Original Article

Presurgical Screening of Fine Needle Aspirates from Thyroid Nodules for *BRAF* Mutations: A Prospective Single Center Experience

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Abstract

Objective: Analysis of BRAF V600E mutation in thyroid fine needle aspirates (FNA) is an important adjunct to cytology, particularly among FNA placed in the "indeterminate category." However, such a prospective evaluation of FNA obtained from patients with thyroid nodules has been lacking from India. **Material and Methods:** FNA from 277 patients were prospectively evaluated for BRAF mutations by Sanger's sequencing. A subset of 30 samples was also analyzed by pyrosequencing using the PyroMark BRAF mutation kit. **Results:** Overall, 27.2% of FNA samples were positive for mutations including 19 (35.8%) of the 53 histologically confirmed papillary thyroid carcinoma (PTC), 2 of the 25 follicular variants of PTC, and 1 anaplastic thyroid carcinoma. Only 1 (2.7%) of the 37 samples in the atypia of undetermined significance/follicular lesion of unknown significance category was BRAF positive. The sensitivity of cytology improved marginally from 67.1% to 68.3% when evaluated with BRAF. Further, a comparison of the clinicopathological characteristics of BRAF positive and negative PTCs showed a significant association ($P = 0.05$) between lymph node metastasis and BRAF positivity. **Conclusion:** BRAF positivity was lower than that reported from East Asia with the test being useful in confirming malignancies among the suspicious of malignancy and malignant categories.

Keywords: Atypia of undetermined significance"/"follicular lesion of uncertain significance, BRAF mutations, cytology, Fine-needle aspiration, papillary thyroid carcinoma

Background

Thyroid nodules are fairly common and are being increasingly detected using improved screening modalities.[1,2] The increased detection of nodules has also highlighted the need for accurate cytological assessment by fine needle aspiration (FNA). Although most thyroid nodules may be benign, current imaging and cytological techniques fail to provide clear differentiation between benign and malignant disorders, in a subset of cases. Approximately 20% of FNAs analyzed are placed under the "indeterminate" category[3-6] requiring surgical excision and definitive histopathology to rule out malignancy, and many are eventually confirmed as benign lesions. This necessitates an additional test that could help diagnose malignancies in the indeterminate category and several molecular makers have been investigated.

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BRAF V600E is the commonest mutation seen in thyroid cancers and is specifically associated with papillary thyroid carcinoma (PTC)[7,8] increasing its potential as an adjunct marker while screening thyroid nodules by cytology. Transversion of thymine to adenine at nucleotide position 1799 in exon 15 of the BRAF resulting in an amino acid change from valine to glutamic acid has been shown to constitutively activate downstream MAPK signaling,[9,10] generally correlating with tumor aggressiveness. The prevalence of BRAF among PTCs has, however, been found to vary in different geographical

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regions, ranging from 30% to 80%,[11-14] thereby raising the debate whether screening for BRAF mutations in settings with lower prevalence could, if at all, aid in increasing the diagnostic accuracy. Several investigators have also argued that if performed presurgically, then BRAF positivity would not only predict thyroid malignancy with a higher specificity but would also potentially guide the extent of initial surgical treatment.[15-18] Therefore, screening for these mutations could be of help in both improving diagnostic accuracy and guiding surgical treatment.

Though several studies have evaluated the role of BRAF retrospectively, few have characterized FNA samples prospectively to assess its true diagnostic potential. In Asia, most studies in this context are from the East, in regions with an established high incidence of BRAF among PTCs.[19-22] Studies from Korea have demonstrated 80% of the PTCs to be positive for BRAF conclusively proving the utility of identifying BRAF positivity along with cytology analysis. The few reports from India that have reported varying percentages of BRAF positivity (25–50%) have been performed using tissues[23,24] and there are no reports from the Indian subcontinent where FNA samples have been prospectively evaluated. Therefore, 277 FNA samples were evaluated prospectively for BRAF mutations and then correlated with cytology results and histology when available.

Materials and Methods

Patients

Over a 24-month period, 277 patients with one or more thyroid nodules were included in the study. The study was approved by the institutional review board and consent was obtained from all patients enrolled in the study. The FNAs were performed by either palpatory or US-guided techniques, as decided by the treating surgeon based on clinical and diagnostic ultrasound features; US guidance was routinely used in the latter part of the study; a total of 60 of the 277 biopsies were US guided. The FNAs were performed by using a 25-ga needle applying the nonaspiration technique of Zajdela, following standard protocol.[25] FNA samples were put on frosted-end glass slides, immediately fixed with 95% alcohol for both Papanicolaou and May–Grunwald–Geimsa staining. FNA samples were obtained by an additional pass after the sample was collected for routine diagnostics and were put in RNA *later* and transported to the laboratory for molecular analysis.

Cytological analysis

The Bethesda System for Reporting Thyroid Cytopathology (BSRTC)[26] was followed for categorization and diagnosis of all FNA samples, and briefly, the following categories were included: "malignant" when suspicious or diagnostic for thyroid cancers, "benign" for hyperplastic/cystic lesions and chronic thyroiditis, "nondiagnostic" when the sample provided too few cells for diagnostic purpose and "indeterminate" which included "atypia of undetermined significance"/"follicular lesion of uncertain significance (AUS/FLUS) when they are not convincingly benign/malignant, or lesions "suspicious of follicular neoplasm" when characterized by architectural atypia and hypercellularity suggestive of follicular adenoma or carcinoma.

DNA isolation and BRAF mutational analysis

About 200 μl of the FNA sample from RNA *later* was centrifuged at 4°C at 8000*g*. About 50 μl of the sediment was used for DNA extraction using the QIAamp DNA micro kit [Qiagen, Hilden, Germany]. The extracted DNA was quantified on the nanodrop [Nanodrop technologies, USA] and stored at −80°C for further use. The BRAF mutational analysis was performed using primers described previously.[27] All reactions were carried in 25 μl volume. The following thermal cycling profile was used for all PCRs: 95°C for 8 min, 95°C for 30 s, optimized anneal for 30 s, 72°C for 1 min, and final extension of 72°C for 10 min. The PCR product was detected using a 2% agarose gel. Sequencing of both the sense and antisense strands of all amplified products was performed with an automated DNA sequencer [ABI PRISM 310 genetic analyzer] using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit [Applied Bio-systems, Foster City, California, USA]. Mutational analysis was performed by comparing the sequence with the wild type and by identifying all known mutations in this exon. DNA from all 277 samples was tested for BRAF mutations. Further, a subset of 30 samples was analyzed by pyrosequencing using the PyroMark BRAF mutation kit [Qiagen, Hilden, Germany]; the concurrence between direct sequencing and pyrosequencing was determined. The pyrosequencing was carried out as described previously.[28] Briefly, 50 ng of DNA was amplified using biotinylated forward and reverse primers and the products electrophoresed on 1.5% agarose gel to verify successful amplification. These products were then immobilized on sepharose beads [GE healthcare, India] in a binding buffer with constant agitation for 15 min at room temperature. The DNA strands were then separated using the PyroMark Q24 vacuum workstation and then transferred to an annealing buffer containing the sequencing primers, incubated at 80°C for 2 min and then cooled to room temperature. Pyrosequencing was performed with the AQ assay using PyroMark gold Q24 reagents [Qiagen, Hilden, Germany] as per the manufacturers' instructions.

Statistical analysis

Descriptive statistics for continuous data was analyzed using mean with standard deviation or median with inter-quartile range. Categorical data were described using frequencies and percentages. Cytological categories were correlated with BRAF mutational analysis using Fischer's exact test and Chi-square test. For all analyses, a $P < 0.05$ was considered statistically significant. Data were analyzed using the SPSS statistics software, version 16.

Results

Correlation between cytology, mutational status, and histology

Fine needle aspirates from 277 thyroid nodules were included for analysis [Figure 1]. Of these, 165 (59.5%) were benign

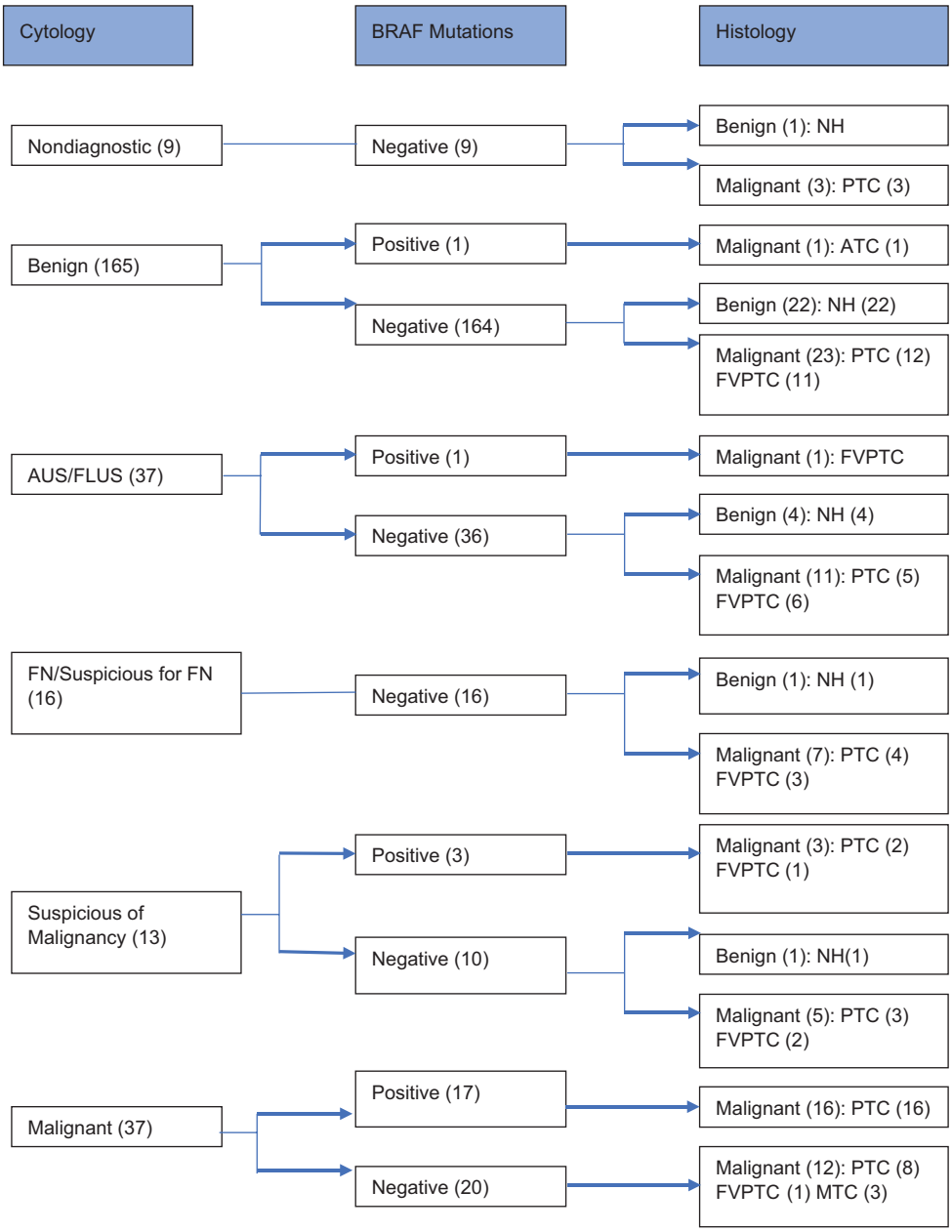

Figure 1: Detection of BRAF V600E in 277 FNA samples by sequencing and comparison with cytology and histology results

and all of these samples except one were negative for BRAF mutations. Similarly, among the 37 (13.3%) samples in the AUS/FLUS category, all except one were negative for BRAF. None of the samples in the follicular neoplasm/suspicious of follicular neoplasm category (n = 16; 5.7%) was positive for BRAF, whereas 23% of those which were suspicious of malignancy (SMC) (n = 13, 4.6%) were positive for mutations. About 46% of all samples in the malignant category (n = 37, 13.3%) were positive for BRAF mutations as shown in Figure 1. Though nine cytologically insufficient/acellular samples (Bethesda Category I) were included for BRAF analysis and provided evaluable DNA, none were positive for mutations.

Of the 165 benign cytology samples, 46 cases were resected, including the lone BRAF-positive sample that was eventually confirmed to be a case of anaplastic thyroid carcinoma (ATC). Of the remaining 45 resected specimens, 22 were cases of nodular hyperplasia (NH) and 23 cases were malignant (PTC [12], follicular variants of PTC FVPTC[11]) but none were BRAF positive [Figure 1]. Fifteen of the 37 patients with AUS/FLUS underwent surgery, and while 4 were benign, 11 were malignant (PTC[5], FVPTC[6]) including the FVPTC sample that was positive for BRAF in this group. Eight of the 16 cases of FN/suspicious of FN category were operated and included one case of NH, the rest were malignant (PTC[4], FVPTC[3]) though none were BRAF positive. Nine of the 13 cases, which were considered to be SMC, were operated where one was NH and the remaining eight were malignant. This included the two PTCs and one

FVPTC that was BRAF positive. Further, 28 of the 37 cases classified as malignant by cytology were resected at our center, whereas the remaining nine were operated elsewhere. Sixteen of the 37 malignant cases were positive for BRAF and all were confirmed as PTCs. The remaining 12 BRAF-negative cases that were operated included three cases of medullary thyroid carcinoma (MTC) apart from eight PTCs and one FVPTC.

Correlation of clinicopathologic and histological characteristics of PTC based on the BRAF mutational status

All 77 histopathologically confirmed cases of PTC included in the study yielded evaluable results and 21 (27.2%) of these were positive for BRAF mutations. A comparison of the clinicopathologic characteristics [Table 1] between the BRAF-positive and -negative groups did not show any significant association by age, gender, tumor size and multifocality, extrathyroidal extension, and capsular invasion. However, a significant association ($P = 0.05$) was seen between lymph node metastasis and BRAF positivity. Further, a comparison of the cell types associated with the PTC did not show any significant association with BRAF positivity, though follicular variant of PTC was less likely to be associated with BRAF positivity ($P = <0.001$).

Comparison of mutational analysis by Sanger's sequencing and pyrosequencing

A subset of 30 samples was tested by pyrosequencing and the results compared with that of Sanger's sequencing [Table 2]. Pyrosequencing additionally picked up three BRAF-positive samples, which were negative for BRAF by Sanger's sequencing. All three samples were confirmed as PTC by histology and were from the benign, SMC, and malignant categories by cytology. Pyrosequencing showed 100% specificity and a high positive predictive value (PPV) (83.3%) and NPV (100%). The agreement between the two tests was also significant with k at 0.862. However, only the results obtained by Sanger sequencing were considered while determining its diagnostic utility in thyroid cancers.

Diagnostic value of BRAF mutational analysis and BRAF along with conventional cytology

The sensitivity and specificity of each test is shown in Table 3. BRAF mutational analysis provided low sensitivity of 25.6% but 100% specificity and PPV. Cytology as standalone test had a sensitivity of 67.1% and a much lower specificity at 79.3%. However, the addition of BRAF mutational analysis helped to only marginally improve the sensitivity of the cytology as a diagnostic aid for thyroid FNAs. Both tests together provided 68.3% sensitivity and there was not significant change in specificity. The PPV remained high at 90.3%.

Table 1: Correlation of clinicopathologic and histological characteristics of PTC based on the BRAF mutational status

	BRAF+	BRAF-	*P*
n	21	66	
Age (yr)			
<45	14 (66.7)	48 (72.7)	0.591
≥45	7 (33.3)	18 (27.3)	
Gender			
Male	10 (47.6)	19 (28.8)	0.121
Female	11 (52.4)	47 (71.2)	
Tumor size (cm)			
<2	3 (21.4)	11 (78.6)	0.695
≥2	5 (16.7)	25 (83.3)	
Extrathyroidal extension	6 (37.5)	4 (7.1)	0.29
Lymph Node metastasis	10 (62.5)	12 (21.4)	0.05
Capsular invasion	1 (6.7)	24 (43.6)	0.46
Multifocality	10 (25.6)	29 (74.4)	0.448
Histology			
Classical	8 (32)	17 (68)	0.010
Tall cell	6 (75)	2 (25)	0.045
Follicular	2 (5.4)	35 (94.6)	0.001
Poorly differentiated	0	2 (100)	0.045

Table 2: Comparison of 30 samples by Sangers sequencing and pyrosequencing for BRAF mutational analysis

Cytology	Histology	Positive by sequencing	Positive by pyrosequencing
Benign (4)	PTC (1) FVPTC (3)	-	1
AUS/FLUS (2)	PTC (2)	-	-
FN/Suspicious of FN (1)	-	-	-
Suspicious of malignancy (11)	PTC (6) FVPTC (2) MTC (1)	4	5
Malignant (12)	PTC (8) MTC (2)	6	7

Table 3: Diagnostic value of cytology and BRAF mutational analysis in detecting thyroid malignancy using FNA samples

	Cytology	BRAF mutations	Both tests
Sensitivity	67.1	25.6	68.3
Specificity	79.3	100	79.3
PPV	90.2	100	90.3
NPV	46.0	32.2	46.9

Discussion

The study attempted to determine the diagnostic potential of BRAF mutational analysis and its utility in presurgical screening of thyroid FNA samples obtained prospectively. This study assumes importance as there is a paucity of information in India in context of BRAF mutational screening from FNA samples. Screening has become essential in the recent years as the "Revised American Thyroid Association Management Guidelines for Patients with Thyroid nodules

and Differentiated Thyroid Cancer" (2009) from the American Thyroid Association (ATA) clearly advocates the use of molecular markers, especially among those samples that demonstrate indeterminate cytology results.[29] Out of a list of suggested possible markers by the ATA, testing for BRAF mutations has gained ground as it has not only been found to be a useful diagnostic marker of PTC but is also associated with virtually 100% specificity (for either PTC or ATC), clearly indicating malignancy among samples that are found to be positive.[22,30-32] This approach has also been found to be meaningful as confirmation of malignancy regardless of the cytological finding has resulted in better surgical management with total thyroidectomies instead of lobectomies.[33]

There were nine samples that were classified as unsatisfactory, though none were found to be BRAF positive despite yielding amplifiable DNA. Further, of the 37 samples in the AUS/FLUS category in the study, only one sample (2.7%) was found to be BRAF positive, clearly highlighting the poor positivity rate in the indeterminate category. However, several studies have demonstrated low positivity (3–4.6%)[34-37] and have argued that BRAF analysis alone might not provide any advantage over the cytology results in the indeterminate category. Yet, there are other reports where >10% of the BRAF-mutated cases were seen in this category.[28,38-42] Ohori *et al.* provide a likely explanation for this difference and argue that this difference is probably due to variability of application of the BSRTC criteria, especially in the demarcation of the AUS/FLUS and SMC and FN/SFN and SMC category that can vary across centers.[43] Further, it has been debated whether the low prevalence of BRAF mutations in the indeterminate category could also be due to more number of follicular adenomas, follicular thyroid carcinomas (FTC), and FVPTC, though 5 of 11 resected cases of AUS/FLUS in this study actually were PTC. Several studies have also argued that the true usefulness of the testing is expected to therefore lie in the SMC category where about 15–20% of them usually appear to carry mutations.[38-42] In this study, 23% of those in the category were positive confirming their malignancy. However, the highest percentage of positivity in the study was, as expected, in the malignant category where nearly 46% of the PTCs were positive for mutations. Such results when available presurgically can perhaps guide intraoperative and postoperative management of patients and prove meaningful.

BRAF mutational analysis in the study was associated with 100% specificity and none of the MTCs and NHs was mutation positive. Mutations were seen in 19 (35.8%) of the 53 histologically confirmed PTCs, 2 (8%) of the 25 FVPTCs, and 1 ATC sample. The percentage of positivity in this study is similar to that reported by Khan *et al*.,[24] but lower than the 50% reported in a retrospective sample set from India recently,[23] though both studies were performed on archived tissues and not from FNA as in this study. About 40–45% of the all PTCs characterized are generally known to be positive for mutations. The prevalence of mutation among PTCs has been found to, however, be highly variable with the PTC subtype, the population characterized, its iodine intake, and the type of mutation assay employed.[34] Also, most (98%) of the PTCs that carry a mutation harbor the V600E mutation. In contrast, very few FVPTCs usually carry mutations and often tend to harbor the K601E mutations. Although both the mutation-positive FVPTC samples in the study harbored the V600E, the FVPTC subtype in this study (32%) was high in contrast to many of the studies from other parts of Asia including Korea where most PTCs are the conventional type[22,44,45] and perhaps might have contributed to the lower overall percentage (27.2%) of BRAF positivity in the study. In fact, Su *et al*. in a recent meta-analysis have highlighted the lack of BRAF positivity among FVPTCs and that this subtype might actually contribute to the false negativity associated BRAF analysis to determine malignancy.[16,46] In fact, FVPTCs and FTCs are known to carry RAS mutations more frequently and are mutually exclusive with BRAF mutations. The presence of a high number of FVPTC as seen in this study, perhaps, is an indicator of the need to look for RAS mutations among BRAF-negative nodules in the AUS/FLUS category, to provide more diagnostic information.[47] Further, the lone ATC sample in the study, categorized by cytology as benign, was found to be BRAF positive. ATCs are known to be positive for BRAF and are presumed to arise from differentiated PTCs with the accumulation of mutations.[47] Such a result, if available presurgically, can be of great help in avoiding two stage surgery in cases of thyroid cancer.

The pooled sensitivity of cytology and BRAF mutational analysis [Table 3], in the study, improved only marginally by 1.2% from that seen by cytology only. However, other studies have shown improved sensitivity,[5,16,22] in fact, Su *et al.* have shown an increased sensitivity by 6% in their meta-analysis. However, Nikiforov *et al*. who assessed the FNA of 1131 patients have also reported lower overall pooled sensitivity of 60%, whereas the pooled sensitivity of this study was 68.3%. This study was associated with higher specificity (79.3%) and PPV (90.3%) though BRAF when evaluated as a standalone test showed 100% specificity and PPV.[32] The lack of improved sensitivity by a combination of cytology and mutational analysis could perhaps be the impact of lower percentage of BRAF positivity seen in this study. There were only two samples, one from the benign and the other from the indeterminate category, that were found to be positive for BRAF. However, this is in line with similar observations in many other studies where the true potential of the test has been shown to be associated more in SMC category.[34-37] A well-known drawback of BRAF analysis as a diagnostic test has always been the fact that all non-PTCs are positive, thereby limiting its utility as the single test for diagnostic purposes.[48,49] Therefore, several investigators argue that BRAF should also be viewed in context of its usefulness in confirming malignancy, eventually resulting in better intra- and postoperative management of such cases.

A comparison of results obtained by Sangers sequencing and pyrosequencing in a small subset of randomly selected

cases ($n = 30$) showed employing pyrosequencing as the primary testing system in the study could have helped to improve the sensitivity of the analysis. Further, the agreement between the two tests was good ($k = 0.862$) and the three additional pick-ups by pyrosequencing were from histologically confirmed cases of PTC. However, in a resource-limited setting, cost is a major limitation, and pyrosequencing though very sensitive proved to be more expensive than Sangers sequencing and was therefore performed only on a subset.

Finally, BRAF has also been investigated in context of its prognostic relevance. Though complete consensus might not exist, several reports have detailed the association of BRAF mutations and the aggressiveness of PTC.[18,50-53] Many features, including capsular invasion, extrathyroidal extension, lymph node metastasis, multifocality, advanced disease stage, radioactive iodine resistance, and size of the tumor, have been investigated as prognostic markers among BRAF-positive PTCs. Although some have conclusively shown the association of BRAF mutations among PTCs with poorer clinical outcomes/recurrence, other studies have failed to find this association.[15] A similar comparison of these features among the BRAF-positive and -negative PTCs in this study showed that BRAF positivity was significantly associated with lymph node metastasis ($P = 0.05$) and the association of BRAF and lymph node metastasis has been established previously.[11,18,30,50] In fact, some authors have shown that preoperative BRAF analysis on FNA can predict occult central lymph node metastasis among PT who are clinically node negative. However, other studies have not found similar associations, which has been explained by Xing *et al*.[18] who have observed that studies with larger sample sizes generally appeared to show an association of BRAF with these features, whereas those with smaller sample sizes did not reflect those findings. The PTCs that were confirmed by histology in this study were also compared in context of BRAF positivity and the cellular variants. None of the cellular variants of PTC were found to be associated with BRAF positivity though analysis showed that FVPTC was more likely to be negative for mutations ($P = 0.001$) which is in line with the data that indicate low prevalence of BRAF among FVPTCs. Various studies have also shown an association between tall-cell variant of PTC and BRAF positivity where ~80% of tall-cell variant PTC are positive[15,34] higher than that seen among classical cell type (40%) and follicular variants (<10%). However, no such associations were established in this study.

Our study has several limitations and could have yielded more information if it included a larger sample set, used a more sensitive assay for detection, and had complete details of the few PTCs that have been operated elsewhere. However, in the absence of data from this region, the study still provides valuable information on prospective presurgical evaluation of BRAF from FNA, the frequency of mutations, and the limitations of BRAF analysis as a diagnostic standalone test, as a test when combined with cytology, and also highlights the utility of the test in confirmation of malignancy. The ATA statement on surgical application of molecular profiling of thyroid nodules also states that for a test to be useful in a given setting, the prevalence of malignancy in each indeterminate category, in one's own institution, should be known.[54] A study of this nature not only helps to determine the prevalence of malignancy but also provides a reasonable estimate of the frequency of mutation in each category.

The study also throws light on BRAF and the clinicopathological features that have been regarded as indicators of prognosis. To the best of our knowledge, this is the first study from India that has prospectively evaluated FNA and the data will be particularly useful for those in the region who are looking to adopt BRAF testing as part of routine diagnostics of thyroid FNA. The study shows that the BRAF test could provide additional information over cytology in a diagnostic role. In addition, the test is useful in the SMC and malignant categories by helping to confirm malignancy in these patients. However, the true potential of this assay lies in utilizing this information for better intraoperative and postoperative management of these patients by pointing to subclinical nodal or extrathyroidal involvement to prompt more extensive surgery. This study provides concrete evidence to surgeons on the possible scenario of BRAF utilization in Indian patients and prompts us to study other markers or a combination of markers in order to provide a better diagnosis of malignancy in the indeterminate cytology category.

Conclusion

Only 35.8% of the histologically confirmed PTC was BRAF positive, much lower than that from studies for East Asia. In addition, the test is useful in the SMC and malignant categories by helping to confirm malignancy in these patients.

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Conflicts of interest

There are no conflicts of interest.

Criteria for authorship

Each author has participated sufficiently in the work and takes public responsibility for appropriate portions of the content of this article.

HR and RP carried out the molecular genetic studies, participated in the analyzing sequences, maintained the database, and drafted the manuscript. MTM analyzed the histopathological aspects of the study. GR participated in the design of the study and performed the statistical analysis.

AJC, DTA, SR, NT, and PR helped with patient recruitment, patient follow-up, and managed the clinical aspects of the study. PMJ helped to conceive the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Each author acknowledges that this final version was read and approved.

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