Details of the research work

Clinical Research Article

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

Introduction: 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

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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 3000g 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 byFNA 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:

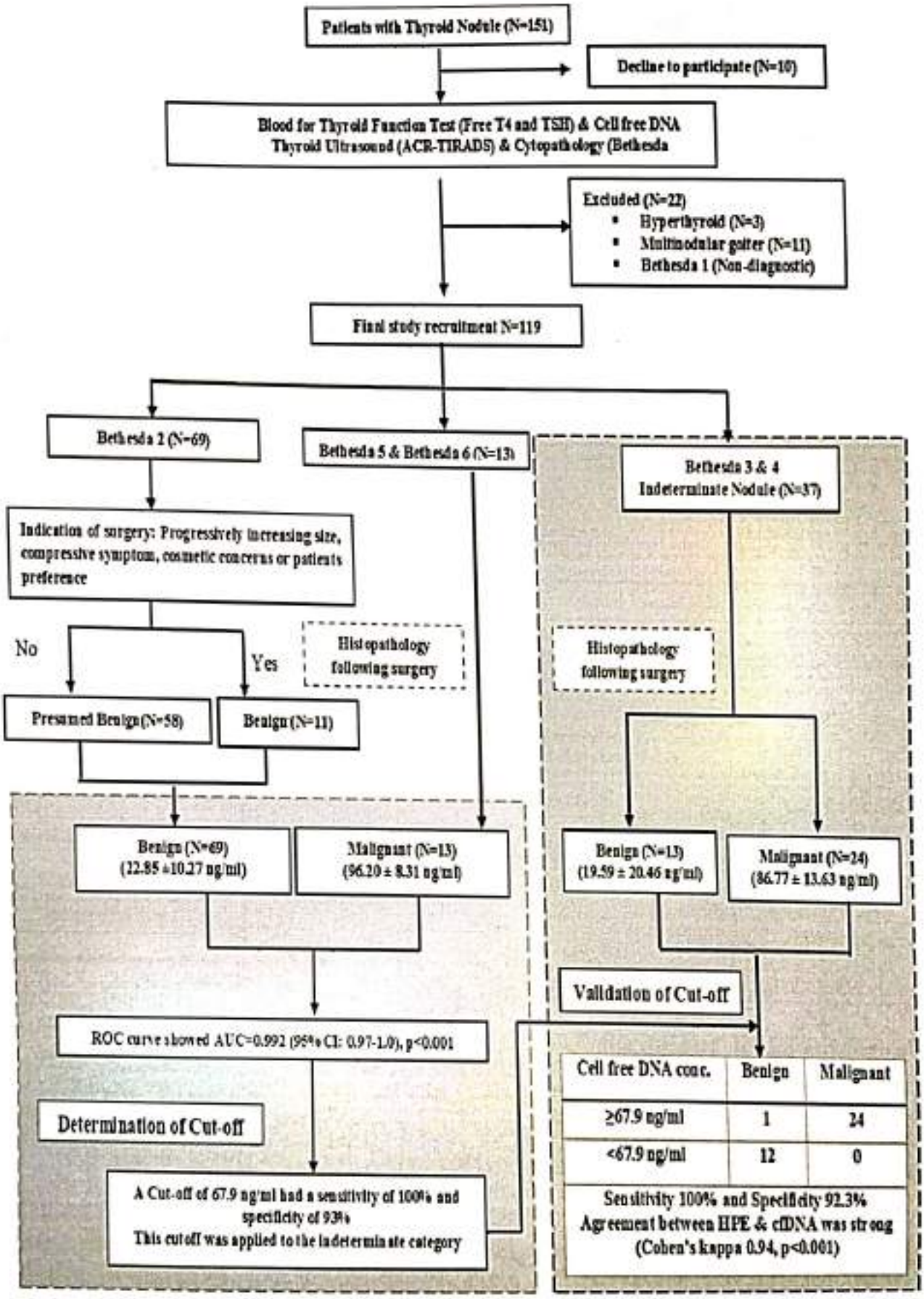

Figure 1. Flowchart illustrating study design, workflow, and results.

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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(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.

<table>
<tr><td colspan="4">Profile of subjects with Solitary Thyroid Nodule (N=119)</td></tr>
<tr><td>Age, mean ± SD, years</td><td colspan="3">34.32 ± 11.25</td></tr>
<tr><td>Gender (Male/ Female)</td><td colspan="3">26/93</td></tr>
<tr><td>Free T4, mean ± SD, ng/dL</td><td colspan="3">1.33 ± 0.40</td></tr>
<tr><td>TSH, mean ± SD, mIU/L</td><td colspan="3">2.72 ± 0.87</td></tr>
<tr><td colspan="4">Radiological Findings of solitary thyroid nodule</td></tr>
<tr><td>TIRADS Score</td><td colspan="2">TIRADS 1 (Benign) no. (%)
TIRADS 2 (Not suspicious) no. (%)
TIRADS 3 (Mildly suspicious) no. (%)
TIRADS 4 (Moderately suspicious) no. (%)
TIRADS 5 (Highly suspicious) no. (%)</td><td>No FNA
2 (1.68%)
48 (40.33%)
65 (54.62%)
4 (3.36%)</td></tr>
<tr><td colspan="4">Cytopathological and Histopathological Findings of solitary thyroid nodule</td></tr>
<tr><td>Cytological Findings</td><td>FNA Categories</td><td>Histopathological Findings</td><td>cfDNA Concentration</td></tr>
<tr><td>Bethesda 1 (Non-diagnostic) (N=8)</td><td>Non-diagnostic (N=8)</td><td>Excluded (Repeat FNAC suggested and Follow up)</td><td>-</td></tr>
<tr><td>Bethesda 2 (Benign) (N=69)</td><td>Benign (N=69)</td><td>Biopsy confirmed (N=11)
FNAC (Presumed benign) (N=58)</td><td>22.85 ± 10.27 ng/ml</td></tr>
<tr><td>Bethesda 3 (AUS/FLUS) (N=17)</td><td rowspan="2">Indeterminate Nodule (N=37)</td><td>Benign (N=13)</td><td>28.58 ± 14.47 ng/ml</td></tr>
<tr><td>Bethesda 4 (Follicular Neoplasm) (N=20)</td><td>Malignant (N=24)</td><td>84.02 ± 13.35 ng/ml</td></tr>
<tr><td>Bethesda 5 (Suspicious for malignancy) (N=11)</td><td rowspan="2">Malignant (N=13)</td><td rowspan="2">Malignant (N=13)</td><td rowspan="2">96.20 ± 8.31 ng/ml</td></tr>
<tr><td>Bethesda 6 (Malignant) (N=2)</td></tr>
</table>

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

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

Determination of Cutoff

Cell-free DNA (cfDNA) concentration was estimated inall subjects at baseline (before surgery). In the first phaseof analysis (determination of cutoff phase) we observed that the mean ± SD of cfDNA concentration for the benigngroup was 22.85 ±10.27 ng/mL and for the malignantgroup was 96.20 ± 8.31 ng/mL. The difference wasstatistically significant ($P <$

0.001). Ratio of absorbance at260 nm and 280 nm was around 1.8. Cell-free DNA concentrationwhen measured by using the Qubit fluorometerwas comparable and consistent with the findings using theNano drop spectrophotometer. Using the Qubit fluorometer,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 predictivevalue (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, RETPTC1, PAX8-PPARγ). 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 differencesin 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.

Variable (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	
Extrathyroidal 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*
	II (N=20)	87.59 ± 12.43	
	III (N=9)	94.44 ± 11.52	

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

All statistical analysis was done using Mann-Whitney-U test. *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 wasused, 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 (non-neoplastic) and Thy4 and Thy5 (suspicious of malignancy and diagnostic of malignancy), according to 2007 cytology guidance fromthe British Thyroid Association (25). However, there wasno 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 indeterminatenodules turned out to have malignant disease. This washigher than that reported in most studies. Most studies suggestthat the risk of malignancy in indeterminate nodulesvaries from 10% to 30% for Bethesda 3 and 25% to 40% for Bethesda 4 (5). We believe this high prevalence of malignancycould be a chance finding. The study of Thakuret 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 hada 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 therequirement 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 ($6.5 \pm 3.7\%$) compared with pre-surgical samples ($43.2 \pm 8.9\%$) ($P < 0.001$).

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 aninferior 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 withthyroid nodules.

Acknowledgments

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