and p.Lys641Glu, respectively, and underwent surgery. Both patients underwent total thyroidectomy and on final histopathology examination were found to be classic PTC. Finally, two patients who had *TP53* (TP005) and TSHR (TS033) oncogenic variants were found. While TP005 underwent hemithyroidectomy and was found to be benign, TS033 is waiting for surgery. Among the nine patients with no significant mutations identified, two patients (DM008 and SR025) underwent hemithyroidectomy due to suspicious sonographic appearance of the nodule (TIRADS 4). Both of them had benign histology on surgical specimens. The remaining seven patients are being managed conservatively on sonographic follow-up [Table 4].

Eight patients in the Bethesda IV category underwent molecular testing, and 6/8 (75%) were positive for clinically significant mutations [Figure 1]. While four patients carried mutations in *RAS* genes (SV021, SS032, TS013, UM011), the two patients were identified with clinically significant fusions (SM023 and VN024). The patients SS032, TS013, and UM011 had p.Gln61Arg mutation in the *HRAS* gene, and SV021 had the same variant in the *NRAS* gene. SS032 is awaiting surgery, TS013 was identified as a Tall Cell sub-type of PTC, and UM011 had follicular nodular disease on final histology following hemithyroidectomy. SS032 and SV021 are awaiting surgery.

The two patients who had high-risk fusions included SM023 with *PAX8/PPARG* fusion who underwent total thyroidectomy and was found to have encapsulated widely angioinvasive follicular thyroid cancer (FTC), and VN024 with *ETV6/NTRK3* fusion underwent complete thyroidectomy and turned out to be FVPTC. Two patients (AN001 and GR015) in this category did not carry any clinically significant mutation. AN001 was highly suspicious on sonography (TIRADS-5) and underwent a complete thyroidectomy. She was found to be FVPTC, and the other is on follow-up [Table 5].

Discussion

A total of 14/35 patients underwent surgery, and an overall sensitivity of 75% was noted with six true positive and two true negative results and five patients with a clinically significant variant and benign nodule histopathology after surgery were considered false positives. 12/35 patients with negative molecular testing who had a stable nodule size on sonographic follow-up were provisionally considered true negative. A negative test is very accurate in identifying a nodule as benign and, thus, avoiding surgery.[8] *RAS* variants alone pose a 37–85% risk of malignancy as per the literature evidence.[1,6,9,10] In our data, 2/6 (33%) patients with *RAS* mutations in thyroid nodules were found to be malignant. It has been noted in earlier studies that *RAS* mutations are not quite as definitive as they can be found in a wide spectrum of thyroid nodule histology ranging from benign to well-differentiated thyroid cancer to poorly differentiated. A significant proportion of *RAS-mutated* resected tumours yielded benign pathology or NIFTP in various published data.[6,7,11] The clinical utility of *RAS* mutation analysis is enhanced when complemented with concurrent sonographic and cytology findings, particularly in cases where the Bethesda system indicates a higher category carrying *RAS* mutations with a greater malignant risk.[12] Variant allele frequency of the *RAS* variants had no effect on the nodules being benign or malignant as also demonstrated by Hudson *et al*.[13] In these data, a 33-year-old female, MK002, with 51mm TIRADS-3, Bethesda III nodule, was on sonographic follow-up for about 18 months. She was found to be positive for *NRAS* mutation on molecular testing and was subjected to hemithyroidectomy. She was confirmed with FVPTC on final histology. This case highlights the importance of molecular tests as an additional

Table 4: Molecular testing results and clinical follow-up summary of Bethesda III nodules (*n*=18)

Subject ID	Bethesda Category	Genomic Results	Gene	Variant	VAF (%)	TERT	Final nodule Histopathology after surgery
SR025	III	Negative	NA	NA	NA	Negative	Benign
AA030	III	Negative	NA	NA	NA	NA	Managed conservatively
AN017	III	Negative	NA	NA	NA	Negative	Managed conservatively
BI034	III	Negative	NA	NA	NA	Negative	Managed conservatively
CP018	III	Positive	*HRAS*	p.Gln61Lys	28.0	Negative	Benign
DM008	III	Negative	NA	NA	NA	Negative	Benign
DS019	III	VUS	*PTEN*	p.Trp111Ser	20.0	Negative	Managed conservatively
KK037	III	Negative	NA	NA	NA	Negative	Managed conservatively
MK002	III	Positive	*NRAS*	p.Gln61Lys	44.0	NA	Follicular Variant of Papillary Thyroid Cancer
PS028	III	Negative	NA	NA	NA	Negative	Managed conservatively
PV003	III	Positive	*NRAS*	p.Gln61Arg	48.0	NA	Benign
RG022	III	Positive	*BRAF*	p.Val640Glu	30.6	Negative	Classic Papillary Thyroid Cancer
RO006	III	Negative	NA	NA	NA	NA	Managed conservatively
RO035	III	Positive	*NRAS*	p.Gln61Lys	12.8	Negative	Awaiting surgery
SL026	III	Positive	*HRAS*	p.Gln61Arg	26.8	Negative	Awaiting surgery
SN029	III	Positive	*BRAF*	p.Lys641Glu	43.0	Negative	Classic Papillary Thyroid Cancer
TP005	III	Positive	*TP53*	p.Arg213Ter	33.0	NA	Benign
TS033	III	Positive	TSHR	p.Ile486Met	47.2	Negative	Awaiting surgery

Table 5: Molecular testing results and clinical follow-up summary of Bethesda IV nodules (*n*=8)

Subject ID	Bethesda Category	Genomic Results	Gene	Variant	VAF (%)	TERT	Final nodule Histopathology after surgery
AN001	IV	Negative	NA	NA	NA	Negative	Follicular Variant of Papillary Thyroid Cancer
GR015	IV	Negative	NA	NA	NA	Negative	Managed conservatively
SM023	IV	Positive	*PAX8/PPARG*	NA	623X	Negative	Follicular Thyroid Cancer
SS032	IV	Positive	*HRAS*	p.Gln61Arg	23.8	Negative	Awaiting surgery
SV021	IV	Positive	*NRAS*	p.Gln61Arg	31.4	Negative	Awaiting surgery
TS013	IV	Positive	*HRAS*	p.Gln61Arg	42.0	Negative	Tall Cell Variant Papillary Thyroid Cancer
UM011	IV	Positive	*HRAS*	p.Gln61Arg	47.0	NA	Benign
VN024	IV	Positive	*ETV6/NTRK3*	NA	7X	Negative	Follicular Variant of Papillary Thyroid Cancer

diagnostic tool in guiding treatment decisions for patients with ITNs who are being managed on sonographic follow-up.

The variants in genes like *BRAF* and *TP53* and high-risk fusions pose a 90–95% risk of malignancy,[1] and in our data, four out of five of patients with high-risk mutations/fusions were found to be cancerous on final histopathology. All (100%) *BRAF*, *PAX8*:*PPARG*, and *ETV6*:*NTRK3* positive patients were found to be malignant. *BRAF* mutation is known to be highly specific for papillary thyroid cancer. As per the meta-analysis by Su *et al*.[14] in 2016, *BRAF* is prevalent in 23% of cytologically ITNs with a specificity of 99% for PTC and a low sensitivity of 40%. In the present study, *BRAF* was present in 13% (2 of 15 Bethesda III or IV) of patients with 100% specificity for papillary thyroid cancer on final histopathology. Gene fusions are potential therapeutic targets in thyroid cancer; however, when detected in ITNs, informed timely management decisions can be taken for aggressive variants of thyroid cancer. *ETV6:NTRK3* is the major NTRK fusion strongly correlated with 2–3% of sporadic PTC cases.[8] In our data, a 51-year-old gentleman, VN024, with 24mm TIRADS-4, Bethesda IV nodule with *ETV6*:*NTRK* fusion, underwent total thyroidectomy instead of a diagnostic hemithyroidectomy due to the high-risk fusion on the NGS and was confirmed to have FVPTC on final histology.

The one false-positive patient TP005 carried *TP53* mutation pre-operatively at a high VAF of 33% and showed benign histology on the nodule. There is a possibility of this mutation being germline; however, confirmation is awaited.

The data regarding diagnostic and prognostic importance of *TP53* mutation in thyroid nodules are scant. It is found to correlate with the malignant transformation of thyroid nodules as well as the aggressive behaviour of thyroid cancer.[15,16] Marcello *et al*.[16] found that *TP53* mutation has more frequent association with malignant thyroid nodules than benign, thereby establishing its utility in the evaluation of ITNs. Nasir *et al*.[17] 2004 demonstrated 90% p53 positivity in FTC and 15% p53 positivity in follicular adenoma.

Among the mutation-negative nodules, only three underwent surgery and one patient was found to have FVPTC on final histopathology. Other mutation-negative nodules were benign

considering the stable size of the nodule on sonographic follow-up. AN001, a 16-year-old female with 35 mm TIRADS-5, Bethesda IV nodule, was tested negative on mutational analysis, however, post-surgery histopathology confirmed FVPTC. This case highlights the importance of a combinational diagnostic approach with clinical and sonographic, cytology, and molecular assessment. None of these approaches should be used in isolation, and each one of them is important for appropriate management decisions.

Molecular analysis using NGS is a promising additional diagnostic tool in the evaluation of thyroid nodules with indeterminate cytology. Thyroseq v2.1 tests 14-point mutations and 46 gene fusions with a sensitivity of 90% and a specificity of 92% with a high negative predictive value of 97%.[5] The test used in our study, Thyrotrack NGS-based assay, showed a sensitivity of 86% and a specificity of 74% for mutation analysis in thyroid nodules. The test demonstrated a high negative predictive value of 94%, making it a reliable and cost-effective option for ruling out malignancy in ITNs in India. It helps avoid unnecessary diagnostic surgeries and, for high-risk mutations or fusions, facilitates planning for total thyroidectomy and more targeted medical interventions. While our study aligns with established literature, it has limitations: It is a retrospective, single-centre study, and histological findings for mutation-negative nodules were not available as they were managed conservatively with sonographic follow-up. These limitations are common in similar studies.

It is essential to highlight the need of collaborative efforts among various centres nationwide to underscore the importance of molecular testing, particularly through NGS, as an adjunct diagnostic tool for managing ITNs effectively. With the availability of cost-effective options, it is imperative to accumulate more data to convincingly demonstrate its clinical utility.

Conclusion

In our study using NGS for thyroid nodules, 57% of nodules were mutation-positive with a sensitivity of 75%. NGS, in combination with sonographic and cytopathology reports, provides valuable data for managing ITNs. It supports active surveillance for mutation-negative cases, hemithyroidectomy for low-risk mutations like RAS, and total thyroidectomy for high-risk mutations such as BRAF or significant fusions.

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Author contributions

SK, VP, S, and SP were involved in developing the study concepts. SK, H, and YD contributed to the design of the study. VB and YD contributed to the definition of intellectual content. Literature search was performed by SK, S, and H. Data acquisition was done by SK, S, A, and H. Data analysis was carried out by H, S, K, and A. Statistical analysis was performed by SP, B, K, and VP. Manuscript preparation was done by H and S, while SK, SP, A, and YD participated in manuscript review. SK served as the guarantor for the study.

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Conflicts of interest

There are no conflicts of interest.

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