European Journal of Endocrinology, 2023, **188**, 1–9
https://doi.org/10.1093/ejendo/lvac018
Advance access publication 10 January 2023
Original Research

Detection of driver mutations in plasma cell–free nucleic acids in differentiated thyroid neoplasm

Susmita Dutta, Soham Tarafdar, Pradip Mukhopadhyay, Nitai P. Bhattacharyya, and Sujoy Ghosh,*

Department of Endocrinology, Institute of Post Graduate Medical Education & Research, Kolkata, India

***Corresponding author:** Department of Endocrinology and Metabolism, IPGME&R, 244, A.J.C Bose Road, Kolkata 700020, India. Email: drsujoyghosh2000@gmail.com

Abstract

Importance: This proof-of-concept paper demonstrates that driver mutations can be detected in plasma in differentiated thyroid tumors, and we were able to detect mutations in upto 80% malignant thyroid nodules. Additionally, cancer subtypes could also be predicted using a 8-gene panel. In almost 90% follicular adenoma, rat sarcoma virus (RAS) mutations were detectable. There was a strong agreement between driver mutations found in plasma samples, FNAC materials, and histopathology samples. This has potential as a noninvasive, preoperative diagnostic tool (particularly of clinical importance in indeterminate nodules) and may help in detection of residual tumor after surgery. Future research is warranted to test the role of this tool to detect tumor recurrence.

Objective: Ultrasonographic (USG) evaluation and fine-needle aspiration (FNA) are cornerstone for evaluation of thyroid neoplasm. Molecular technique including detection of driver mutation from FNA cytology (FNAC) material is an established modality. In this study, we explored the feasibility of using plasma cell–free nucleic acids to identify known driver mutations in differentiated thyroid neoplasm.

Design: Patients presenting with thyroid nodules underwent USG with Thyroid Image Reporting and Data Systems scoring and FNAC (Bethesda classification). All patients in Bethesda 3, 4, 5, 6 underwent surgery and histopathological confirmation. Patients in Bethesda 2 (cosmetic concerns, compressive symptoms) underwent surgery, and rest were presumed benign on the basis of USG, FNAC features, and clinical followup.).

Setting: Endocrinology clinic.

Participants: Subjects with thyroid nodule.

Intervention(s) or Exposure(s): None.

Main Outcome(s) and Measure(s): Plasma sample, FNA, and histopathology material were evaluated for driver mutations (8-gene panel comprising BRAF-V600E, RET/PTC3, RET/PTC1, TERT promoter, HRAS, NRAS, KRAS, and PAX8-PPARG).

Results: A total of 223 subjects were recruited; of these 154 were benign and 69 had differentiated thyroid cancer. We were able to detect driver mutation from plasma in 55 subjects (79.71%) of all malignant patients, and 11 patients in benign category had RAS mutation (follicular adenoma). Rest of the benign nodules did not have any detectable driver mutations.

Conclusions and Relevance: Plasma might be a viable noninvasive alternative source for detection of driver mutations (8-gene panel) in subjects with differentiated thyroid tumors and may have significant clinical utility.

Keywords: liquid biopsy, differentiated thyroid tumors, driver mutation and indeterminate nodule

Significance

This is a proof-of-concept paper that demonstrates that driver mutations can be detected in plasma in subjects with differentiated thyroid tumors, and we were able to detect driver mutations in upto 80% patients with malignant thyroid nodules; additionally, cancer subtypes could also be predicted using a 8-gene panel. In almost 90% subjects with follicular adenoma, rat sarcoma virus (RAS) mutations were detectable. There was a strong agreement between driver mutations found in plasma samples, FNAC materials, and histopathology samples. This has potential as a noninvasive, preoperative diagnostic tool (particularly of clinical importance in indeterminate nodules) and may have role in detection of residual tumor after surgery. Future research is warranted to test the role of this tool to detect tumor recurrence.

Introduction

Thyroid malignancy is the commonest endocrine malignancy.[1] Current protocol for evaluation of thyroid nodules includes a thyroid function test, ultrasonography (USG) of the thyroid gland, followed by USG-guided fine needle aspiration (FNA) cytology in those indicated.[2–4]

Knowledge gaps exist in areas of diagnosis and management of thyroid nodules, particularly indeterminate nodules (Bethesda 3 and 4). Several molecular techniques, including detection of mutations and presence of specific panel of micro-RNAs, have been used.[5] Limited availability, costs, and need for FNA material are limitations of currently used

Received: August 9, 2022. **Revised:** December 15, 2022. **Editorial Decision:** December 16, 2022. **Accepted:** December 27, 2022

molecular tests. These tests have limited role in detecting residual tumor or disease recurrence.

In recent years, liquid biopsy has been found to be useful for cancer diagnosis, prognosis, and follow-up. Circulating cell-free nucleic acids (cell-free DNA (cfDNA), cell-free RNA, microRNA (miRNA), long non-coding RNA (lnc RNA)) has been investigated in plasma or serum of cancer patients affected by malignancies of breast, prostate, bladder, ovary, and colon.[6] In malignancies circulating DNA, RNA represents short fragments of nucleic acids released from tumors, which may contain tumor-specific somatic mutations.[6,7] These cell-free genetic materials can be analyzed for cfDNA content and tumor-specific driver mutations. Recently published papers have specifically looked at driver mutations from circulating cfDNA in anaplastic thyroid cancer (ATC) and medullary thyroid cancer.[8–13] Isolated studies have tested for detection of single driver mutations, mostly detection of BRAF V600E.[14–16] Additionally, there is a published systematic review[17] of attempts of detection of driver mutations, DNA integrity, and cfDNA quantification from plasma. To the best of our knowledge, no single clinical paper (with documented histopathology) has attempted to detect a comprehensive panel of mutations from plasma in differentiated thyroid neoplasm (including papillary thyroid cancer [PTC), follicular thyroid cancer [FTC], and follicular adenoma [FA]).

In addition to preoperative prediction of histopathological diagnosis, detection of driver mutations in plasma (postoperatively) may help in detection of residual tumor and tumor recurrence in a noninvasive manner.[18–20]

Our group has already published data regarding use of noninvasive molecular methods for diagnosing thyroid cancer.[21] CfDNA quantification from plasma showed high degree of sensitivity (100%) and specificity (92.7%) for diagnosis of thyroid cancer. cfDNA concentration was able to differentiate malignant from benign thyroid nodules but could not predict subtype of cancer. The increased concentration of cfDNA in patients with cancer represents short fragments released from tumors that may contain tumor-specific somatic driver mutations.

With this background, we embarked upon to test whether such driver mutations are detectable in differentiated thyroid cancer (DTC) and benign thyroid nodules from plasma cell–free nucleic acids (including cfDNA and cfRNA), and we also tested the agreeability of mutation detected from plasma, FNA cytology (FNAC) sample, and histopathology tissue. Secondly, we wanted to evaluate if presence of specific mutations in preoperative plasma sample was able to predict subtype of cancer/tumor.

We also compared our preoperative plasma cfDNA concentration data (previously published), with the driver mutations detected from plasma cell–free nucleic acids to identify specific tumor subtypes based on unique molecular signatures.

Materials and methods

This was a single-center prospective study, approved by Institutional Ethics Committee, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India. The research was conducted in compliance with the Declaration of Helsinki. Written consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used. Consecutive patients presenting to Endocrinology Outpatient Department with solitary thyroid nodule were selected for our study. The study design and workflow are illustrated in Figure 1.

Patients with hyperthyroidism, multinodular goiter, Thyroid Image Reporting and Data Systems (TIRADS) 1 and 2 on USG, nondiagnostic (Bethesda 1) results on FNAC, and anaplastic and medullary thyroid cancer were excluded from final analysis. Patients with past/current history of known malignancies were also excluded.

Free T4 and TSH assay

Serum thyroid-stimulating hormone (TSH) and free T4 were estimated by chemiluminescence immunoassay using commercially available kits from Siemens Diagnostics (Germany) with Immulite-1000 analyzer.

USG and USG-guided FNAC procedure

Patients underwent high resolution USG (PHILIPS HD7 using a 3-12 MHz broadband linear array transducer with selectable frequency and electronic focus). USG was performed by one of two dedicated radiologists and reported according to the American College of Radiology TIRADS (2017) classification. USG-guided FNAC performed in patients fulfilling inclusion criteria and cytological examination was reported as per Bethesda classification. Histopathology was reported according to World Health Organization guidelines for classification of endocrine tumors.[22]

Venous blood samples were collected in ethylenediamine tetraacetic acid (EDTA) vials for plasma cell–free nucleic acid analysis before FNAC. We preserved tissue samples from thyroid nodules that underwent surgery. We detected mutations from plasma cell–free nucleic acids and corroborated findings with mutations found on FNAC and histopathology. Histopathology served as gold standard of diagnosis. Blood sample was collected postoperatively (2-3 weeks postsurgery) to reassess presence of mutations that had been found preoperatively.

We used an 8-gene panel of mutations, commonly detected in DTC. Nikiforov et al.[7,23,24] used a panel of 7-gene for FNA samples to differentiate benign from malignant thyroid nodule in indeterminate category. The panel contained proto-oncogenes related to classical PTC like BRAF (V600E), RET/PTC1, and RET/PTC3 rearrangements and mutations related to follicular tumors such as HRAS, NRAS, KRAS (codons 12, 13, and 61), and PAX8-PPARG. TERT promoter mutations (TERTC250T and TERTC228T)[25] were also included as they are a marker of aggressive DTC.

Circulating cell-free nucleic acids analysis

Circulating cfDNA extraction

Blood samples were collected in EDTA vials and plasma was separated by centrifugation at 4 °C at 3 000×*g* for 15 minutes and plasma portion was recentrifuged at 3000×*g* for 15 minutes at 4 °C to obtain cell-free plasma (repeated 2times) and stored at −80 °C until further analyses. CfDNA extraction from 200 µL of plasma samples using ZYMO DNA kit (Quick-cfDNA Serum and Plasma Kit Cat No: D-4076).

DNA extraction from FNAC materials and histopathology samples

FNA was performed using a 22-gauge needle fitted to a 10 mL syringe. Most of the material (about two thirds) was used for cytological examination, and remaining was used for DNA isolation after needle washing with 1 mL of the normal saline. The sample was frozen for DNA extraction subsequently, using ZYMO DNA kit (Quick-DNA Micro prep Kit Cat No: D3021).

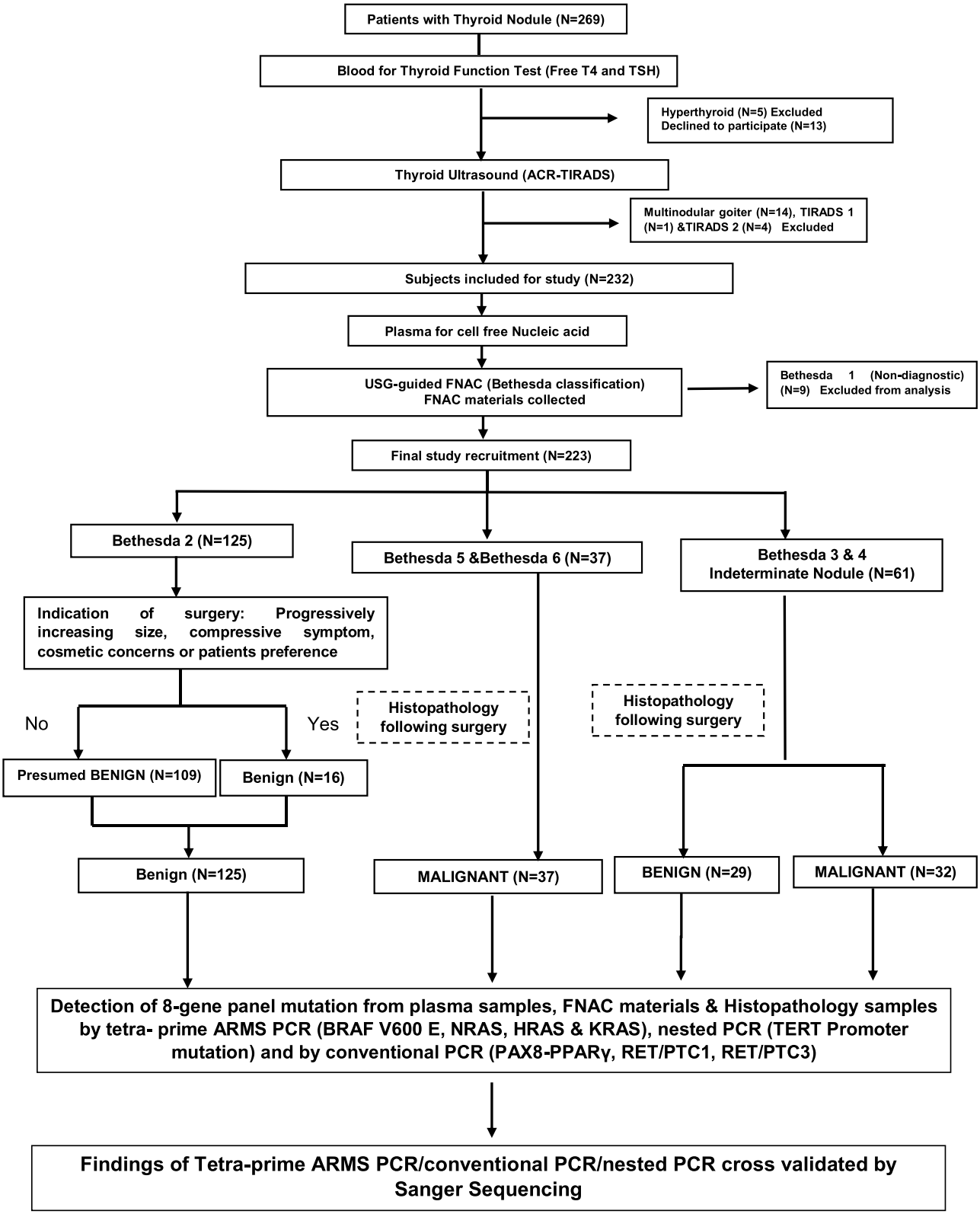

Figure 1. Study design and workflow.

Postoperatively, a representative tissue sample was taken from the thyroid nodule in normal saline and stored at −80 °C. Stored tissue sample was grounded to a fine powder in liquid nitrogen, using a mortar and pestle. DNA from tissue samples was extracted using NucleoSpin® Tissue kit (Cat No: 740952.50).

RNA extraction from plasma, FNAC materials, and histopathology samples

RNA was extracted from 400 µL of plasma samples, FNAC, and histopathology samples by using Trizol reagent (RNAiso plus TAKARA Cat No: 9108) RNA extraction from plasma: methodology: Supplementary File S1.

Quantification and purity

Quantification and purity of the isolated DNA and RNA from plasma, FNAC, and tissue samples were determined in duplicate by measuring absorbance at 260 and 280 nm using a Bio Spectrometer (Eppendorf Bio Spectrometer ® basic). Concentration of DNA and RNA was recorded in ng/mL. To check the purity of DNA, a ratio of absorbance at 260 and 280 nm ranging ~1.8 and ranging ~2.0 for RNA. DNA concentration was also measured using a Qubit fluorometer (Qubit 4 Fluorometer Invitrogen by Thermo Fisher Scientific). Preoperative benign samples had a mean cfDNA concentration of 22.85 ± 10.27 ng/mL, while malignant cases had a mean cfDNA concentration of 96.20 ± 8.31 ng/mL.

BRAF and RAS (HRAS, NRAS, and KRAS) mutation analysis

Tetra-prime amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) is also referred to as allele-specific oligonucleotide PCR.[26–28] Tetra prime ARMS PCR has comparable accuracy in identifying mutations when compared with next-generation sequencing (NGS) and digital PCR.[29–33]

Additionally, Sanger sequencing was performed using ABI 3730 XL DNA Analyzer and BigDye® Terminator v3.1Cycle Sequencing Kit in all positive cases (Validation of the assay). Primers were designed by using freely available online software (http://primer1.soton.ac.uk/primer1.html).[34] "BLAST" (basic local alignment search tool) program (http://www.ncbi.nlm.nih.gov/blast) was used to check for specificity of the primers. Supplementary Table shows the primer sequences and their annealing temperature (Tm value).

PCR cycling was performed, and the PCR products were separated by running on 2% agarose gel with DNA size marker of 50-1000 bp followed by staining with ethidium bromide and visualized and semi-automatically analyzed by the gel documentation system.

TERT promoter mutation analysis

We detected TERT promoter mutation hotspot TERTC250T and TERTC228T, which were amplified using nested PCR on 50 ng of DNA extracted from plasma, FNAC, and tissue samples. The first PCR used pair primers (5′ACGAACG TGGCCAGCGGCAG3′ [sense] and 5′CTGGCGTCCCT GCACCCTGG3′ [antisense]) in a Premix Taq™ DNA Polymerase (TaKaRa Taq™ Version 2.0). It was performed with an initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of 94 °C for 30 seconds, 62 °C annealing for 30 seconds, 72 °C elongation for 45 seconds, and completion with an elongation at 72 °C for 15 minutes. The second PCR used a dilution (1:50) of the first PCR product. The primers used were 5′AGTGGATTCGCGGGCACAGA3′ (sense) and 5′CAGCGCTGCCTGAAACTC3′ (antisense) in a Premix Taq™ DNA polymerase (TaKaRa Taq™ Version 2.0). PCR was performed, and the reaction mixtures were then electrophoresed against 2% to visualize the amplicon products.[35,36]

RET/PTC and PAX8-PPARG rearrangement analysis

For RET/PTC and PAX8-PPARG rearrangement, complementary DNA (cDNA) samples were analyzed using PCR with primers flanking the fusion point between H4 and ret genes to detect RET/PTC1 and with primers flanking the fusion point between the ELE1 (RET fused gene) and ret genes to detect RET/PTC3. Mean RNA concentration in benign cases were 29.38 ± 3.40 ng/μL and in malignant cases were 29.61 ± 3.73 ng/μL. The PCR conditions were 95 °C for 5 minutes followed by 35 cycles of amplification (95 °C for 40 seconds, 50-62 °C for 40 seconds, and 72 °C for 40 seconds) with final extension at 72 °C for 5 minutes.[37] cDNA samples (100 ng) were also analyzed for the PAX8-PPARG rearrangement by PCR with the upstream primers located in exons 7, 8, or 9 of PAX8 paired with a downstream primer in exon 1 of PPARG. PCR conditions were 94 °C for 10 seconds, 60 °C for 15 seconds, and 70 °C for 15 seconds for 40 cycles. Primers for RET/PTC1, RET/PTC3 and PAX8-PPARG rearrangement were designed based on previous studies.[38,39]

Sample size

For sample size calculation, we assumed a prevalence of DTC in thyroid nodule as 15%.[40] In order to detect a prevalence even as low as 5% with mutation in thyroid nodule with 95% confidence, we calculated that at least 204 subjects with thyroid nodule needs to be recruited assuming a type 1 error of 5%.[41]

Statistical analysis

Statistical analyses were performed using SPSS software (version 26; SPSS, Inc. Chicago, IL, USA). Categorical variables were expressed as frequencies and percentages. In the case of a normal distribution, continuous data are presented as mean ± SD. Fleiss kappa analysis was performed to check for agreement of the presence of mutation among the three groups (cell-free nucleic acid, FNAC materials, and histopathology samples) ($\kappa < 0.20$: poor, $\kappa = 0.21$-0.40: fair, $\kappa = 0.41$-0.60: moderate, $\kappa = 0.61$-0.80: good and $\kappa = 0.81$-1.00: very good).

Results

A total of 269 subjects with clinically solitary thyroid nodule were evaluated. Following were excluded: hyperthyroid ($N = 5$), multinodular goiter ($N = 14$), TIRADS 1 and 2 ($N = 5$), declined to participate for the study ($N = 13$), and non-diagnostic (Bethesda 1) results on FNAC ($N = 9$). Finally, 223 patients were included. Basic demographic profile and evaluation of USG and FNA results are summarized (Table 1).

Patients in Bethesda class 5 and 6 ($N = 37$) underwent surgery, and all had DTC on histopathology. Of them 31 were classic PTC, 4 were follicular variant PTC (FVPTC), and 2 were FTC. Patients in Bethesda 3 and 4 ($N = 61$) also underwent surgery as per institutional protocol. Of them, 29 were benign and 32 were malignant (27 were PTC, 4 were FVPTC, and 1 were FTC). Patients in Bethesda class 2 ($N = 109$) who had not undergone surgery were followed up clinically and radiologically with guided FNAC if required. Some ($N = 16$) in Bethesda class 2 underwent surgery (progressively increasing size with compressive symptoms, cosmetic concerns, high-risk features in USG, and patient's choice) were proven to be benign on histopathological examination (HPE). Histopathology was considered gold standard for diagnosis. Patients in Bethesda 2 category who did not undergo surgery were presumed to be benign based on absence of high-risk features on USG, a benign cytology, and nonprogression during a 1-year follow-up period. Mutation of 8 genes

Table 1. Demographic, clinical, radiological, cytopathological, and histopathological findings of solitary thyroid nodule.

Profile of subjects with solitary thyroid nodule (*N* = 223)		
Age, mean ± SD, years	36.46 ± 12.50	
Gender (male/female)	1/6.9	
Free T4, mean ± SD, ng/dL	1.21 ± 0.38	
TSH, mean ± SD, mIU/L	2.07 ± 0.97	
Radiological findings of solitary thyroid nodule		
TIRADS score	TIRADS 3 (mildly suspicious), *n* (%)	97 (43.49)
	TIRADS 4 (moderately suspicious), *n* (%)	94 (42.15)
	TIRADS 5 (highly suspicious), *n* (%)	32 (14.34)
Cytopathological findings of solitary thyroid nodule		
Bethesda classification	Bethesda 2 (benign), *n* (%)	125 (56.05)
	Bethesda 3 (AUS/FLUS), *n* (%)	20 (8.96)
	Bethesda 4 (follicular neoplasm), *n* (%)	41 (18.38)
	Bethesda 5 (suspicious for malignancy), *n* (%)	35 (15.69)
	Bethesda 6 (malignant), *n* (%)	2 (0.89)
HPE findings of solitary thyroid nodule (*N* = 114)		
HPE findings (*N* = 114)	Benign (*N* = 45)	
	Adenomatoid goiter, *n* (%)	5 (11.11)
	Colloid goiter, *n* (%)	15 (33.33)
	Follicular adenoma, *n* (%)	13 (28.88)
	Hashimoto thyroiditis, *n* (%)	9 (19.99)
	Nodular hyperplasia, *n* (%)	2 (4.44)
	Hurthle cell adenoma, *n* (%)	1 (2.22)
	Malignant (*N* = 69)	
	Papillary carcinoma, *n* (%)	58 (84.05)
	Follicular variant papillary carcinoma, *n* (%)	8 (11.59)
	Follicular carcinoma, *n* (%)	3 (4.34)

Total sample (*N* = 223): presumed benign (*N* = 109), benign (HPE proved [*N* = 16]), indeterminate nodule (HPE proved [*N* = 6]), and suspicious for malignancy and malignant (HPE proved [*N* = 37]).
Abbreviations: AUS, Atypia of undetermined significance; FLUS: follicular lesion of undetermined significance; HPE, histopathologic; TIRADS, Thyroid Image Reporting and Data Systems; TSH: thyroid-stimulating hormone.

(*BRAF V600E, HRAS, NRAS, KRAS, TERT Promoter mutations and gene rearrangements PAX8-PPARG, RET/PTC1 [RET-ccd6*], *RET/PTC3 [RET-NCO4*]) was analyzed, and the panel was presumed to be positive if any one mutation on the panel was positive.

Of 223 subjects, 69 were malignant and 154 were benign. Among DTC patients, we detected driver mutations in 55 (79.1%). Of which, most were PTC-related mutation (BRAF V600 E: 42, RET/PTC3: 1 and TERT: 1), and 11 subjects had follicular tumor specific mutations in FVPTC and FTC cases (HRAS: 6, NRAS: 1 and KRAS: 1). The patient with TERT mutation had distant metastasis. In our cohort, 11 patients with RAS related mutations turned out to be FAs (HRAS: 7, NRAS: 3 and KRAS: 1). Driver mutations were not detected in 14 (20.9%) of patients with DTC. It is a common knowledge that up to 25% of all malignancies do not have known driver mutations.[6] The plasma mutation analysis of all the subjects included in our study is schematically shown in Figure 2.

Commonest mutation identified was BRAF V600E. These patients had a diagnosis of PTC (*N* = 42). The second commonest mutation detected was RAS mutation (FA [*N* = 11], FVPTC [*N* = 8], and FTC [*N* = 3]). RET/PTC3 was detected in one patient and TERT was positive in one patient with PTC. PAX8-PPARG was not detected in any patient.

Comparison of detection of mutations in plasma, FNA, and histopathology

For comparison of diagnostic accuracy of mutations identified from plasma, FNAC, and tissue sample, we used Fleiss kappa. There was a high degree of agreement, and Fleiss kappa was 0.880 (95% CI: 0.876-0.883) ($P < .001$) among 3 groups. We had 114 patients who underwent surgery, of which 45 were benign and 69 were malignant.

Of 69 malignant cases, 55 (79.71%) were mutant positive in plasma samples, 56 (81.15%) were mutant positive in FNAC materials, and 58 (84.05%) were mutant positive in tissue samples.

Using our 8 gene mutation panel in patients with indeterminate nodule, our 8-gene panel of mutation from plasma, FNAC, and histopathology samples to predict histopathological diagnosis (Tables 2, 3, and 4) and our 8-gene panel of mutation from plasma was able to rule out malignancy in the indeterminate category with specificity of 72.50%, positive predictive value 76.47% and rule in malignancy with sensitivity of 81.25% and negative predictive value 77.78%.

We had determined cfDNA concentration of our patients, and a cutoff value >67.9 ng/mL was used in differentiating benign and malignant thyroid nodules (based on previous publication).[21] The mutation analysis complemented with cfDNA (Figure 3) could help in predicting malignancy even in those without driver mutations. Presence of RAS mutation with low cfDNA concentration (<67.9 ng/mL) was able to predict a diagnosis of benign FA. A high cfDNA (>67.9 ng/mL) with RAS mutation was found in FVPTC and FTC.

Postoperative plasma samples were collected in all patients with HPE proven malignancy. Postoperative mutation analysis revealed persistent BRAF V600E with high cfDNA in 3 patients. These patients had significant residual tissue, had local lymph node metastasis, and underwent repeat surgical intervention. Subsequently, when we repeated mutation analysis following second surgery, one patient revealed absence of any mutations and cfDNA concentration was 29.8 ng/mL.

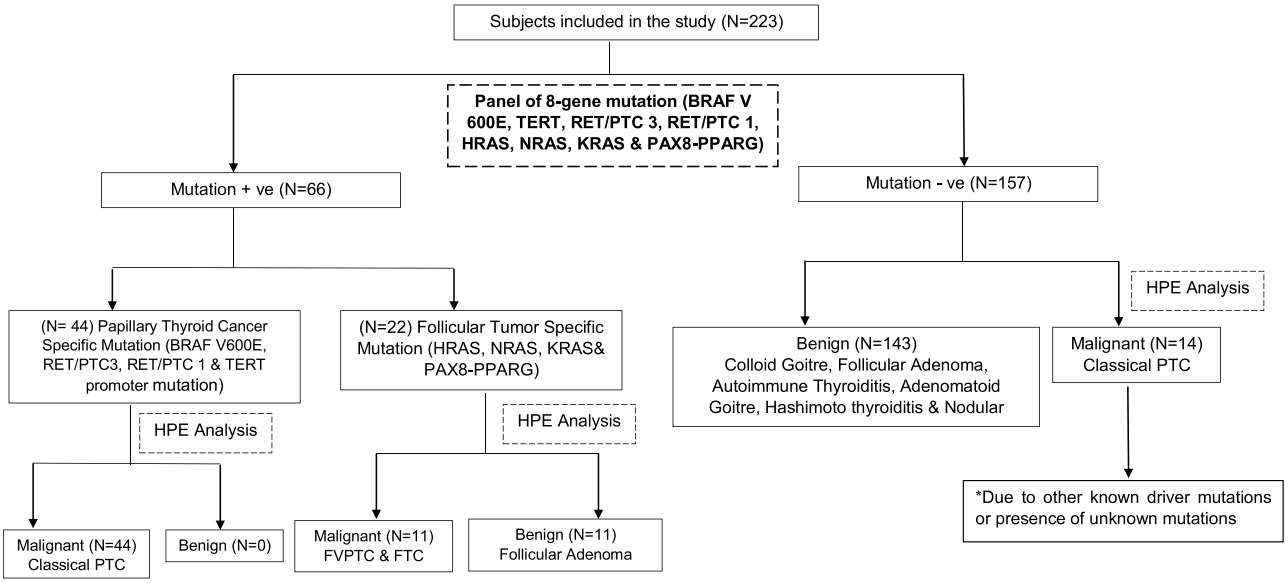

Figure 2. Detection of somatic mutations in plasma samples.

Table 2. Performance of mutation panel in plasma to predict histopathological diagnosis in patients with indeterminate nodules undergoing surgery.

Indeterminate nodule				
Plasma sample	**Histopathology (*N* = 61)**			
Mutation (BRAF, NRAS, KRAS, HRAS, TERT, RET-PTC3, RET-PTC1, and PAX8-PPARG)	Malignant	Benign	Total	*P*-value
POSITIVE	26	8	34	<.001
NEGATIVE	6	21	27	
Total	32	29	61	

Sensitivity 81.25%, specificity 72.41%, positive predictive value 76.47%, and negative predictive value 77.78%.

Table 3. Performance of mutation panel in FNAC to predict histopathological diagnosis in patients with indeterminate nodules undergoing surgery.

Indeterminate nodule				
FNAC SAMPLE	**Histopathological report (*N* = 61)**			
Mutation (BRAF, NRAS, KRAS, HRAS, TERT, RET-PTC3, RET-PTC1, and PAX8-PPARG)	Malignant	Benign	Total	*P*-value
POSITIVE	27	6	33	<.001
NEGATIVE	5	23	28	
Total	32	29	61	

Sensitivity 84.38%, specificity 79.31%, positive predictive value 81.82%, and negative predictive value 82.14%.

Discussion

In our study, 69 patients had proven malignancy (histopathological diagnosis). Of them 55 (79.71%) were harboring positive driver mutations, and 14 (20.28%) were mutation negative. This is comparable with data published by Nikiforov et al.,[6] who were able to detect mutations up to 70% of DTC patients based on 7-gene panel from FNA sample. It has been reported previously that up to 25% of all malignancies do not have known driver mutations.[6] In the clinical validation study of Thyroseq version 3 genomic classifier, Nikiforov et al. have demonstrated a sensitivity of 94.1% and specificity of 81.6%, and they failed to detect any mutations in 7.7% of malignant thyroid nodules.[42]

Among benign tumors, only FAs have known driver mutations. In our cohort, we had 12 FAs for which we were able to detect known driver mutations in 11. The rest of the benign tumors did not have known driver mutations.

Molecular tests are of greatest clinical relevance in individuals with indeterminate nodules. In this category, we were able to detect driver mutation from plasma successfully; establishing the proof of concept and the performance of the tests was very encouraging.

Our study indicates that in indeterminate category almost 50% turned out to be malignant, which is not surprising, given the fact that ours is a tertiary care hospital wherein patients with greater likelihood of malignancy were referred on for second opinion and decision for surgery. These subjects in indeterminate category had relatively higher risk stratification on USG. Finally, the proportion of individuals in Bethesda 6 was possibly lower than reported in most series. This again reflects referral bias, as most of the highly suspicious nodules would have already been operated at secondary care hospital.

We were able to detect somatic driver mutations from plasma cell–free nucleic acids in patients with DTC and FAs. Previous attempts to isolate driver mutations from cell-free nucleus acids have yielded mixed results. Fussey et al.[17] have published a review of nine relevant studies, with varying methodologies, which evaluated utility of cfDNA in DTC. These studies have mostly attempted to identify BRAF V600E mutation form circulating tumor DNA/cfDNA. To the best of our knowledge, this is the first study that has evaluated the 8-gene panel in cell-free nucleic acids from plasma. Pupilli et al.[43] analyzed the diagnostic capability of BRAF V600E in differentiating PTC with benign disease. They demonstrated a higher concentration of BRAF V600E expression in patients

Table 4. Performance of mutation panel in tissue samples to predict histopathological diagnosis in patients with indeterminate nodules undergoing surgery.

Indeterminate nodule				
Tissue sample	**Histopathological report ($N = 61$)**			
Mutation (BRAF, NRAS, KRAS, HRAS, TERT, RET-PTC3, RET-PTC1, and PAX8-PPARG)	Malignant	Benign	Total	*P*-value
POSITIVE	29	6	35	<.001
NEGATIVE	3	23	26	
Total	32	29	61	

Sensitivity 90.63%, specificity 79.31%, positive predictive value 82.86%, and negative predictive value 88.46%.

with PTC compared to benign thyroid disease and normal controls. Hu et al.[44] determined methylation of cfDNA, specifically 5 genes: *CALCA, CDH1, TIMP3, DAPK,* and *RARβ2*. Combining all 5 genes, they achieved a diagnostic sensitivity of 68% and a specificity of 95%, with a preoperative diagnostic accuracy of 77%. Salvianti et al.[45] were able to demonstrate a statistically significant difference in cfDNA integrity index between those with Thy2 nodules and those with Thy 3 and Thy4/5 nodules (0.67, 0.83, 1.02, respectively, $P < .001$). Kim et al.[46] demonstrated presence of BRAF V600E in higher concentration among PTC patients compared to benign thyroid disease. They had 3 patients who had persistently high BRAF V600E postoperatively, and these patients had lung metastasis and advanced stage of disease. Contrary to other studies, Kwak et al.[47] had failed to detect BRAF V600E from serum samples of 93 patients with known papillary thyroid carcinoma with presence of BRAF V600E in tumor tissue. Low concentration of BRAF could be because of 67 patients 71.3% patients had a micropapillary carcinoma, which is a low risk lesion with low circulating tumor DNA levels. In another study, Condello et al.[48] were not able to detect BRAF V600E in the plasma of patients with PTC from ctDNA. In that study, commercially available kits meant for use in tissue and FNA samples were used for detection of mutations in plasma samples. This methodological difference may have resulted in dissimilar results.

In our study, there was strong agreement of mutation findings between cell-free nucleic acid, FNAC, and histopathology samples; Fleiss kappa was 0.880 (95% CI: 0.876-0.883). Yu-Qin et al.[10] detected driver mutations from cfDNA via NGS in patients with ATC. They detected a concordance rate between cfDNA and matched tissue for BRAF V600E mutation of 82.1%. Iyer et al.[11] detected BRAF V600E mutations from cfDNA in 44 patients with ATC. They found a concordance of 93% between cfDNA and tumor tissue.

Mutation detected from plasma helped to predict subtype of tumor including classic PTC, FVPTC, FA, and FTC preoperatively. Detection of tumor subtypes could have ramifications in extent of surgery, postoperative management, and risk of recurrence.

Use of cfDNA in our cohort of patients has shown promising results in differentiating malignant from thyroid nodules. Previously published data[21] have established cfDNA concentration >67.9 ng/mL as cutoff for detecting malignancy. Preoperatively, benign samples had a mean cfDNA concentration of 22.85 ± 10.27 ng/mL, while malignant cases had a mean cfDNA concentration of 96.20 ± 8.31 ng/mL. Using cfDNA concentrations along with mutation analysis, we could differentiate benign and malignant tumors in patients with RAS mutations. This could have future implications in management.

One patient had TERT positive mutation and had distant metastasis. This is in keeping with existing literature that PTC with TERT positivity usually has a more aggressive course.

In postoperative samples, 3 patients had persistent BRAF V600E positivity and high cfDNA. These patients had residual tumor, which highlights possible utility of detection of

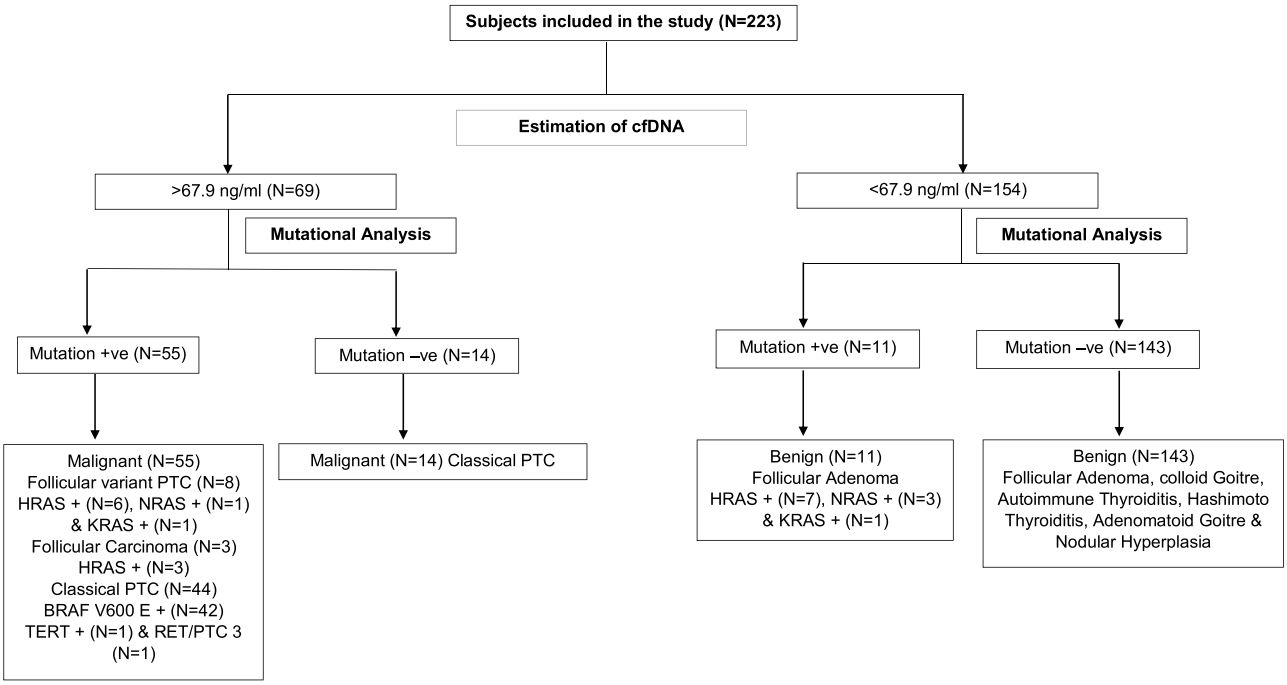

Figure 3. CfDNA concentration along with mutational analysis.

mutation from plasma in detecting residual tumor and even possible detection of recurrence.

Limitations

All patients in Bethesda 2 did not undergo surgery for ethical reasons. We excluded subjects with other overt malignancies; however, we did not overzealously investigate to rule out malignancies (eg, did not perform colonoscopy). We also did not include noninvasive follicular thyroid neoplasm with papillary-like nuclear features, well differentiated thyroid tumor of uncertain malignant potential and follicular tumor of uncertain malignant potential, or any borderline tumors. This is a proof of concept paper, so we first embarked upon choosing only solitary thyroid nodules for better validation of molecular tests (in case of multiple nodules with multiple FNA, could complicate interpretation of results).

Conclusion

Using liquid biopsy from plasma circulating cell-free nucleic acids, we were able to detect known driver mutations, which could serve as a useful noninvasive marker for preoperative diagnosis of thyroid nodules and aid in decision-making for further management and follow-up of the patients. Ours is a proof of concept study to demonstrate plasma as potential source for identification of driver mutations. Plasma as a source of nucleic acid has the advantage of greater ease of sampling and could be used to detect possible residual disease and also detect possible recurrence. FNA sampling is dependent on operator. In addition, it has no role in follow-up of thyroid cancer patients.

We have demonstrated a comparable accuracy for detection of mutations from cf DNA, FNA, and histopathology samples. This has potential as a noninvasive, preoperative diagnostic tool (particularly of clinical importance in indeterminate nodules) and may have role in detection of residual tumor after surgery. Future research is warranted to test the role of this tool to detect tumor recurrence.

Acknowledgments

We would like to acknowledge our lab members (Madhurima Basu, Astha Mukhopadhyay, Sreya Bhattacharya), the technicians, and staffs of the Department of Radiology, Pathology, Surgery and Endocrinology, IPGME&R, SSKM Hospital, and Kolkata for their cooperation and support.

Authors' contribution

Conceptualization of the study and overseeing: S.G. Patient selection and conduct of test: S.T. and S.D. Data analysis: P.M., S.D., and S.T. Manuscript preparation: S.G., N.P.B., S.D., S.T., and P.M.

Supplementary material

Supplementary material is available in the *European Journal of Endocrinology* online.

Funding

This work was supported by the Endocrine Society of Bengal (ESB/7/2018), Endocrine Society of India (ESI/5/2019), and DST/INSPIRE (IF180262) for funding this project.

Conflicts of interest: None declared.

Data availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Mazzaferri E, delos Santos E, Rofagha-Keyhani S. Solitary thyroid nodule: diagnosis and management. *Med Clin North Am*. 1988;72(5):1177-1211. https://doi.org/10.1016/S0025-7125(16)30736-2
2. Cooper D, Doherty G, Haugen B, *et al*. Management guidelines for patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines taskforce. *Thyroid*. 2006;16(2):109-142. https://doi.org/10.1089/thy.2006.16.109
3. Frates M, Benson C, Doubilet P, *et al*. Prevalence and distribution of carcinoma in patients with solitary and multiple thyroid nodules on sonography. *J Clin Endocrinol Metab*. 2006;91(9):3411-3417. https://doi.org/10.1210/jc.2006-0690
4. Barbosa T, Junior C, Graf H, *et al*. ACR TI-RADS and ATA US scores are helpful for the management of thyroid nodules with indeterminate cytology. *BMC Endocr Disord*. 2019;19(1):112. https://doi.org/10.1186/s12902-019-0429-5
5. Silaghi CA, Lozovanu V, Georgescu CE, *et al*. Thyroseq v3, afirma GSC, and microRNA panels versus previous molecular tests in the preoperative diagnosis of indeterminate thyroid nodules: A systematic review and meta-analysis. *Front Endocrinol (Lausanne)*. 2021;12:649522. https://doi.org/10.3389/fendo.2021.649522
6. Nikiforov Y, Ohori N, Hodak S, *et al*. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab*. 2011;96(11):3390-3397. https://doi.org/10.1210/jc.2011-1469
7. Cantara S, Capezzone M, Marchisotta S, *et al*. Impact of proto-oncogene mutation detection in cytological specimens from thyroid nodules improves the diagnostic accuracy of cytology. *J Clin Endocrinol Metab*. 2010;95(3):1365-1369. https://doi.org/10.1210/jc.2009-2103
8. Alexander E, Kennedy G, Baloch Z, *et al*. Preoperative diagnosis of benign thyroid nodules with indeterminate cytology. *N Engl J Med*. 2012;367(8):705-715. https://doi.org/10.1056/NEJMoa1203208
9. Sandulache V, Williams M, Lai S, *et al*. Real-time genomic characterization utilizing circulating cell-free DNA in patients with anaplastic thyroid carcinoma. *Thyroid*. 2017;27(1):81-87. https://doi.org/10.1089/thy.2016.0076
10. Qin Y, Wang J, Wang Y, *et al*. Clinical utility of circulating cell-free DNA mutations in anaplastic thyroid carcinoma. *Thyroid*. 2021;31(8):1235-1243. https://doi.org/10.1089/thy.2020.0296
11. Iyer P, Cote G, Hai T, *et al*. Circulating BRAF V600E cell-free DNA as a biomarker in the management of anaplastic thyroid carcinoma. *JCO Precis Oncol*. 2018;2(2):1-11. https://doi.org/10.1200/PO.18.00173
12. Cote G, Evers C, Hu M, *et al*. Prognostic significance of circulating RET M918T mutated tumor DNA in patients with advanced medullary thyroid carcinoma. *J Clin Endocrinol Metab*. 2017;102(9):3591-3599. https://doi.org/10.1210/jc.2017-01039
13. Raffaele C, Cristina R, Teresa R, *et al*. Pre- and post-operative circulating tumoral DNA in patients with medullary thyroid carcinoma. *J Clin Endocrinol Metab*. 2022;107(8):e3420-e3427. https://doi.org/10.1210/clinem/dgac222
14. Vdovichenko K, Markova S, Belokhvostov A. Mutant form of BRAF gene in blood plasma of cancer patients. *Ann N Y Acad Sci*. 2004;1022(1):228-231. https://doi.org/10.1196/annals.1318.035
15. Cradic K, Milosevic D, Rosenberg A, Erickson L, McIver B, Grebe S. MutantBRAFT1799A can be detected in the blood of papillary thyroid carcinoma patients and correlates with disease Status. *J*

Clin Endocrinol Metab. 2009;94(12):5001-5009. https://doi.org/10.1210/jc.2009-1349
16. Chuang T, Chuang A, Poeta L, Koch W, Califano J, Tufano R. Detectable BRAF mutation in serum DNA samples from patients with papillary thyroid carcinomas. *Head Neck*. 2010;32(2):229-234. https://doi.org/10.1002/hed.21178
17. Fussey JM, Bryant JL, Batis N, *et al.* The clinical utility of cell-free DNA measurement in differentiated thyroid cancer: a systematic review. *Front Oncol*. 2018;8:132. https://doi.org/10.3389/fonc.2018.00132
18. Carbonare L, Frigo A, Francia G, *et al.* Runx2 mRNA expression in the tissue, serum, and circulating non-hematopoietic cells of patients with thyroid cancer. *J Clin Endocrinol Metab*. 2012;97(7):E1249-E1256. https://doi.org/10.1210/jc.2011-2624
19. Yu S, Liu Y, Wang J, *et al.* Circulating MicroRNA profiles as potential biomarkers for diagnosis of papillary thyroid carcinoma. *J Clin Endocrinol Metab*. 2012;97(6):2084-2092. https://doi.org/10.1210/jc.2011-3059
20. Milas M, Shin J, Gupta M, *et al.* Circulating thyrotropin receptor mRNA as a novel marker of thyroid cancer. *Ann Surg*. 2010;252(4):643-651. https://doi.org/10.1097/SLA.0b013e3181f5ba51
21. Dutta S, Tarafdar S, Mukhopadhyay P, Bhattacharyya N, Ghosh S. Plasma cell-free DNA to differentiate malignant from benign thyroid nodules. *J Clin Endocrinol Metab*. 2021;106(5):e2262-e2270. https://doi.org/10.1210/clinem/dgab030
22. Lloyd RV, Osamura RY, Klöppel G, Rosai J, eds. *WHO Classification of Tumours of Endocrine Organs*. 4th ed. IARC; 2017:65–67.
23. Kim S, Lee J, Kim J, *et al.* BRAFV600E Mutation analysis in fine-needle aspiration cytology specimens for evaluation of thyroid nodule: a large series in aBRAFV600E-prevalent population. *J Clin Endocrinol Metab*. 2010;95(8):3693-3700. https://doi.org/10.1210/jc.2009-2795
24. Nikiforov Y, Steward D, Robinson-Smith T, *et al.* Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. *J Clin Endocrinol Metab*. 2009;94(6):2092-2098. https://doi.org/10.1210/jc.2009-0247
25. Liu R, Xing M. TERT Promoter mutations in thyroid cancer. *Endocr Relat Cancer*. 2016;23(3):R143-R155. https://doi.org/10.1530/ERC-15-0533
26. Huang T, Zhuge J, Zhang W. Sensitive detection of BRAF V600E mutation by amplification refractory mutation system (ARMS)-PCR. *Biomark Res*. 2013;1(1):3. https://doi.org/10.1186/2050-7771-1-3
27. Chen Q, Lu P, Jones A, Cross N, Silver R, Wang Y. Amplification refractory mutation system, a highly sensitive and simple polymerase chain reaction assay, for the detection of JAK2 V617F mutation in chronic myeloproliferative disorders. *J Mol Diagn*. 2007;9(2):272-276. https://doi.org/10.2353/jmoldx.2007.060133
28. Duletić AN, Dekanić A, Hadzisejdić I, *et al.* JAK2-v617F mutation is associated with clinical and laboratory features of myeloproliferative neoplasms. *Coll Antropol*. 2012;36(3):859-865.
29. Ye P, Cai P, Xie J, Wei Y. The diagnostic accuracy of digital PCR, ARMS and NGS for detecting KRAS mutation in cell-free DNA of patients with colorectal cancer: A systematic review and meta-analysis. *PLoS One*. 2021;16(3):e0248775. https://doi.org/10.1371/journal.pone.0248775
30. Li C, He Q, Liang H, *et al.* Diagnostic accuracy of droplet digital PCR and amplification refractory mutation system PCR for detecting EGFR mutation in cell-free DNA of lung cancer: a meta-analysis. *Front Oncol*. 2020;10:290. https://doi.org/10.3389/fonc.2020.00290
31. Feng WN, Gu WQ, Zhao N, *et al.* Comparison of the SuperARMS and droplet digital PCR for detecting EGFR mutation in ctDNA from NSCLC patients. *Transl Oncol*. 2018;11(2):542-545. https://doi.org/10.1016/j.tranon.2018.02.007
32. Gorgannezhad L, Umer M, Islam MN, Nguyen NT, Shiddiky MJA. Circulating tumor DNA and liquid biopsy: opportunities, challenges, and recent advances in detection technologies. *Lab Chip*. 2018;18(8):1174-1196. https://doi.org/10.1039/C8LC00100F
33. Wan R, Wang Z, Lee JJ, *et al.* Comprehensive analysis of the discordance of EGFR mutation status between tumor tissues and matched circulating tumor DNA in advanced non-small cell lung cancer. *J Thorac Oncol*. 2017;12(9):1376-1387. https://doi.org/10.1016/j.jtho.2017.05.011
34. Ke X, Collins A, Ye S. PCR Designer for restriction analysis of various types of sequence mutation. *Bioinformatics*. 2002;18(12):1688-1689. https://doi.org/10.1093/bioinformatics/18.12.1688
35. Giorgenon T, Carrijo F, Arruda M, *et al.* Preoperative detection of TERT promoter and BRAFV600E mutations in papillary thyroid carcinoma in high-risk thyroid nodules. *Arch Endocrinol Metab*. 2019;63(2):107-112. https://doi.org/10.20945/2359-3997000000116
36. Trung N, Hoan N, Trung P, *et al.* Clinical significance of combined circulating TERT promoter mutations and miR-122 expression for screening HBV-related hepatocellular carcinoma. *Sci Rep*. 2020;10(1):8181. https://doi.org/10.1038/s41598-020-65213-8
37. Guerra A, Rosaria Sapio M, Marotta V, *et al.* Prevalence of RET/PTC rearrangement in benign and malignant thyroid nodules and its clinical application. *Endocr J*. 2011;58(1):31-38. https://doi.org/10.1507/endocrj.K10E-260
38. Cheung L, Messina M, Gill A, *et al.* Detection of the PAX8-PPARγ fusion oncogene in both follicular thyroid carcinomas and adenomas. *J Clin Endocrinol Metab*. 2003;88(1):354-357. https://doi.org/10.1210/jc.2002-021020
39. Kroll T, Sarraf P, Pecciarini L, *et al.* PAX8-PPAR Γ 1 fusion in oncogene human thyroid carcinoma. *Science*. 2000;289(5483):1357-1360. https://doi.org/10.1126/science.289.5483.1357
40. Schmidbauer B, Menhart K, Hellwig D, Grosse J. Differentiated thyroid cancer—treatment: state of the art. *Int J Mol Sci*. 2017;18(6):1292. https://doi.org/10.3390/ijms18061292
41. Sullivan L. Power and sample size determination. Accessed March 3, 2022. https://sphweb.bumc.bu.edu/otlt/MPH-Modules/BS/BS704Power/BS704_Power4.html.
42. Nikiforov YE, Baloch ZW. Clinical validation of the ThyroSeq v3 genomic classifier in thyroid nodules with indeterminate FNA cytology. *Cancer Cytopathol*. 2019;127(4):225-230. https://doi.org/10.1002/cncy.22112
43. Pupilli C, Pinzani P, Salvianti F, *et al.* CirculatingBRAFV600Ein the diagnosis and follow-up of differentiated papillary thyroid carcinoma. *J Clin Endocrinol Metab*. 2013;98(8):3359-3365. https://doi.org/10.1210/jc.2013-1072
44. Hu S, Ewertz M, Tufano R, *et al.* Detection of serum deoxyribonucleic acid methylation markers: a novel diagnostic tool for thyroid cancer. *J Clin Endocrinol Metab*. 2006;91(1):98-104. https://doi.org/10.1210/jc.2005-1810
45. Salvianti F, Giuliani C, Petrone L, *et al.* Integrity and quantity of total cell-free DNA in the diagnosis of thyroid cancer: correlation with cytological classification. *Int J Mol Sci*. 2017;18(7):1350. https://doi.org/10.3390/ijms18071350
46. Kim B, Kim I, Lee B, *et al.* Detection of plasma BRAF(V600E) mutation is associated with lung metastasis in papillary thyroid carcinomas. *Yonsei Med J*. 2015;56(3):634. https://doi.org/10.3349/ymj.2015.56.3.634
47. Kwak J, Jeong J, Kang S, *et al.* Study of peripheral BRAF(V600E) mutation as a possible novel marker for papillary thyroid carcinomas. *Head Neck*. 2012;35(11):1630-1633. https://doi.org/10.1002/hed.23195
48. Condello V, Macerola E, Ugolini C, *et al.* Analysis of circulating tumor DNA does not improve the clinical management of patients with locally advanced and metastatic papillary thyroid carcinoma. *Head Neck*. 2018;40(8):1752-1758. https://doi.org/10.1002/hed.25155

The Journal of Clinical Endocrinology & Metabolism, 2021, Vol. 106, No. 5, e2262–e2270
doi:10.1210/clinem/dgab030
Clinical Research Article

Clinical Research Article

Plasma Cell-Free DNA to Differentiate Malignant from Benign Thyroid Nodules

Susmita Dutta,* Soham Tarafdar,* Pradip Mukhopadhyay, Nitai P. Bhattacharyya, and Sujoy Ghosh

Department of Endocrinology and Metabolism, Institute of Post-Graduate Medical Education and Research and Seth Sukhlal Karnani Memorial Hospital, Kolkata 700020, India

ORCiD number: 0000-0001-5397-961X (S. Ghosh).

*Both authors have equally contributed.

Abbreviations: AUC, area under the curve; FNA, fine needle aspiration; FNAC, fine needle aspiration cytology; GC, Genomic Classifier; ROC, receiver operator characteristic; T4, thyroxine; TIRADS, Thyroid Imaging, Reporting and Data System; TSH, thyrotropin (thyroid-stimulating hormone); USG, ultrasonography.

Received: 25 November 2020; Editorial Decision: 11 January 2021; First Published Online: 21 January 2021; Corrected and Typeset: 25 February 2021.

Abstract

Background: Molecular testing is increasingly used to identify malignancy in thyroid nodules (especially indeterminate category). Measurement of cell-free DNA (cfDNA) levels from plasma has been useful in diagnosis of cancers of other organs/tissues; herein we analyze cfDNA levels in patients with thyroid nodules to explore the possibility of establishing a cutoff for identification of malignancy.

Methods: Patients underwent ultrasonography (USG) and USG-guided fine needle aspiration as well as surgery, where indicated. Cell-free DNA was extracted from plasma and quantified. In initial analysis (determination of cutoff), cfDNA levels were compared between Bethesda 2 and Bethesda 5 &6 to establish a cutoff value that could differentiate malignant from benign nodules. In the subsequent analysis, the aforementioned cutoff was applied (validation of cutoff) to those with indeterminate nodules to check ability to predict malignancy.

Results: Fine needle aspiration (n = 119) yielded patients with Bethesda 2 (n = 69) Bethesda 5 & 6 (n = 13) who underwent histopathological confirmation. Cell-free DNA levels in these 2 groups were 22.85 ± 1.27 and 96.20 ± 8.31 (ng/mL) respectively. A cfDNA cutoff of 67.9 ng/mL, with area under the curve of 0.992 (95% CI, 0.97-1.0) with 100% sensitivity and 93% specificity was established to identify malignant lesions. Indeterminate group (Bethesda 3 & 4) underwent surgery (malignant n = 24), (benign n = 13), and using the previously identified cutoff for cfDNA, we were able to identify malignant lesions with a sensitivity of 100% and specificity of 92.3%. There was a very strong agreement between cfDNA-based classification with histopathology-based classification of benign and malignant nodules (Cohen's kappa 0.94; $P < 0.001$)

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Conclusion: Plasma cfDNA estimation could help differentiate malignant from benign thyroid nodules.

Key Words: thyroid nodule, cell-free DNA, indeterminate nodule, differentiated thyroid cancer

Thyroid malignancy is the most common endocrine malignancy, which usually presents as a solitary thyroid nodule. Studies have suggested that 5% to 15% of all thyroid nodules evaluated are malignant (1-3). The current protocol for evaluation of thyroid nodules includes initial thyroid function test, ultrasonography (USG) of the thyroid gland, followed by USG-guided fine needle aspiration (FNA) cytology (FNAC) in those for whom it is indicated (4). USG results are reported according to the American College of Radiology (ACR) Thyroid Imaging, Reporting, and Data System (TIRADS) classification guidelines (4). This helps in stratifying risk of malignancy in thyroid nodules and in selecting patients for FNAC. The most widely used reporting system for thyroid FNAC is the Bethesda system for reporting thyroid cytopathology (5). Cytopathology results are categorized as Bethesda categories 1 to 6. Results of cytopathology as categorized by Bethesda has been proven to be highly reliable in categories 2 (benign) and categories 5 and 6 in decision making for surgery. Categories 3 and 4 are indeterminate lesions in which we need to exclude the possibility of malignancy. Current management protocol warrants a surgical biopsy to establish the nature of the lesion (benign or malignant) in this category (6).

This indeterminate category presents a management dilemma. It would be useful to develop tools and markers to predict nature of these (indeterminate) lesions, so that unnecessary surgery could be avoided in many patients, and even if surgical intervention is needed, the extent/nature of surgery could be preplanned appropriately and repeat surgical interventions could be avoided.

Several molecular techniques, including determination of the presence of mutations from FNA samples from thyroid nodules, or even from circulating cell-free DNA (cfDNA), have been explored to help in diagnosis of indeterminate thyroid nodules.

In malignancies of different organs/systems, circulating cfDNA represents short fragments of DNA released from tumors that may contain tumor-specific somatic mutations and circulate in plasma, serum, and other body fluids, including urine, saliva, breast milk, and spinal and amniotic fluid (7-9). The quantity of cfDNA in circulation varies according to the nature of neoplasm (10). It has been observed that patients with benign lesions or with early-stage cancer may have lower amounts of cfDNA compared with patients with advanced or metastatic tumors of comparable size. These findings suggest that the level of cfDNA shed by tumors differ in various stages of cancer (11). Following surgery, the levels of cfDNA in cancer patients with localized disease may decrease to levels that are observed in healthy individuals, reconfirming that the possible source of the raised cfDNA was indeed the malignant lesion (12).

The utility of cfDNA in malignancy could be manifold. It can be used for detection of specific somatic mutations, evaluation of integrity, or estimation of total cfDNA. Previous studies in thyroid nodules have demonstrated/detected individual somatic mutations like *BRAF* V600E from cfDNA (11). Multiple driver mutations are responsible for development of differentiated thyroid cancer and detection of all these mutations from cfDNA in all suspicious or indeterminate lesions could miss hitherto unknown mutations and be laborious and expensive. Hence, total cfDNA concentration could be a viable possible alternative marker to evaluate thyroid nodules for suspected differentiated thyroid cancer, especially in indeterminate lesions. Cell-free DNA has been evaluated as a biomarker in pancreatic, colon, and breast cancer previously (13). In our study, we have undertaken quantitative estimation of the concentration of cfDNA rather than any molecular DNA assessment of mutation. To the best of our knowledge, measurement of total cfDNA has not been evaluated comprehensively as a marker to help differentiate benign from malignant thyroid nodules. This could especially be of clinical relevance in patients presenting with indeterminate nodules.

Materials and Methods

This was a single-center prospective observational study, approved by Institutional Ethics Committee, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India. Consecutive patients presenting to Endocrinology Outpatient Department (OPD) with a clinically solitary thyroid nodule were recruited. The patients underwent a thyroid function test (free thyroxine [T4] and thyrotropin [TSH]). Serum TSH and free T4 were estimated by chemiluminescence immunoassay using commercially available kits from Siemens Diagnostics (Germany) with Immulite-1000 analyzer. The analytical sensitivity and total precision values (as given by the providers) for TSH were 0.01 µIU/mL and 2.2%, respectively, and for free T4 assays they were 0.35 ng/dL and 2.7%, respectively. The laboratory reference range for TSH was 0.4 to 4 µIU/mL, and that for free T4 was 0.8 to 1.9 ng/dL,

and the inter-assay coefficients of variation for the assays were 8.9% and 5.5%, respectively (as determined locally).

USG was performed by 1 of 2 dedicated radiologists and reported according to the American College of Radiology TIRADS (2017) classification. USG-guided FNAC was done from all patients with thyroid nodules and cytological examination was performed and reported as per Bethesda classification. Histopathology was reported according to the latest World Health Organization guidelines for classification of endocrine tumors (14).

Patients who declined consent for inclusion in the study, those with hyperthyroidism (by thyroid function test), those who had multinodular disease on USG (patients with >1 nodule >1cm), and those who had nondiagnostic report on FNA (Bethesda 1) were excluded from the study. Patients with anaplastic cancer and medullary thyroid cancer were not included in final analysis. Patients with past/current history of any known malignancies were also excluded.

Peripheral blood (5 mL) was collected in EDTA tubes from all subjects. Plasma was separated by centrifugation at 4 °C at 3000*g* for 15 minutes. Plasma was isolated and stored at –80 °C. Cell-free DNA extraction from plasma was performed using ZYMO DNA kit (Quick-cfDNA Serum & Plasma Kit Cat No: D-4076) according to the protocol provided by the manufacturer. For extraction of cfDNA, 200 µL of plasma was taken and eluted in 30 µL elution buffer for each sample. Quantification and purity of the isolated cfDNA was determined in duplicate by measuring absorbance at 260 nm and 280 nm using a BioSpectrometer (Eppendorf BioSpectrometer basic). The cfDNA concentration was also measured using a Qubit fluorometer (Qubit 4 Fluorometer Invitrogen by Thermo Fisher Scientific) in some cases, for cross-validation of results obtained using BioSpectrometer. Concentration of cfDNA was recorded in ng/mL. For determination of purity, a ratio of absorbance at 260 nm and 280 nm was ~1.8. All samples in our study satisfying this criterion were included.

Surgery was performed in all cases suspicious of malignancy (Bethesda 5, 6), indeterminate nodules (Bethesda 3, 4) and selected cases of patients with Bethesda 2 who had progressively increasing size, compressive symptoms, cosmetic concerns, or as per patient preference. All patients with Bethesda 2 lesions who did not undergo surgery (clinically not indicated) were presumed to have histological diagnosis of benign lesion. For all patients undergoing surgery, histopathology was considered as gold standard for diagnosis.

A blood sample was also collected 2 weeks after surgery in subjects who were diagnosed to have a differentiated thyroid cancer and cfDNA was re-estimated in them.

In the first phase of analysis (determination of cutoff), we included patients who had Bethesda 2, 5, and 6 (ie, those with benign and malignant lesions as suggested by FNA and thereafter confirmed by histopathology following surgery [for benign lesions see above]).

We performed a receiver operating characteristics curve (ROC) to help obtain a suitable cutoff of cfDNA levels to help differentiate between benign and malignant lesions.

In the second phase of analysis (validation of cutoff) we utilized the cutoff obtained from the analysis phase and applied it to subjects with an FNA diagnosis of Bethesda 3, 4 (indeterminate lesions) to test the predictive value of the cutoff in differentiating benign from malignant lesions.

Statistical Analysis

The data were tested for normality using the Kolmogorov–Smirnov test. Categorical variables are expressed as frequencies and percentages. In the case of a normal distribution, continuous data are presented as mean value ± standard deviation. For nonparametric comparisons, Mann-Whitney U tests of 2 independent variables were performed. The cutoff for cfDNA concentration for differentiating malignant from benign nodule was calculated by the ROC curve.

Based on the cutoff for cfDNA, indeterminate nodules were classified as benign and malignant during the validation phase. Sensitivity and specificity of that cutoff for indeterminate nodules were calculated using histopathology-based classification as gold standard. Cohen's kappa (k) was also applied for assessing the agreement between cfDNA-based classification and the histopathology-based classification in the categorical format.

Sample size was calculated using Medcalc (15) software 19.5.6 for the first phase of the study (determination of cutoff) with the assumptions of beta of 0.2 and ratio of sample sizes in benign: malignant groups to be 20 and area under curve (AUC) of 0.95. With the mentioned assumptions the number of cases needed was calculated to be a minimum of 63 cases. Medcalc (15) software 19.5.6 was also used for calculation of sample size for the second phase of study (validation of cutoff) with assumptions of beta of 0.2 and assumption that 25% of all indeterminate nodules are likely to be malignant. With the mentioned assumptions, the number of cases needed was calculated to be a minimum of 29 cases.

All statistical analyses were performed using SPSS software (version 23; SPSS, Inc. Chicago, IL, USA).

Results

The study design, workflow, and results are illustrated in Fig. 1. The total number of patients presenting with a clinically solitary thyroid nodule evaluated for possible recruitment in the study was 151 patients; 3 were hyperthyroid, 11 patients had significant multinodularity, 10 patients declined to enroll for the study, and 8 patients yielded nondiagnostic

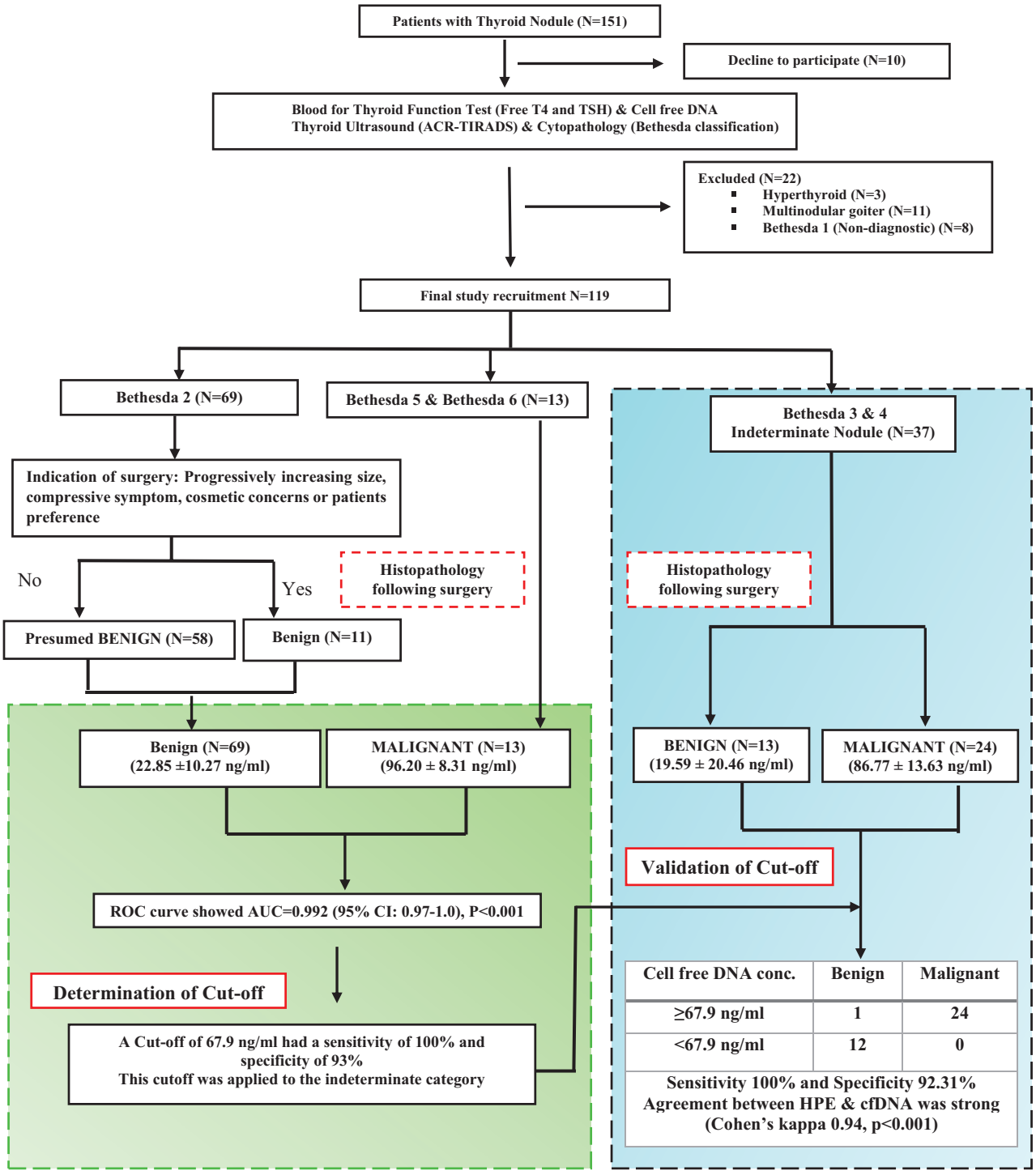

Figure 1. Flowchart illustrating study design, workflow, and results.

(Bethesda 1) results on FNAC. Thus, a total of 119 patients were included in the study. The basic demographic profile and evaluation of subjects (ultrasound and USG-guided thyroid FNA) results are summarized in Table 1.

Determination of Cutoff

Cell-free DNA (cfDNA) concentration was estimated in all subjects at baseline (before surgery). In the first phase of analysis (determination of cutoff phase) we observed that the mean ± SD of cfDNA concentration for the benign group was 22.85 ± 10.27 ng/mL and for the malignant group was 96.20 ± 8.31 ng/mL. The difference was statistically significant ($P < 0.001$). Ratio of absorbance at 260 nm and 280 nm was around 1.8. Cell-free DNA concentration when measured by using the Qubit fluorometer was comparable and consistent with the findings using the Nano drop spectrophotometer. Using the Qubit fluorometer,

Table 1. Demographic, Clinical, Radiological, Cytopathological,and Histopathological Findings of Solitary Thyroid Nodule

Profile of subjects with solitary thyroid nodule (N = 119)			
Age, mean ± SD, years	34.32 ± 11.25		
Gender (male/ female)	26/93		
Free T4, mean ± SD, ng/ dL	1.33 ± 0.40		
TSH, mean ± SD, mIU/L	2.72 ± 0.87		
Radiological findings of solitary thyroid nodule			
TIRADS score	TIRADS 1 (benign), no. (%)	No FNA	
	TIRADS 2 (not suspicious), no. (%)	2 (1.68%)	
	TIRADS 3 (mildly suspicious), no. (%)	48 (40.33%)	
	TIRADS 4 (moderately suspicious), no. (%)	65 (54.62%)	
	TIRADS 5 (highly suspicious), no. (%)	4 (3.36%)	
Mean size of nodule (based on USG) (N = 119)	3.1 cm		
Cytopathological and histopathological findings of solitary thyroid nodule			
Cytological findings	FNA categories	Histopathological findings	cfDNA concentration
Bethesda 1 (nondiagnostic) (N = 8)	Nondiagnostic (N = 8)	Excluded (repeat FNAC suggested and follow-up)	-
Bethesda 2 (benign) (N = 69)	Benign (N = 69)	Biopsy confirmed (N = 11) FNAC (presumed benign) (N = 58)	22.85 ± 10.27 ng/mL
Bethesda 3 (AUS/FLUS) (N = 17)	Indeterminate nodule (N = 37)	Benign (N = 13)	28.58 ± 14.47 ng/mL
Bethesda 4 (follicular neoplasm) (N = 20)		Malignant (N = 24) (16 papillary thyroid carcinoma, 3 follicular variant papillary carcinoma, 4 follicular carcinoma and 1 papillary micro-carcinoma)	84.02 ± 13.35 ng/mL
Bethesda 5 (suspicious for malignancy) (N = 11)	Malignant (N = 13)	Malignant (N = 13) (papillary thyroid carcinoma)	96.20 ± 8.31 ng/mL
Bethesda 6 (malignant) (N = 2)			
Mean size of malignant tumor (max diameter of tumor based on histopathology) (N = 37)	3.8 cm		

Abbreviations: AUS/FLUS, atypia of undetermined significance / follicular lesion of undetermined significance; FNAC, fine needle aspiration cytology; T4, thyroxine; TIRADS, Thyroid Imaging, Reporting, and Data System; TSH, thyrotropin (thyroid-stimulating hormone); USG, ultrasonography;

the cfDNA concentration for the benign group (n = 42) was 25.89 ± 9.27 ng/mL and the malignant group (n = 13) was 93.20 ± 7.39 ng/mL.

The ROC curve obtained by utilizing this data was statistically significant in differentiating malignant and benign thyroid nodules with AUC of 0.992 (95% CI, 0.97- 1.0; $P < 0.001$). A cfDNA concentration of 67.9ng/mL had a sensitivity of 100% and a specificity of 93% for detecting malignant lesions. This cutoff has a positive predictive value (PPV) of 96% and a negative predictive value (NPV) of 100%.

Validation of Cutoff

In the second phase of analysis (validation phase), we utilized the cutoff obtained from the first phase (determination of cutoff phase) in the patients with indeterminate lesions (Bethesda categories 3 and 4) to predict the nature of lesion

(benign or malignant, as established by histopathology following thyroid surgery). In our study, 37 indeterminate nodules were identified on cytology (Bethesda categories 3 and 4). Of these, 13 were proven to be benign on histopathological examination and 24 were found to be malignant (differentiated) thyroid cancer. On applying this cfDNA concentration of 67.9 ng/mL as a cutoff, all 24 malignant were identified and 12/13 benign nodules were identified. Thus, a cfDNA concentration of 67.9 ng/mL to diagnose thyroid cancer had a sensitivity of 100% and specificity of 92.3%. There was a very strong agreement between cfDNA-based classification with histopathology-based classification of benign and malignant nodules (Cohen's kappa 0.94; $P < 0.001$). Using this cutoff, all patients with thyroid cancer were correctly detected among the indeterminate category of nodules.

Comparison of cfDNA Concentrations Before and After Surgery in Patients With Thyroid Cancer

Those patients who underwent surgery and had a histopathological diagnosis of thyroid cancer were reevaluated after surgery. The cfDNA was re-estimated 2 weeks after surgery. The mean cfDNA values were 19.59 ± 20.46 ng/mL 2 weeks after surgery compared with 86.77 ± 13.63 ng/mL before surgery ($P < 0.001$). This result suggests that the source of the preoperatively elevated cfDNA in the cases of thyroid malignancy was the malignant thyroid tumor.

Detection of Mutation From cfDNA in Subjects Detected With Malignancy

We had preserved the cfDNA of all subjects included in the study. Following histopathology, 37 patients in the entire cohort were diagnosed to have differentiated thyroid cancer. In these patients, we undertook evaluation of known driver mutations from the cfDNA. In 31 out of 37 patients known driver mutations were detected (including *BRAF*, *NRAS*, *KRAS*, *HRAS*, *RET-PTC3*, *TERT*, *RET-PTC1*, *PAX8-PPARϒ*). We reconfirmed the presence of these driver mutations additionally from FNA material.

The detection of these driver mutations in the cfDNA, along with normalization of cfDNA levels following surgery in patients with differentiated thyroid cancer, strongly suggests that the source of raised cfDNA in patients with cancer is indeed the malignant thyroid tumor.

cfDNA Levels and Association With Histopathological Features and Staging in Patients With Differentiated Thyroid Cancer

We analyzed cfDNA levels in patients with differentiated thyroid cancer to explore any possible differences in cfDNA levels based on patient age, gender, tumor size, multifocality of lesion, lymph node involvement, capsular invasion, lymphovascular invasion, extra-thyroidal invasion, and TNM staging of the tumor. The results are summarized in Table 2.

Table 2. Relationship Between Cell-Free DNA Concentrations With Histopathological Parameters of Thyroid Cancer

Variables (N = 37)		Cell-free DNA Concentration (ng/ml)	*P* value
		Mean ± SD	
Age	≤30 (N = 20)	84.41 ± 13.35	0.251
	>30 (N = 17)	89.58 ± 13.85	
Gender	Female (N = 30)	87.37 ± 13.02	0.624
	Male (N = 7)	82.25 ± 19.06	
Multifocality	Yes (N = 20)	87.96 ± 14.67	0.245
	No (N = 17)	82.73 ± 7.34	
Lymph node	Present (N = 10)	96.54 ± 12.03	0.005
	Absent (N = 27)	82.70 ± 12.29	
Lymphovascular invasion	Present (N = 11)	97.09 ± 10.99	<0.001
	Absent (N = 26)	81.83 ± 12.04	
Capsular invasion	Present (N = 9)	98.54 ± 10.84	<0.001
	Absent (N = 28)	82.53 ± 12.06	
Extra-thyroidal Extension	Present (N = 5)	104.54 ± 3.34	<0.001
	Absent (N = 32)	82.56 ± 10.43	
Tumor size	≤2.0 (N = 6)	76.53 ± 14.50	0.060
	>2.0 (N = 31)	88.97 ± 12.64	
pTNM staging	I (N = 8)	69.72 ± 5.77	0.005[a]
	II (N = 20)	87.59 ± 12.43	
	III (N = 9)	94.44 ± 11.52	

All statistical analysis was done using Mann-Whitney-U test.
[a]Analysis was done using Kruskal-Wallis tests.

Discussion

Diagnostic and management dilemma arises in indeterminate nodules (Bethesda 3 and 4). It has been suggested that various molecular techniques (with variable sensitivity and specificity) have been utilized to predict possibilities of malignancies in these nodules (6). All of the currently available molecular methods focus on somatic mutation identification from FNA sample from nodules obtained under ultrasound guidance (16).

Thyroseq version 3 is a DNA and RNA-based next-generation sequencing assay that analyses 112 genes for a variety of genetic alterations, including point mutations, gene fusions, copy number alterations, and abnormal gene expression and it uses a Genomic Classifier (GC) to differentiate malignant from benign lesions. For establishing the cutoff for GC, a training set of 238 thyroid tissues was

used, which showed a sensitivity of 93.9% and a specificity of 89.4%. On validation of these finding in 175 FNA samples, the GC showed sensitivity of 98.0% and specificity of 81.8% (17).

The Afirma gene expression classifier (18) is an upgrade to the gene expression classifier that utilizes next-generation RNA sequencing to identify specific oncogenes responsible for thyroid cancer. The current generation of Afirma has a sensitivity of 100% and specificity of 94% in Bethesda 3 and 100% sensitivity and 86% specificity in Bethesda 4 in identifying benign gene expression from FNA samples in thyroid nodules.

Although very sensitive, both of these commercially available tests are expensive, logistically difficult, and may not be feasible in a resource-poor setting. In addition, these tests entail the requirement of collection of FNA samples (possibly even repeat FNA) for isolation of genetic material, which is an invasive and operator-dependent process that might give rise to inaccurate results.

Previous studies have suggested that cfDNA may predict neoplastic disease by increased cfDNA concentration or identification of somatic driver mutations from cfDNA of cancer patients (19, 20). Schwarzenbach et al (8) observed cfDNA in 55 patients with advanced colorectal cancer compared with 14 healthy individuals. The cfDNA was extracted from plasma using specific kits and quantification and the quality of the isolated DNA was spectrophotometrically determined at 260 and 280 nm, on a NanoDrop photometer. The mean cfDNA content in the malignant group was 1157 ng/mL and among healthy individuals it was 8 ng/mL. Chun et al (21) followed a similar methodology to determine cfDNA level in patients with prostate cancer and benign hypertrophy of prostate. The median concentration of cfDNA in the cancer group was 709 ng/mL and in the benign group was 267 ng/mL.

Kim et al (22) measured cfDNA concentrations in plasma samples of patients with breast cancer and found that it was significantly higher in breast cancer patients compared with benign breast disease. Findings from Seyedolmohadessin et al (23) indicated significant differences between cfDNA levels of patients with localized and metastatic prostate cancer.

Salvianti et al (24) used a quantitative real-time PCR approach based on the quantification of 2 amplicons of different length (67 and 180 bp, respectively) to evaluate the integrity index 180/67 from plasma cfDNA. Cell-free DNA integrity was highest in those with thyroid malignancy, lower in those with benign disease, and the least in a control group who had no thyroid illness. In their study, 2 major groups for comparison were Thy2 (nonneoplastic) and Thy4 and Thy5 (suspicious of malignancy and diagnostic of malignancy), according to 2007 cytology guidance from the British Thyroid Association (25). However, there was no validation of their findings in the indeterminate category of nodules cytology Thy3 (follicular lesions). In our study, we have established the significant difference between the benign and malignant category of thyroid nodules by quantifying cfDNA. We have also validated these findings in the indeterminate category.

Thakur et al (26) reported cell-free DNA integrity using ALU 115/247 primer and quantitative PCR. They evaluated 67 patients with 100 nodules, all belonging to AUS category (Bethesda 3). On surgery and histopathological examination, 38 patients had malignancy and 29 had a benign diagnosis. Integrity of cell-free DNA (determined by ALU segments 115 and 247) was evaluated in all patients prior to surgery. They noted that there was no significant difference between individuals with benign and malignant categories.

In our study, almost 65% of patients with indeterminate nodules turned out to have malignant disease. This was higher than that reported in most studies. Most studies suggest that the risk of malignancy in indeterminate nodules varies from 10% to 30% for Bethesda 3 and 25% to 40% For Bethesda 4 (5). We believe this high prevalence of malignancy could be a chance finding. The study of Thakur et al however reported a risk of malignancy of 58%, demonstrating the variability in prevalence of malignancy, possibly due to small sample size.

Pupilli et al (11) performed a study in which they recruited patients with different ultrasound-guided FNA cytology. They prospectively detected the proportion of wild-type and mutant and *BRAF* (V600E) in plasma cfDNA and observed a statistically significant elevated proportion of circulating mutant allele over wild-type allele in patients with Thy3 (follicular lesions) (18.7%) and Thy4 and Thy5 (suspicious of malignancy and diagnostic of malignancy) (27.1%) cytology (2007 British Thyroid Association guidance) (25) compared with healthy individuals (1.7%).

In our study, we measured cfDNA concentration in patients with thyroid nodules using a BioSpectrometer. It was selected based on its cost effectiveness and efficiency. We also validated our cfDNA concentration by comparing with that obtained using the Qubit fluorometer, and the results were comparable as mentioned in "Results."

The cfDNA level in the benign group was 22.85 ± 10.27 ng/mL, which was significantly ($P < 0.001$) lower than in the malignant group (96.20 ± 8.31 ng/mL). An ROC curve was made for determination of cutoff for distinguishing malignant from benign nodules. We selected a cutoff 67.9 ng/mL, as at this value the sensitivity was 100%, with a specificity of 92.8% for detecting malignant lesions. We validated this cutoff in the indeterminate category of patients with histopathology serving as the gold standard. The cfDNA concentration had

a strong agreement with the histopathological diagnosis and a cutoff of 67.9 ng/mL had 100% sensitivity and 92.3% specificity in differentiating malignant from benign lesion in the indeterminate category.

Among patients with differentiated thyroid cancer, a higher cell-free DNA concentration was also significantly associated with high-risk features of malignancy, such as the presence of lymph node, lympho-vascular, or capsular invasion, extra-thyroidal extension, and a higher tumor stage. Hence, a preoperative higher cfDNA may help in planning the extent of surgery in advance preventing the requirement of a repeat surgery.

The detection of driver mutations in cfDNA, along with normalization of cfDNA levels following surgery in patients with differentiated thyroid cancer strongly suggests that the source of raised cfDNA in patients with cancer is indeed the malignant thyroid tumor.

Similar decreases in cell-free DNA were also noted by Pupilli et al (11), who showed a significant decrease in circulating *BRAF* V600E percentage in the postsurgical blood draw (6.5 ± 3.7%) compared with presurgical samples (43.2 ± 8.9%) ($P < 0.001$).

In patients with locally advanced rectal cancer, the cfDNA was measured before treatment and during follow-up after adjuvant chemo-radiotherapy and surgery. Initial high cfDNA predicted a higher risk of recurrence and a shorter time to recurrence (27).

Tie et al (28) showed that, among colon cancer patients treated with chemotherapy, the presence of cfDNA after completion of chemotherapy was associated with an inferior recurrence-free survival (hazard ratio 11; 95% CI, 1.8-68; $P = 0.001$). Cell-free DNA detection after stage II colon cancer resection provides direct evidence of residual disease and identifies patients at very high risk of recurrence.

Patients with differentiated thyroid cancer traditionally have been treated with radioiodine (iodine-131), according to risk categorization, and are followed up by serial measurements of serum thyroglobulin levels with imaging including neck ultrasound or iodine-131 scan when indicated. Serum thyroglobulin has been used as a prognostic marker, as well as a marker for detection of residual disease and disease recurrence. However, a serum thyroglobulin measurement has certain limitations including possibilities of interference with presence of anti-thyroglobulin antibodies. Additionally, there are issues related to measurement of thyroglobulin, whether to be tested while on levothyroxine replacement, or after stimulation by recombinant TSH or whether after temporarily stopping levothyroxine replacement, which is inconvenient for the patient. Further studies on estimation of cfDNA may serve as an alternate marker for prognostication, detection of residual disease, or early detection of disease recurrence.

Our data supports the concept that cfDNA could serve as an accurate, efficient, and inexpensive biomarker for identifying malignancy in the indeterminate category of thyroid nodules. The number of patients in our study in the malignant group is much lower than the benign group in our study which is in keeping with population prevalence. We validated our cell-free DNA data in a Qubit fluorometer and cross-validated the results in a proportion of our patients using real-time PCR.

Thyroseq and/or Afirma remain the current gold standard for evaluation of indeterminate thyroid nodules. Our study is essentially a proof-of-concept study which explores the possibility of a newer alternative that needs to be validated in a much larger and diverse population to be considered for clinical usage.

Conclusion

Plasma cell-free DNA estimation could be a useful method for prediction of malignancy in patients presenting with thyroid nodules.

Acknowledgments

Financial Support: Endocrine Society of Bengal, ESB/7/2018, granted to Susmita Dutta. Endocrine Society of India, ESI/5/2019, granted to Soham Tarafdar.

Additional Information

Correspondence: Sujoy Ghosh, MD, DM, FRCP, Department of Endocrinology and Metabolism, Institute of Post-Graduate Medical Education and Research and Seth Sukhlal Karnani Memorial Hospital, 244 A J C Bose Road, 4th Floor, Ronald Ross Building, Kolkata 700020, India. Email: drsujoyghosh2000@gmail.com.

Disclosures: None declared.

Data Availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Mazzaferri EL, de los Santos ET, Rofagha-Keyhani S. Solitary thyroid nodule: diagnosis and management. *Med Clin North Am*. 1988;**72**(5):1177-1211.
2. Cooper DS, Doherty GM, Haugen BR, et al.; American Thyroid Association Guidelines Taskforce. Management guidelines for patients with thyroid nodules and differentiated thyroid cancer. *Thyroid*. 2006;**16**(2):109-142.
3. Frates MC, Benson CB, Doubilet PM, et al. Prevalence and distribution of carcinoma in patients with solitary and multiple thyroid nodules on sonography. *J Clin Endocrinol Metab*. 2006;**91**(9):3411-3417.
4. Barbosa TLM, Junior COM, Graf H, et al. ACR TI-RADS and ATA US scores are helpful for the management of thyroid

nodules with indeterminate cytology. *BMC Endocr Disord*. 2019;**19**(1):112.
5. Cibas ES, Ali SZ. The 2017 bethesda system for reporting thyroid cytopathology. *J Am Soc Cytopathol*. 2017;**6**(6):217-222.
6. Nikiforov YE, Ohori NP, Hodak SP, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab*. 2011;**96**(11):3390-3397.
7. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;**11**(6):426-437.
8. Schwarzenbach H, Stoehlmacher J, Pantel K, Goekkurt E. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. *Ann N Y Acad Sci*. 2008;**1137**:190-196.
9. Song Q, Zhang Y, Liu H, Du Y. Potential of using cell-free DNA and miRNA in breast milk to screen early breast cancer. *Biomed Res Int*. 2020;**2020**:1-11.
10. Corcoran R, Chabner B. Application of cell-free DNA analysis to cancer treatment. *Obstet Gynecol Surv*. 2019;**74**:204-206.
11. Pupilli C, Pinzani P, Salvianti F, et al. CirculatingBRAFV600Ein the diagnosis and follow-up of differentiated papillary thyroid carcinoma. *J Clin Endocrinol Metab*. 2013;**98**:3359-3365.
12. Catarino R, Ferreira MM, Rodrigues H, et al. Quantification of free circulating tumor DNA as a diagnostic marker for breast cancer. *DNA Cell Biol*. 2008;**27**(8):415-421.
13. Bettegowda C, Sausen M, Leary R, et al. Detection of circulating tumor DNA in early and late stage human malignancies. *Neuro Oncol*. 2014;**16**(l3):iii7.
14. Lloyd RV, Osamura RY, Klöppel G, Rosai J (editors). *WHO Classification of Tumours of Endocrine Organs*. 4th edn. Lyon, France: IARC; 2017.
15. MedCalc® Statistical Software version 19.5.6 (MedCalc Software Ltd, Ostend, Belgium; 2020. https://www.medcalc.org. Accessed November 12, 2020.
16. Macerola E, Basolo F. Current methodologies for molecular screening of thyroid nodules. *Gland Surg*. 2018;**7**(Suppl 1):S1-S7.
17. Nikiforova MN, Mercurio S, Wald AI, et al. Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. *Cancer*. 2018;**124**(8):1682-1690.
18. Chudova D, Wilde JI, Wang ET, et al. Molecular classification of thyroid nodules using high-dimensionality genomic data. *J Clin Endocrinol Metab*. 2010;**95**(12):5296-5304.
19. Swarup V, Rajeswari MR. Circulating (cell-free) nucleic acids–a promising, non-invasive tool for early detection of several human diseases. *FEBS Lett*. 2007;**581**(5):795-799.
20. van der Vaart M, Pretorius PJ. Is the role of circulating DNA as a biomarker of cancer being prematurely overrated? *Clin Biochem*. 2010;**43**(1-2):26-36.
21. Chun FK, Müller I, Lange I, et al. Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. *BJU Int*. 2006;**98**(3):544-548.
22. Kim S, Sim U, Ri C, et al. Analytical study on relationship between cell-free plasma DNA concentration and clinicopathological features in breast cancer. *Arch Intern Med Res*. 2020;**03**(01). Doi: 10.26502/aimr.0020
23. Seyedolmohadessin SM, Akbari MT, Nourmohammadi Z, Basiri A, Pourmand G. Assessing the diagnostic value of plasma-free DNA in prostate cancer screening. *Iran Biomed J*. 2018;**22**(5):331-337.
24. Salvianti F, Giuliani C, Petrone L, et al. Integrity and quantity of total cell-free DNA in the diagnosis of thyroid cancer: correlation with cytological classification. *Int J Mol Sci*. 2017;**18**(7):1350.
25. British Thyroid Association, Royal College of Physicians. Diagnostic categories. *Guidelines for the Management of Thyroid Cancer*. 2nd ed. Suffolk, UK: Lavenham Press; 2007:10.
26. Thakur S, Tobey A, Daley B, et al. Limited utility of circulating cell-free DNA integrity as a diagnostic tool for differentiating between malignant and benign thyroid nodules with indeterminate cytology (Bethesda Category III). *Front Oncol*. 2017;**9**:905.
27. Schou JV, Larsen FO, Sørensen BS, et al. Circulating cell-free DNA as predictor of treatment failure after neoadjuvant chemoradiotherapy before surgery in patients with locally advanced rectal cancer. *Ann Oncol*. 2018;**29**(3):610-615.
28. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci Transl Med*. 2016;**8**(346):346ra92.

Original Article

Comparison between Sonographic Features and Fine Needle Aspiration Cytology with Histopathology in the Diagnosis of Solitary Thyroid Nodule

Debanu De*, Susmita Dutta*, Soham Tarafdar[1], Suvrendu S. Kar[3], Utpalendu Das, Keya Basu[2], Pradip Mukhopadhyay[1], Sujoy Ghosh[1]

Departments of Radiodiagnosis, [1]Endocrinology and [2]Pathology, IPGME&R, [3]Department of Medicine, R.G. KAR Medical College, Kolkata, West Bengal, India

*These authors have contributed equally to this work

Abstract

Background: High resolution ultrasonography (USG) is the first-line investigation in evaluation of euthyroid nodules. Thyroid imaging reporting and data system (TIRADS) is an USG-based risk stratification system for classifying thyroid nodules. Subjects with high-risk category of TIRADS undergo fine needle aspiration cytology (FNAC) and FNAC findings are reported according to Bethesda classification. Bethesda categories are used for determining risk of malignancy. Data regarding sonographic classification of thyroid nodule and its cytological association with respect to final histopathological diagnosis remains scarcely available in India. **Aims and Objective:** The study evaluated euthyroid nodules for risk of malignancy and compared sonographic features and FNAC (Bethesda classification) findings with histopathology of excised samples. **Material and Methods:** This was a single-center observational study on 137 consecutive subjects of solitary euthyroid nodule. All subjects underwent USG according to TIRADS and FNAC where applicable. Surgical biopsy report was used as a gold standard. **Results:** The sensitivity, specificity, accuracy, positive predictive and negative predictive value of FNAC were 80%, 90%, 85%, 86%, and 86.6% and TIRADS were 80%, 47.2% 61%, 51.3%, and 77.3%, respectively. FNAC classification was equally sensitive and more specific than TIRADS. Among individual USG parameters, micro-calcification was most sensitive (80%) and specific (86%). Irregular margin and taller-than-wider shape had a specificity of 89% and 92%, respectively. 3 patients (14.28%) with benign cytology and suspicious USG features (specifically TIRADS 4 & 5) undergoing surgery had malignancy in final HPE. **Conclusions:** USG and FNAC are equally sensitive in diagnosing malignant thyroid nodule but FNA is more specific (90%). It's a minimally invasive method which can be used to distinguish malignant from benign lesions with a high degree of accuracy (85%). In patient having high risk feature on USG, a benign cytology needs to be repeat FNAC and they should undergo surgical biopsy for confirmation.

Keywords: Fine needle aspiration cytology, histopathology, thyroid swelling, TIRADS

Introduction

Thyroid nodule is defined as a focal well-defined area of altered echogenicity within thyroid gland that is radiologically distinct from surrounding normal thyroid parenchyma.[1] Worldwide, thyroid nodule occurs with relatively high frequency in general population with an estimated prevalence of 4–8% by palpation alone and 19–67% by ultrasound examination. In India, thyroid nodules are seen in about 8.5% of the population.[2] An increase in the incidence of thyroid carcinoma has been noted in the recent decades due to markedly improved USG surveillance and USG-guided FNAC of thyroid nodules.

Majority of thyroid nodules are benign, but malignancy is found in approximately 5–15% of cases, high risk features like age, sex, radiation exposure history, family history, and

Access this article online

Quick Response Code:

Website: www.ijem.in

DOI: 10.4103/ijem.IJEM_349_20

Address for correspondence: Dr. Sujoy Ghosh, Department of Endocrinology, IPGME&R, Kolkata - 700 020, West Bengal, India.
E-mail: drsujoyghosh2000@gmail.com

Submitted: 08-Jun-2020 **Revised:** 26-Jun-2020
Accepted: 11-Jul-2020 **Published:** 27-Aug-2020

How to cite this article: De D, Dutta S, Tarafdar S, Kar SS, Das U, Basu K, *et al*. Comparison between sonographic features and fine needle aspiration cytology with histopathology in the diagnosis of solitary thyroid nodule. Indian J Endocr Metab 2020;24:349-54.

other factors warrants further evaluation.[3-5] Epidemiological studies have shown that thyroid nodules are more common in women. Despite their low prevalence in men, nodules are more aggressive with higher risk of malignancy.[6]

Evaluation of a patient with thyroid nodule requires detailed history and imaging. High resolution ultrasonography (USG) is the first line investigations in clinically detected thyroid nodules who are biochemically euthyroid. Thyroid imaging recording and data system (TIRADS) is a risk stratification system for classifying thyroid nodules similar to BIRADS scoring for breast lesions. It was first proposed by Horvath *et al*.[7] in the year of 2009 with modified recommendation from Kwak JY *et al*.[8] Recently, thyroid nodules have been classified into 5 TIRADS categories based on 5 descriptors (composition, echogenicity, shape, margin, echogenic foci/calcification).Each descriptor gives a point, adding all points of all descriptors a numerical value is calculated which gives the TIRADS score. Sonographic findings suggestive of malignancy are solid nodules, hypoechogenicity, irregular margins, microcalcifications, and a shape taller than wide on a transverse view.

Fine needle aspiration cytology (FNAC) is considered as an essential tool in providing a rational approach to the clinical management of thyroid nodules and determines the correct surgical procedure when surgery is needed. Similar to other clinical tests in medicine, it is expected that thyroid FNA should demonstrate high degree of sensitivity and specificity. Therefore, it is prudent that thyroid FNA reporting should be close to uniform among pathologists to give the path for rational management strategies and avoid confusion among clinicians.[9,10] According to current standards of thyroid cytopathology, Bethesda classification is used for determining which patients should undergo surgery. Thyroid is a superficial structure and thus easily accessible to invasive and noninvasive procedures. USG-guided FNA has been done routinely for proper localization of nodule.

Studies have been done worldwide regarding stratification of risks of malignancy in subjects with thyroid nodule by ultrasound and cytological examination. Kwak *et al*.[8] have proposed a TIRADS score by retrospective analysis of thyroid nodules in ultrasound and FNA, using five ultrasound criteria that can be used during thyroid evaluation. This article describes that a malignancy risk of[11] 0% is expected for TIRADS 2, 1.7% for TIRADS 3, a risk of 3.3–72.4% for TIRADS 4, and of 87.5% for TIRADS 5.Srinivas *et al*.[12] also concluded that the risk of malignancy for TIRADS categories1, 2, 3, 4A, 4B, 4C, and 5 was 0, 0, 0.64, 4.76, 66.67, 83.33, and 100%, respectively. But still data regarding sonographic classification of thyroid nodule and its cytological correlation remains scarcely available in India.

In this background, the aim of this study was to compare high resolution USG by TIRADS scoring and cytological diagnosis by Bethesda scoring with histopathological diagnosis in subjects with solitary thyroid nodule. We evaluated all consecutive subjects of solitary thyroid nodules by TIRADS Scoring, and cytopathology (if done) by Bethesda classification and compared the TIRADS and Bethesda classification with final diagnosis as reported by HPE (gold standard) in those who had excisional biopsy done. The sensitivity, specificity, PPV, and NPV of TIRADS and Bethesda scoring in detecting malignancy (confirmed by HPE) were also evaluated.

Materials and Methods

This was a single center observational study in which 150 patients of was recruited of which 137 subjects with thyroid nodules giving informed consent for this study were selected. Demographic and other clinical data were collected from the subjects according to specified protocol and all subjects with clinically detected nodule underwent thyroid function tests.

Serum TSH and free T4 were estimated by chemiluminescence technique (CLIA) using commercially available kits from Siemens Diagnostics (Germany) with Immulite-1000 analyzer. The analytical sensitivity and total precision values (as given by the providers) for TSH were 0.01 μIU/ml and 2.2%, respectively, and for freeT4 assays were 0.35 ng/dl and 2.7%, respectively. The laboratory reference ranges for TSH was 0.4–4 μIU/ml, for free T4 was 0.8–1.9 ng/dl and the inter-assay coefficients of variation (CV) for the assays were 8.9% and 5.5%, respectively (as determined locally).

Those who had normal TSH and free T4 levels were included in the study and they subsequently underwent high resolution USG and USG-guided FNAC. All USG were performed by one of two dedicated persons who were trained in the subject. Ultrasound report was prepared according to the TIRADS Score. TIRADS 1: Benign (No FNA), TIRADS 2: Not Suspicious (No FNA), TIRADS 3: Mildly Suspicious (FNA if ≥2.5 cm and follow-up if ≥1.5 cm), TIRADS 4: Moderately Suspicious (FNA if ≥1.5 cm and follow-up if ≥1 cm), and TIRADS 5: Highly Suspicious (FNA if ≥1 cm and follow-up if ≥0.5 cm). Then USG-guided FNAC done from thyroid nodules if indicated.

Cytopathology reports were prepared according to Bethesda classification as Bethesda 1 (non-diagnostic), Bethesda 2 (Benign), Bethesda 3 (AUS/FLUS), Bethesda 4 (follicular neoplasm), Bethesda 5 (suspicious for malignancy), and Bethesda 6 (malignant). All FNA slides were examined by a single cytopathologist oriented about the protocol and nature of the study.

Of the total 137 subjects with thyroid nodules, 61 underwent surgical biopsy, and hence histopathological examination. Evaluation of all histopathology slides was done by a single pathologist. Out of 61 biopsy proved subjects, 21 with "Bethesda 2" cytology and thus having a benign etiology were also operated due to increasing size, compressive symptoms as described by patient or due to cosmetic

concern.

Written informed consent was taken from all subjects. The study was approved by Institutional Ethics Committee of Institute of Post Graduate Medical Education and Research. Kolkata, West Bengal, India.

Statistical analysis

All the data collected was compiled in MS Excel. Appropriate statistical methods like Fisher exact test and Chi-square test were used. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each of major ultrasound features that highly suggest malignancy (irregular margin, taller than wider shape, presence of micro calcifications and hypoechogenecity). Odds ratio (risk estimates) were calculated and presented using 95% confidence interval (CI) statistic. The risk of malignancy of each TIRADS category and Bethesda category was determined with respect to histopathological reports. All statistical analysis was performed using the software IBM SPSS 20.0 and Graph Pad Prism 8.0.

Results

In this study, 150 subjects of thyroid nodules were recruited of which 137 subjects fulfilled our inclusion criteria. The basic demographic profile of solitary thyroid nodule is shown the [Table 1]. Mean age of subjects is 40.18 ± 13.64 years. Distribution pattern of solitary thyroid nodule in various age groups was 3.64% (5 patients) in age group between 18 and 20 years, 54% cases (i.e., 74 patients) were in between 21 and 40 years group, 24 (17.51%) cases were recorded in age group 41–50, and least in age group 51–60 with 34 cases, that is, 24%. Our study population had a female predominance with 119 (86.6%) female and 18 (13.1%) male subjects.

Table 1 also shows the percentages of subjects in different TIRADS category and percentage of subjects with different FNA finding as per Bethesda classification. TIRADS 3 was the most common category. Subjects with TIRADS categories 3, 4, 5 underwent USG-guided FNAC. Of total 137 subjects, 61 had undergone surgery with 36 subjects having benign pathology and 25 having thyroid malignancy on HPE.

Table 2 shows final histopathological diagnosis in different categories of USG findings as per TIRADS. The risk malignancy increased with the TIRADS categories 3–5.The incidence of malignancy in solitary thyroid nodule was more in females. Out of 25 malignant patients 8 were males (32%) and 17 were females (68%).

Preoperative investigation of TIRADS score with histopathological findings is shown in Table 3. Combining TIRADS 4 and 5 (Moderately and highly suspicious lesion) as probably malignant US findings, and TIRADS 3 as probably benign US findings and the sensitivity, specificity, PPV, and NPV were respectively 80%, 47.2%, 51.28%, and 77.27%. The overall accuracy of ultrasound was 61%. The risk of malignancy in our study for TIRADS 3, TIRADS 4, and TIRADS 5 were 22.7%, 29.16% and 86.66%, respectively.

From this data it is clear that USG is a good initial screening test but has poor specificity.

On analysis of this subgroup of subjects using HPE as the gold standard, the individual parameters of TIRADS like shape, echogenicity, and presence of microcalcification were statistically significant, and serves to differentiate benign from malignant nodule. The nodule margin and consistency were not statistically significant. Major ultrasound features according to TIRADS Score are shown in Table 4. Sensitivity, specificity, PPV, NPV, OR, and likelihood ratio were calculated for each feature and tabulated in Table 5. Combining TIRAD 4 and 5 together, a sensitivity of 80% in diagnosing thyroid cancer was documented. Table 5 show the different statistical analysis of the major ultrasound features suggestive of malignancy with respect to histopathological reports, and their respective performance. Major ultrasound features like microcalcification is highly sensitive (80%) and specific (86.11%) parameter, taller than wider shape is highly specific (92%) but low sensitivity (36%) parameter, hypoechogenecity is also specific (78%) but not very sensitive (68%) parameter and irregular margin is highly specific (89%) but not sensitive (28%) in differentiation of malignant and benign thyroid nodule.

A total of 61 out of 137 cases (44.5%) underwent surgery after FNA procedures, which included 20 benign (Bethesda 2), 1 non-diagnostic (Bethesda 1), 14 AUS/FLUS(Bethesda 3), 12 FN/ SFN (Bethesda 4), and 14 were suspicious for malignancy (Bethesda 5). In the "indeterminate" cytological categories of Bethesda 3, out of 14 cases, 10 were benign and 4 malignant (3 were papillary thyroid carcinoma and 1 was follicular thyroid carcinoma) in histopathological diagnosis. Out of 12 cases of Bethesda 4 cytology, 4 were papillary thyroid carcinoma, 1 was minimally invasive follicular carcinoma, 2 follicular carcinoma, and 5 were benign by histopathology.

In Bethesda 3 category we had 14 patients. Out of those 14 samples 4 were malignant. 2 subjects with malignant histopathology were TIRADS 4, 1 subject with malignant histopathology as TIRADS 3, and 1 subject with malignant histopathology was TIRADS 5 by sonography. TIRADS did not help in the prediction of malignancy in patients with Bethesda 3.

Considering Bethesda 3 and 4 as indeterminate group we had 26 patients with an indeterminate cytology. In these patients, TIRADS has shown little benefit in influencing the final diagnosis. Only TR 5 had some predictive value in diagnosing malignancy.

Out of 14 cases of suspicious for malignancy (Bethesda 5), 10 cases were papillary thyroid carcinoma and 2 cases were diagnosed as follicular carcinoma by histopathology, whereas 2 were benign. Table 6 shows comparison between Bethesda

Table 1: Baseline characteristics of demographic, clinical, radiological, cytological and histopathological features

Profile of subjects with Solitary Thyroid Nodule (*n*=137)		
Clinical Features	**Sub-features**	**No. (%)**
Composition	Completely solid	119 (86.86%)
	Mixed solid & cystic	18 (13.13%)
Echogenicity	Hyperechoic	62 (45.25%)
	Isoechoic	51 (37.22%)
	Hypoechoic	24 (17.51%)
Calcification	None or comet-tail artifacts	95 (69.34%)
	Macro calcification	20 (14.59%)
	Micro calcification	14 (10.21%)
	Rim calcification	8 (5.83%)
Shape	Wider than taller	123 (89.78%)
	Taller than wider	14 (10.21%)
Margin	Smooth	118 (86.13)
	Ill-defined	6 (4.37)
	Lobulated/irregular	13 (9.48)
TIRADS Score	TIRADS 1 (Benign)	No FNA
	TIRADS 2 (Not suspicious)	No FNA
	TIRADS 3 (Mildly suspicious)	91 (64.96)
	TIRADS 4 (Moderately suspicious)	29 (22.62)
	TIRADS 5 (Highly suspicious)	17 (12.40)
BETHESDA Classification	BETHESDA 1 (Non-diagnostic)	4 (2.91)
	BETHESDA 2 (Benign)	85 (62.04)
	BETHESDA 3 (AUS/FLUS)	20 (14.59)
	BETHESDA 4 (Follicular neoplasm)	13 (9.48)
	BETHESDA 5 (Suspicious for malignancy)	14 (10.21)
	BETHESDA 6 (Malignant)	1 (0.72)
Surgical Histopathology (*n*=61)	Benign	36 (59.01)
	Malignant	25 (40.98)

Table 2: Proportion of malignancy as per TIRADS Score

TIRADS Score	Histopathology		Total	Risk of malignancy (%)
	Malignant	Benign		
3	5	17	22	22.72%
4	7	17	24	29.16%
5	13	2	15	86.66%
Total	25	36	61	40.98%

Table 3: Comparison of TIRADS with risk of malignancy

Pre-operative investigation	Histopathology			*P*
Conventional USG	Malignant	Benign	Total	
TIRADS 4,5	20 (58.3%)	19 (48.7%)	39 (64%)	
TIRADS 3	5 (22.7%)	17 (77.3%)	22 (36%)	0.03
Total	25 (41%)	36 (59%)	61 (100%)	

classifications with histopathological reports. The sensitivity, specificity, PPV, and NPV were respectively 80%, 90%, 86%, and 86%. The overall accuracy of FNAC was 85%.According to histopathological report, the risk of malignancy in case of indeterminate thyroid nodule (Bethesda 3 and 4) was 38.46%.

We had 21 patients with Bethesda 2 of whom 3 harbored malignancy (papillary thyroid carcinoma) on HPE and rest were benign. In one case of malignancy TR 5 was suggestive of high risk, but other two cases were having TR 3. Despite an initial Bethesda 2 on FNAC, high risk features on USG and a higher TIRADS score compelled us to repeat this apparently benign cytology or to undergo a surgical biopsy for confirmation.

Discussion

Euthyroid nodule was commonly seen in females compared to males in this study, suggestive of female predominance and was almost 86.86% of total study population ($N = 137$). This is in accordance with earlier studies,[13] in which aprevalence of thyroid nodule in females was 86% ($N = 50$).

In the present study highest number of cases were reported in the 21–40 years age group (74 cases, 54%), followed by 51–60 years (34 cases, 24%). The results were in accordance to reports published earlier,[13] in which they noted that majority of the cases were between 21 and 40 years age group (80%, $N = 50$).

Kwak *et al*.[8] have proposed a TIRADS score by retrospective analysis of thyroid nodules in ultrasound and FNA, using five ultrasound criteria that can be used during thyroid evaluation.

This article describes that a malignancy risk of 0% is expected for TIRADS 2, 1.7% for TIRADS 3, a risk of 3.3-72.4% for TIRADS 4, and of 87.5% for TIRADS 5. Our study has shown 22.72% malignancy risk for TIRADS 3. The risk of malignancy in our study for TIRADS 4 and TIRADS 5 were 29.16% and 86.66%, respectively. According to another Indian study by Srinivas et al.,[12] it was concluded that the risk of malignancy for TIRADS categories1, 2, 3, 4A, 4B, 4C, and 5 was 0, 0, 0.64, 4.76, 66.67, 83.33, and 100%, respectively. Our results are within the range suggested by Kwak *et al*.[8] and two other studies based on Indian population [Table 2].The sensitivity, specificity, PPV, and NPV of TIRADS versus histopathology were respectively 80%, 47.2%, 51.28%, and 77.27%. The overall accuracy of ultrasound was 61%.

It is intriguing that a malignancy rate of 22.7% was found in subjects with TR 3 in our study. We have used the latest ACR TIRADS criteria 2017. Other studies have used previous versions of TIRADS classification (ACR TIRADS 2009, K-TIRADS 2017 and EU-TIRADS 2017). A study using the latest ACR TIRADS 2017 criteria conducted by Barbosa *et al*.[11] reported a percentage of malignancy of 23.3% in subjects with TR 3. Similar finding is reported in our study also. As per ACR TIRADS criteria 2017, the four possible scenarios classified as TR3 are as follows: (i) solid and hyperechoic; (ii) solid and isoechoic; (iii) mixed solid cystic and hyperechoic with macrocalcification; (iv) mixed solid cystic and hypoechoic. This might explain the higher rate of malignancy in TR3, as individual features like macrocalcifiation or hyperechoic nature of the nodule are not included as possible features to predict malignancy in previous systems. This might be one of the drawbacks of ACR TIRADS 2017.

Paradoxically, the specificity of TIRADS score in this study was only 47%. According to latest guidelines of TIRADS,[14] a patient with solid nodule (2 points) which is hyperechoic (1 point) and having macrocalcification (1 point) is labelled as TIRADS 4. Sixteen such subjects in our study had a benign cytology on HPE. So this can be a limitation of the current TIRADS scoring.

The limitation of FNAC includes false-negative result and false positive results. A comparative study was done by Bloch[15] between FNAC and histopathology and found that the accuracy of FNAC was 91.6%. Handa *et al*.[16] have a similar study in which FNAC revealed a sensitivity of 97%, specificity 100% a PPV of 96% and a NPV of 100%. Mundasad *et al*.[17] had done similar study and identified that FNAC had a sensitivity (52.6%), specificity (86.6%) and accuracy (79.1%) for thyroid malignancy. According to histopathological diagnosis the risk of malignancy was calculated in case of indeterminate thyroid nodule (Bethesda 3 and 4) was 38.46%. In our study sensitivity of FNAC was 80%, specificity was 90%, positive and negative predictive value was 86%, and the overall diagnostic accuracy was 85%.

Table 4: Major Ultrasound features and histopathology results

Major ultrasound Features	Histopathology ($n=61$)		Total	*P*
	Malignant ($n=25$)	Benign ($n=36$)		
Taller than Wider				
Present	9	3	12	0.01
Absent	16	33	49	
Hypoechogenecity				
Present	17	8	25	<0.01
Absent	8	28	36	
Microcalcification				
Present	20	5	25	<0.0001
Absent	5	31	36	
Irregular margin				
Present	7	4	11	0.17
Absent	18	32	50	

Conclusion

We can conclude that FNAC and TIRADS both are highly sensitive (80%) but FNA is more specific (90%) and accurate test (85%) in identifying thyroid cancer. Among individual USG parameters, micro-calcification was most sensitive (80%) and specific (86%). Irregular margin and taller-than-wider shape had a specificity of 89% and 92%, respectively. In patient having high risk feature on USG (TIRADS 5), a benign cytology dose not completely rule out risk of malignancy and they should undergo surgical biopsy for further confirmation. A benign FNAC diagnosis should be viewed with caution as false-negative results do occur and these subjects should be followed up and any clinical suspicion of malignancy even in the presence of benign FNAC requires surgery. USG features like taller-than-wide and irregular margins are specific for malignancy but have poor sensitivity. The suspicious indeterminate results prove to be an area of uncertainty which can be resolved by surgical resection and biopsy.

Declaration of patient consent

The authors certify that they have obtained all appropriate

Table 5: Diagnostic attributes of individual USG finding

Features	Sensitivity	Specificity	PPV	NPV	OR (95%CI)	Likelihood Ratio
Taller than wider	36%	92%	75%	67.4%	6.19 (1.57 to 22.78)	4.32
Hypoechogenecity	68%	78%	68%	77%	7.44 (2.47 to 23.61)	3.06
Microcalcification	80%	86.11%	80%	86%	24.80 (6.129 to 86.37)	5.76
Irregular margin	28%	89%	64%	64%	3.11 (0.78 to 10.33)	2.52

Table 6: Comparison of FNAC with histopathology

Fine-needle aspiration assay	Malignant	Benign	Total
BETHESDA 5,6	12 (86%)	2 (14.28%)	14 (4%)
BETHESDA 2	3 (14.28%)	18 (86%)	21 (6%)
Total	15 (43%)	20 (57.14%)	35 (100%)

participant consent forms. In the form, the participants have given their consent for clinical information to be reported in the journal. The participants understand that their names will not be published and due efforts will be made to conceal their identity.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Cooper D, Doherty G, Haugen B, Kloos R, Lee S, Mandel S, *et al*. Revised american thyroid association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid 2009;19:1167-214.
2. Hegedüs L. The thyroid nodule. N Engl J Med 2004;351:1764-71.
3. Desforges J, Mazzaferri E. Management of a solitary thyroid nodule. N Engl J Med 1993;328:553-9.
4. Tan G. Thyroid incidentalomas: Management approaches to nonpalpable nodules discovered incidentally on thyroid imaging. Ann Intern Med 1997;126:226-31.
5. Gharib H, Papini E. Thyroid nodules: Clinical importance, assessment, and treatment. Endocrinol Metab Clin North Am 2007;36:707-35.
6. Rahbari R, Zhang L, Kebebew E. Thyroid cancer gender disparity. Future Oncol 2010;6:1771-9.
7. Horvath E, Majlis S, Rossi R, Franco C, Niedmann J, Castro A, *et al*. An Ultrasonogram reporting system for thyroid nodules stratifying cancer risk for clinical management. J Clin Endocrinol Metab 2009;94:1748-51.
8. Kwak J, Han K, Yoon J, Moon H, Son E, Park S, *et al*. Thyroid imaging reporting and data system for US features of nodules: A step in establishing better stratification of cancer risk. Radiology 2011;260:892-9.
9. Yoon J, Lee H, Kim E, Moon H, Kwak J. Thyroid nodules: Nondiagnostic cytologic results according to thyroid imaging reporting and data system before and after application of the bethesda system. Radiology 2015;276:579-87.
10. Singh Ospina N, Brito J, Maraka S, Espinosa de Ycaza A, Rodriguez-Gutierrez R, Gionfriddo M, *et al*. Diagnostic accuracy of ultrasound-guided fine needle aspiration biopsy for thyroid malignancy: Systematic review and meta-analysis. Endocrine 2016;53:651-61.
11. Barbosa T, Junior C, Graf H, Cavalvanti T, Trippia M, da Silveira Ugino R, *et al*. ACR TI-RADS and ATA US scores are helpful for the management of thyroid nodules with indeterminate cytology. BMC Endocrine Disorders 2019;19.
12. Srinivas M, Amogh V, Gautam M, Prathyusha I, Vikram N, Retnam M, *et al*. A prospective study to evaluate the reliability of thyroid imaging reporting and data system in differentiation between benign and malignant thyroid lesions. J Clin Imaging Sci 2016;6:5.
13. Muthu S, Saravanakumar R. A prospective study of incidence of malignancy in solitary nodule of thyroid. Int J Contemporary Med Res 2019;6:E24-6.
14. Tessler F, Middleton W, Grant E, Hoang J. Re: ACR thyroid imaging, reporting and data system (TI-RADS): White paper of the ACR TI-RADS committee. J Am College Radiol 2018;15:381-2.
15. Bloch M. Fine needle aspiration biopsy of head & neck masses. Otolaryngol Head Neck Surg 1997;89:62-8.
16. Garg S, Mohan H, Nagarkar N, Handa U. Role of fine needle aspiration cytology in diagnosis and -management of thyroid lesions: A study on 434 patients. J Cytol 2008;25:13.
17. Hirachand S, Maharjan M, Lakhey M, Thapa R, Kafle S. Accuracy of fine needle aspiration cytology in diagnosis of thyroid swelling. J Pathol Nepal 2013;3:433-6.