Molecular Testing for \textit{BRAFV600E} and \textit{RAS} Mutations from Cytoscrapes of Thyroid Fine Needle Aspirates: A Single-Center Pilot Study

Ojas Gupta\(^1\), Upasana Gautam\(^1\), Muralidaran Chandrasekhar\(^1\), Arvind Rajwanshi\(^2\), Bishan Dass Radotra\(^2\), Roshan Verma\(^2\), Radhika Srinivasan\(^1\)

\(^1\)Department of Cytology and Gynecological Pathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, \(^2\)Department of Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Abstract

\textbf{Context and Aim:} Molecular testing of thyroid FNA has been advocated in the indeterminate categories of The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) 2018. The utility of cytoscrapes of thyroid FNA samples for \textit{BRAFV600E} and \textit{RAS} mutations was evaluated in this pilot study. \textbf{Methods and Materials:} Thyroid FNA samples between 2015 and 2018 from TBSRTC categories 3–6 were included. DNA was extracted from one to two representative smears (cytoscope). Real-time PCR for \textit{BRAFV600E} and \textit{RAS} (\textit{KRAS}, \textit{NRAS}, and \textit{HRAS}) gene mutations was performed. Histopathology correlation was available in 44 cases. \textbf{Statistical Methods:} Chi-square test and calculation of sensitivity, specificity, and positive/negative predictive values were performed. \textbf{Results:} A total of 73 thyroid FNA cases and 11 nodal metastases of papillary thyroid carcinoma (PTC) were evaluated. The DNA yield ranged from 1.9 to 666 ng/\(\mu\)l (mean 128 ng/\(\mu\)l) in 80 cases and was insufficient in four cases. Overall, mutations were seen in 45 (56.25\%) cases with \textit{BRAFV600E}, \textit{NRAS}, \textit{HRAS}, and \textit{KRAS} in 21 (46.7\%), 19 (42.2\%), 4, and 1 cases, respectively. \textit{BRAFV600E} mutation was seen in PTC (11/18, 61\%), nodal PTC metastases (5/10, 50\%), and occasionally in TBSRTC category 3 (1/18, 5.5\%). \textit{NRAS} mutations were seen across all categories and were maximum in the AUS/FLUS group (6/18, 33\%). \textit{BRAFV600E}/\textit{RAS} testing had an overall sensitivity, specificity, and positive and negative predictive values of 61.7\%, 80\%, 91.3\%, and 38\%, respectively, for the detection of malignancy. In indeterminate thyroid nodules, the sensitivity, specificity, PPV, and NPV were 56.2\%, 80\%, 81.8\%, and 53.3\%, respectively. \textbf{Conclusion:} \textit{BRAFV600E}/\textit{RAS} mutation testing from cytoscrapes are useful as a rule-in test for indeterminate thyroid nodules and provide molecular confirmation in nodal metastases of PTC.

\textbf{Keywords:} Bethesda system, \textit{BRAFV600E}, fine needle aspiration, indeterminate cytology, molecular testing, \textit{RAS}, thyroid

Introduction

Fine needle aspiration cytology (FNAC) is a reliable technique for diagnosing malignant thyroid nodules with papillary thyroid carcinoma (PTC), the most common thyroid cancer in our setup.\(^{1-3}\) The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) is the commonly used classification system for reporting thyroid FNAC and categories 3, 4, and 5 of TBSRTC are regarded as grey-zone areas with intermediate malignancy risk.\(^{1-3}\) The recent TBSRTC system has advocated the application of molecular studies to identify the genetic mutations/gene rearrangements in category 3 or AUS (atypia of undetermined significance) in line with the guidelines of the American Thyroid Association.\(^{4}\) This would potentially identify the patients for surgery which includes near-total thyroidectomy or lobectomy. The most common alteration involves the B-type RAF kinase (\textit{BRAF}) and the \textit{RAS} genes followed by rearrangements of \textit{RET}/PTC

Address for correspondence: Dr. Radhika Srinivasan, Professor and Head, Department of Cytology and Gynecological Pathology, Postgraduate Institute of Medical Education and Research, Chandigarh - 160012, India. E-mail: drsradhika@gmail.com

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Gupta O, Gautam U, Chandrasekhar M, Rajwanshi A, Radotra BD, Verma R, \textit{et al}. Molecular testing for \textit{BRAFV600E} and \textit{RAS} mutations from cytoscapes of thyroid fine needle aspirates: A single-center pilot study. J Cytol 2020;37:174-81.

Submitted: 09-Aug-2020, Revised: 17-Sep-2020, Accepted: 23-Sep-2020, Published: 07-Nov-2020
and \(PAX8/PPAR\) genes. \(BRAFV600E\) mutation is present in 45–80% of classical PTC, 5–25% of follicular variant of PTC or FVPTC, 1.4% of follicular thyroid carcinoma, 5–15% of poorly differentiated thyroid carcinoma or PDC, and 10–50% of anaplastic thyroid carcinoma.[5,6] The rat sarcoma (\(RAS\)) oncogene family includes three genes \(HRAS\), \(NRAS\), and \(KRAS\). The prevalence of \(RAS\) mutations in thyroid cancer is 20–40% and is found in follicular lesions including adenomas, carcinomas, and FVPTCs.[6,7] The \(PAX8/PPAR\) gene fusion is present in thyroid follicular lesions, both in adenoma and in carcinoma, while the \(RET/PTC\) gene rearrangement is seen in 5–35% of PTC.[5,6]

We aimed to evaluate the utility of detection of \(BRAFV600E\) and \(RAS\) (\(HRAS\), \(KRAS\), and \(NRAS\)) gene mutations by real-time polymerase chain reaction across the TBSRTC spectrum and with special focus on its value for the indeterminate thyroid nodules including categories 3, 4 and 5 of TBSRTC. We also included cases of nodal metastases of PTC in this analysis. We exclusively used DNA obtained by cytoscrapes of representative smears of the thyroid lesions to confirm its feasibility.

**Materials and Methods**

This was a retrospective analysis of cases referred to the Department of Cytology & Gynaec Pathology, PGIMER, Chandigarh, for FNAC of thyroid swelling between July 2015 and June18. The approval for the study was obtained from the Institute Ethics Committee vide letter no. INT/IEC/2019/001424 dated 18.07.19 with a waiver for obtaining individual consent. A total of 84 cases in category 3–6 of TBSRTC were included in the study. The number of cases was restricted to 84 based on the resources available. The inclusion criteria was a TBSRTC category 3–6 with adequate material available on the smears. All unsatisfactory and benign thyroid aspirates (category 2) were excluded for molecular testing. In all these cases, FNAC was routinely done using a 23G needle attached to a 20-ml syringe in the specially designed holder (Camco, AB Taby) and May Grünwald–Giemsa (MGG) stained air-dried smears and Haematoxylin & Eosin stained alcohol fixed smears were routinely evaluated. The histopathological (HPE) follow-up of these cases wherever available was also recorded and compared with cytology findings.

**Extraction of DNA:** Genomic DNA was extracted generally from air-dried, May-Grünwald–Giemsa stained smears of the cases included. The slides chosen were moderately cellular containing at least 10 clusters of cells with a minimum of 20 cells. If the cellularity was low, then one more smear was used. The entire slide was dipped in water for 2–3 seconds and scraped manually using a sterile scalpel blade and the material was transferred to an Eppendorf tube. This was referred to as “cytoscrape.” Genomic DNA was extracted from the material in the Eppendorf tube using a commercially available DNA extraction kit (Qiagen DNeasy Blood and Tissue kit, GmBH, Hilden, Germany) following the manufacturer’s protocol. The DNA yield was quantified with a spectrophotometer by measuring the absorbance at 260 nm. The purity of DNA was determined by the ratio of A260/280 and a ratio of 1.8 to 2.0 was taken as an acceptable value for DNA. Two microliters of DNA was also run on a 2% agarose gel to check for its quality.

**Real-Time PCR:** Real-time PCR (Agilent technologies—AriaMx Real-time PCR system, Agilent Technologies, Santa Clara, USA) was performed using an EntroGen thyroid mutation analysis kit (THDNA-RT64, Entrogen Inc, CA, USA). Each reaction well contains primer sets and probes for the detection of somatic mutations as well as an internal control gene. The assay works by amplifying the mutant-specific sequences in the samples that contain a mixture of mutant and wild-type DNA. The probes were used labeled with fluorochromes, Fluorescein amide (FAM) and VIC (2′-chloro-7′phenyl-1,4-dichloro-6-carboxyfluorescein). The method was standardized using 20 ng of DNA for each gene mutation. Each reaction contained 15 μl of the reaction mix, 1.5 μl of 20 ng of DNA, 6 μl of primer, and 7.5 μl of water in a final volume of 30 μl. The RT-PCR was carried out using the following run conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 secs, and 60°C for 60 secs. The results were analyzed using the AGILENT AriaMx 1.0 software and called out as positive or negative for mutation based on the cycle threshold (Ct) values for the two probes used as per the manufacturer’s instructions. This assay uses two probes, VIC and FAM which detect the internal control and test mutation, respectively. A cycle threshold (Ct) value of \(\leq 38\) for FAM and \(\geq 25\) for VIC indicate positivity for mutation (internal control and test positive); values of \(<38\) for FAM and \(<25\) for VIC indicated excess DNA; values \(>38\) for FAM and \(<31\) for VIC indicate negativity for the mutation, and values \(>38\) for FAM and \(<31\) for VIC indicated insufficient DNA.

**Statistical analysis:** The statistical analysis was carried out using the IBM Statistical Package for Social Sciences software version 22. Qualitative data or categorical variables were described as frequencies and proportions. Proportions were compared using a Chi-square test. A \(P\) value \(<0.05\) was considered to be statistically significant.

**Results**

A total of 84 cases were included in the study. There were 64 females and 20 males with a mean age of 42.5 (2–70) years and a median age of 43 years. These included 73 primary thyroid lesions and 11 cases of lymph nodal metastases from PTC. The break-up of cases as per TBSRTC categories along with the histopathology follow-up and mutations observed are detailed in Table 1. The cohort included 28 thyroid malignant neoplasms (including 19 conventional PTC and one follicular variant ofPTC), 11 nodal metastases of PTC, and the remaining 45 cases were indeterminate thyroid nodules with 20, 9, and 16 cases of TBSRTC categories 3, 4, and 5, respectively.
The histopathology outcomes in 44 of these cases is also shown [Table 1].

**DNA extraction from cytocles and its quantitation in various categories**

Overall, sufficient DNA was available for molecular analysis in 80 cases, and in the remaining four cases, it was insufficient and hence were excluded from the study. The quantity of DNA extracted from the cytocles ranged from 1.9 to 666 ng/µl, with a mean value of 128 ng/µl. The range and mean DNA extracted for each of the TBSRTC categories is shown in Table 1. Category 5 cases showed the lowest mean DNA extracted followed by the nodal metastases group and categories 3, 6, and 4.

**Mutational analysis for BRAF and RAS genes**

Molecular testing could be performed in 80 cases, and 45 cases (54.9%) showed positivity for BRAF/RAS gene mutations whereas the remaining 35 cases were negative [Table 2]. The most common mutation was BRAFV600E mutation which is seen in 21 cases (46.7%) followed by NRAS mutation in 19 cases (42%), HRAS mutation in four cases (8.9%), and KRAS mutation in only one case (2%). The cases which showed BRAFV600E mutation were mostly in cases confirmed as PTC and in nodal metastases of PTC and a single case in category 5, suspicious of malignancy. It was also detected in one case in the AUS/FLUS group which did not have a histopathology follow-up. A representative case of PTC positive for BRAF mutation is illustrated in Figure 1 (a–c). On the other hand, RAS gene mutations were spread across categories 3–6. NRAS gene mutations were seen in 19 cases and across all categories with six, three, four, and five cases in categories 3, 4, 5, and 6, respectively, and in one case of nodal metastases [Tables 1 and 2]. In eight cases that were positive for NRAS mutation, follow-up histopathology revealed malignancy in seven cases with five follicular carcinomas, one FVPTC, and one conventional PTC. Hence, detection of NRAS gene mutation had a high (87.5%) predictive value for malignancy.

In 35 cases, no mutations were detected [Table 2]. Four cases were nodal PTC metastases. Histopathological follow-up was benign in eight cases (two nodular goiters, one Hürthle cell adenoma, and five follicular adenomas) and malignant in 13 cases (five conventional PTC, two nodal PTC metastases, three FVPTC, one follicular carcinoma, one Hürthle cell carcinoma, and one poorly differentiated carcinoma), thereby indicating the low predictive value of a negative test.

**TBSRTC category 3, 4, and 5 or indeterminate thyroid nodules**

Molecular testing could be performed in 18 of 20 cases of AUS/FLUS from cytocles of representative smears and 9/18 (50%) tested positive. Fifteen cases showed architectural atypia (AA), two cases showed cytological atypia (CyA), and one showed both (CyA/AA). The most common mutation was in the NRAS gene (six cases, 33.3%), followed by one case each positive for BRAFV600E (5.6%), HRAS (5.6%), and KRAS (5.6%) mutations. Of the 15 cases subcategorized as AA, no mutations were detected in seven cases, NRAS mutations were seen in four cases, and HRAS and BRAFV600E mutations were seen in one case each, respectively; in two cases, molecular testing was technically compromised. Among two cases with CyA, one showed NRAS mutation while others showed no mutations. The CyA/AA case showed NRAS mutation. Follow-up histopathology was available in ten cases with one
PTC (BRAFV600E mutation positive), one FVPTC (NRAS mutation positive), five follicular adenomas (one HRAS, one NRAS and three no mutations), one case each of Hurthle cell adenoma, colloid goiter, and follicular hyperplasia that were negative for any mutation. A representative case of FLUS (follicular lesion of undetermined significance) with NRAS mutation whose histopathology revealed follicular adenoma is shown in Figure 1 (d–f).

There were nine cases in category 4, of which three showed HRAS and NRAS mutation each and the rest three were negative. Of the three cases with no detectable mutations, follow-up histopathology revealed FVPTC, follicular carcinoma, and poorly differentiated carcinoma in one case each, respectively. Follicular carcinoma was seen on histopathology in two out of three cases that showed NRAS and HRAS mutations, respectively, and HPE was not available in the remaining cases. A representative case of follicular neoplasm on cytology with HRAS mutation whose histopathology revealed follicular carcinoma is shown in Figure 1 (g–i).

Among 16 cases in category 5, suspicious for malignancy, seven mutations (43.8%) were seen with four NRAS (25%) and three (18.8%) BRAFV600E mutations. The histological outcome was conventional PTC in four cases (one BRAFV600E and three no mutations), FVPTC in one case (no mutation), follicular carcinoma in two cases with NRAS mutation, and follicular adenoma in two cases with no mutations detected. Two representative cases in category 5, suspicious for PTC, one with no mutations detected and FVPTC on histopathology

| TBSRTC                  | No. of Cases | No mutation detected | Mutation detected | BRAF | NRAS | HRAS | KRAS |
|-------------------------|--------------|----------------------|-------------------|------|------|------|------|
| Cat 3                   | 18           | 9                    | 9 (50%)           | 1    | 6    | 1    | 1    |
| Cat 4                   | 9            | 3                    | 6 (66.7%)         | 0    | 3    | 3    | 0    |
| Cat 5                   | 16           | 9                    | 7 (43.75%)        | 3    | 4    | 0    | 0    |
| Cat 6                   | 27           | 10                   | 17 (66.7%)        | 12   | 5    | 0    | 0    |
| Nodal metastases       | 10           | 4                    | 6 (55.6%)         | 5    | 1    | 0    | 0    |
| Total                   | 80           | 35                   | 45 (56.25%)       | 21   | 19   | 4    | 1    |

*cases with technically noncontributory results excluded
and the second with BRAFV600E and PTC on histopathology are depicted in Figure 2 (a–c and d–f).

**Mutational analysis of nodal metastases (N = 11)**

Mutational analysis was performed from 10 out of 11 lymph nodal metastases of PTC as in one case the results were technically noncontributory. Overall, 5/10 (50%) showed BRAFV600E mutation; NRAS mutation was present in one case and none detected in four cases [Table 1]. In all these cases which initially presented as nodal metastases and diagnosed by FNA, the thyroid primary lesion was detected subsequently by imaging and ultrasound-guided FNA confirmed PTC of the thyroid. HPE correlation was available for seven cases, which showed PTC with lymph nodal metastases, six conventional and one FVPTC. A representative case positive for BRAFV600E mutation is illustrated in Figure 2 (g–i).

**Histopathological outcome and correlation to gene mutational analysis**

The gene mutations in histologically proven cases (N = 44) are shown in Table 3. It was observed that BRAFV600E mutation was restricted to PTC including FVPTC; however, NRAS mutation was seen in follicular neoplasms as well as few cases of PTC. All cases reported as follicular adenoma and Hürthle cell adenoma were assumed to have a benign outcome for calculation of the sensitivity and specificity of molecular testing for BRAF and RAS gene mutations. The results of the correlation of gene mutation detection with the histological outcomes are tabulated in Table 4A and 4B. There was a positive correlation of gene mutation detection with the overall malignant histological outcome (Chi-square, 5.402; $P = 0.02$). However, in the indeterminate category, this association was not seen ($p = 0.06$). The sensitivity of molecular testing for

| Histopathology       | BRAF V600E | NRAS | HRAS | None detected |
|----------------------|------------|------|------|---------------|
| PTC (13)             | 7 (53.8%)  | 1 (7.6%) | 0 (0%) | 5 (38.4%)     |
| Nodal PTC (6)        | 4 (66.7%)  | 0    | 0    | 2 (33.3%)     |
| FVPTC (5)            | 1 (20%)    | 1 (20%) | 0    | 3 (60%)       |
| Follicular (8)       | 0          | 5 (62.5%) | 2 (25%) | 1 (12.5%)    |
| Carcinoma PD carcinoma (1) | 0    | 0    | 0    | 1 (100%)    |
| Hürthle cell (1) Carcinoma | 0    | 0    | 0    | 1 (100%)    |
| Follicular (7)       | 0          | 1 (14.3%) | 1 (14.3%) | 5 (71.4%)    |
| Adenoma              | 0          | 0    | 0    | 1 (100%)     |
| Hürthle cell (1) Adenoma | 0    | 0    | 0    | 1 (100%)     |
| Multinodular (2) Goiter | 0    | 0    | 0    | 2 (100%)     |
| Total (44)           | 12         | 8    | 3    | 21           |

PTC- Papillary thyroid carcinoma; FVPTC- Follicular variant of PTC; PD- poorly differentiated

**Figure 2:** Each row represents one illustrative case. a–c, Suspicious for PTC on cytology (category 5) with no mutations detected and FVPTC on histopathology; d–f, Suspicious for PTC on cytology (category 5), positive for BRAFV600E mutation, and PTC on histopathology; g–i, Metastatic PTC in lymph node on cytology, positive for BRAFV600E mutation, and confirmed on histopathology [a, g x400, d x200– May-Grünwald–Giemsa stain; c x400, f, i x200– Hematoxyline-Eosin stained section; b, e, and h—screenshots of plots obtained]
**Table 4: Correlation of gene mutations with histological outcomes**

**A: Overall histological outcome**

| Histological Outcome | Mutation Positive | Mutation Negative | Total |
|----------------------|-------------------|-------------------|-------|
| Benign               | 2                 | 8                 | 10    |
| Malignant            | 21                | 13                | 34    |
| Total                | 23                | 21                | 44    |

chi-square = 5.402; p-value = 0.0201

| Histological Outcome | Mutation Positive | Mutation Negative | Total |
|----------------------|-------------------|-------------------|-------|
| Benign               | 2                 | 8                 | 10    |
| Malignant            | 9                 | 7                 | 16    |
| Total                | 11                | 15                | 26    |

Chi-square = 3.313; p-value = 0.068. * includes Follicular and Hürthle cell adenoma

**BRAF** and **RAS** genes was 61.7%, specificity was 80%, positive predictive value (PPV) was 91.3%, and negative predictive value (NPV) was 38%. In the indeterminate category, the sensitivity of molecular testing for **BRAF** and **RAS** genes was 56.2% with a specificity of 80%, PPV of 81.8%, and NPV of 53.3%.

**DISCUSSION**

The current version of TBSRTC recommends molecular testing for category 3 (atypia of undetermined significance) in line with the guidelines of the American Thyroid Association.[2] In the present study, we evaluated a commercially available simple 5-gene mutation kit in TBSRTC categories 3–6 and nodal metastases of PTC. The material for molecular testing may be obtained as a direct sample at the time of performing the FNA. However, this is operator dependent and may not be taken at the time of FNA due to the paucity of the sample. The cytoscrape technique was therefore employed in this retrospective analysis, wherein DNA was extracted from one representative moderately or highly cellular air-dried May-Grünwald–Giemsa stained smear, thereby ensuring lesional representation. The quantity of DNA for molecular testing was insufficient in four cases with adequate DNA in the remaining 80 cases. Category 5 smears showed the least amount of DNA obtained followed by category 3. The reason for this was the lower smear cellularity and in these two categories, we used two smears generally to obtain adequate DNA. DNA in direct cytology smears is easily extractable and is stable for six months to a few years.[3] The use of stained rather than unstained smears offers the advantage of ensuring lesional representation. Diff-Quik stained smears can provide high-quality DNA for sophisticated molecular studies.[8,9] Further, DNA appears to be preserved better in such smears as compared to Papanicolaou-stained smears, where DNA degradation occurs as a function of aging.[9] DNA extracted from microdissected direct cytologic smears from various sites like lung, lymph node, liver, soft tissue, and thyroid was also found suitable for next-generation sequencing.[10] In another study, Ferraz et al. have also extracted RNA from air-dried smears of thyroid aspirates for molecular testing of gene rearrangement.[11] We reaffirm that cytoscrapes from air-dried MGG stained smears provide sufficient DNA for molecular testing in thyroid aspirates in all categories. The RT-PCR based kit employed required only 100 ng per sample for testing five gene mutations (**BRAF**-V600E, **NRAS**, and **HRAS**, and two mutations in **KRAS** genes) which is an advantage in cytology samples. This is a sensitive assay with a quick turnaround time of 3–4 hours and cost per test being approximately INR 3000 makes it affordable to our patients with limited resources.

PTC can present as metastasis in the cervical lymph node with or without a palpable thyroid lesion. In all the cases of nodal metastases included in this cohort, the thyroid primary was not clinically palpable. Of 11 cases, molecular testing was possible in ten cases and five showed **BRAF**-V600E mutation and one showed **NRAS** mutation. The thyroid primary lesion was confirmed subsequently upon imaging and ultrasound-guided fine-needle aspiration cytology. In such a clinical scenario, confirmation of the cytomorphological diagnosis by molecular testing can be a useful ancillary technique.

The overall mutation (**BRAF** or **RAS**) positive cases were 54.9%. Taking histopathology as the gold standard, the sensitivity, specificity, PPV, and NPV of detecting a malignant lesion with any of these mutations were 61.7%, 80%, 91%, and 38%, respectively. Eszlinger et al.[12] showed a sensitivity of 75.3% and 90.4% specificity for detecting the malignant lesions by combining the FNA and molecular studies. Similar results were seen in literature where the presence of any mutations has been found to be a good predictor of malignancy.[5,13,14] Studies in the literature have also shown that by performing molecular tests, the sensitivity of detecting malignant thyroid nodules increases to 80–90%.[14,15]; however, these studies have also included detection of **RET-PTC** and **PAX/PPARy**. The detection of gene rearrangements requires the extraction of total RNA from the sample which was not performed in this study. Few authors have demonstrated the feasibility of extracting RNA from routine air-dried FNA smears for the detection of **PAX8/PPARG** and **RET/PTC** rearrangements with RT-qPCR.[11,12] This will add to the overall cost of the test as cDNA would need to be synthesized from the extracted mRNA followed by appropriate controls and capillary sequencing of any gene fusion detected by qRT-PCR.

**BRAF**-V600E mutation was seen in 53.8% of histologically proven PTC which is concordant with several studies and is reviewed by Nikiforov.[15] In category 3, **BRAF**-V600E mutation was seen in just one case (5%) but was seen in 19% in category 5; none was seen in category 4. Studies have shown 15–39% **BRAF** positivity in indeterminate/nondiagnostic cases.[15,17] In a meta-analysis, Su et al. reported **BRAF** positivity...
rates of 43.2% in suspicious for malignancy, 13.77% in AUS/FLUS, and 4.43% in follicular neoplasm categories.[18] However, it is highly specific for a diagnosis of PTC and in another meta-analysis, the prediction of malignancy (POM) of BRAF for PTC was found to be 99.8%.[17] In suspected nodal metastases of PTC, BRAF mutation was positive in just 50% cases, which was lower than expected as BRAFV600E-positive PTCs are well-known to have aggressive behavior and nodal metastases.[19] 

NRAS mutation was seen predominantly in the category 3 (AUS/FLUS) group with a 66.7% positivity rate. Histology follow-up of eight NRAS mutation–positive cases revealed malignancy in seven cases with follicular carcinoma most commonly observed with just a single case of follicular adenoma. HRAS mutation positivity was restricted to Bethesda categories 3 and 4 with either follicular carcinoma or adenoma outcome. Some studies have shown that NRAS and HRAS–positive follicular adenomas are precursors for follicular carcinoma.[6,7,11,20] However, in a recent meta-analysis, Nabhan et al. reported the wide variability in the frequency of RAS mutations in the indeterminate cytology category. They concluded that future studies are required to clarify the management of RAS-mutated nodules and to understand the potential pathways from a RAS-mutated benign neoplasm to invasive carcinoma.[21] 

In the present study, FVPTC cases (two cytologically suspected and five histologically proven) showed NRAS and BRAFV600E mutations in one case each which was concordant with the other similar studies.[6,7,22] Interestingly, we did not encounter any histologically proven case of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) in this cohort of patients which differs from the previously reported histological outcome for the indeterminate category from other parts of the world.[7,12,23-25] A total of 35 cases did not reveal any mutations in the BRAF/RAS genes with an equal probability of benign or malignant outcome clearly implying the low negative predictive value of this molecular assay. Further, no mutations were detected in 21/43 (48.8%) cases in the indeterminate cases. Hwang et al.[24] reported 21.3% of indeterminate cases were negative for NRAS or BRAF mutations. Nikiforov et al.[15,15] studied seven gene panels including RET/PTC1, RET/PTC3, and PAX8/PPARG rearrangements and showed only 6% of indeterminate nodules were negative for these mutations. This clearly implies the need for an extended panel to include gene fusions to resolve a greater number of cases. However, this requires RNA extraction from the sample which was not performed due to limited resources. In a recent report by Bellevicine et al.[26] a seven-gene panel was tested in 162 AUS/FLUS cases of which, only four gene fusions (PAX8/PPARG and RET/PTC) were detected, implying their rarity in this category; RAS gene mutations were the most common supporting our observations. A correlation of BRAFV600E with a CyA qualifier was observed. Hence, testing for 5-gene mutations only may be justified in the indeterminate nodules. The limitations of this study are the small number of subjects who were tested only for gene mutations and not evaluated for RET-PTC and PAX8-PPARG gene rearrangements. To conclude, this is the first study from India to demonstrate the application of molecular testing in FNA of thyroid using cytoscrapes. The commercially available multiplex RT-PCR assay kit testing for 5-genes requires only a small amount of DNA and is easy to perform in routine molecular cytology laboratories with modest resources, as it is a low cost procedure with a quick turnaround time. Among the indeterminate thyroid categories, this 5-gene molecular assay showed a PPV of 81% making it a good rule-in test. 

Financial support and sponsorship

The study was supported by a Departmental grant, PGIMER, Chandigarh.

Conflicts of interest

There are no conflicts of interest.

Presented at: International Cytology Congress, Sydney, 2019.

REFERENCES

1. Mahajan S, Sinrivasan R, Rajwanshi A, Radotra B, Panda N, Dey P, et al. Risk of malignancy and risk of neoplasia in the Bethesda indeterminate categories: Study on 4,532 thyroid fine-needle aspirations from a single institution in India. Acta Cytol 2017;61:103-10.

2. Cibas ES, Ali SZ. The 2017 Bethesda system for reporting thyroid cytopathology. Thyroid 2017;27:1341-6.

3. Bongiovanni M, Spitale A, Faquin WC, Mazzucchelli L, Baloch ZW. The Bethesda system for reporting thyroid cytopathology: A meta-analysis. Acta Cytol 2012;56:333-9.

4. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association Management Guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: The American Thyroid Association Guidelines Task Force on thyroid nodules and differentiated thyroid cancer. Thyroid 2016;26:1-133.

5. Nikiforov YE. Molecular analysis of thyroid tumors. Mod Pathol 2011;24(Suppl 2):S34-43.

6. Lloyd RV, Osamura RY, Klöppel G, Rosai J, editors. WHO Classification of Tumours of Endocrine Organs. 4th ed. Lyon: IARC; 2017.

7. An JH, Song KH, Kim SK, Park KS, Yoo YB, Yang JH, et al. RAS mutations in indeterminate thyroid nodules are predictive of the follicular variant of papillary thyroid carcinoma. Clin Endocrinol (Oxf) 2015;82:760-6.

8. Knoepp SM, Roh MH. Ancillary techniques on direct-smear aspirate slides: A significant evolution for cytopathology techniques. Cancer Cytopathol 2013;121:120-8.

9. Killian JK, Walker RL, Suuriniemi M, Jones L, Scurruci S, Singh P, et al. Archival fine-needle aspiration cytology (FNAC) samples: Untapped resource for clinical molecular profiling. J Mol Diagn 2010;12:739-45.

10. Roy-Chowdhuri S, Goswami RS, Chen H, Patel KP, Routbort MJ, Singh RR, et al. Factors affecting the success of next-generation sequencing in cytology specimens. Cancer Cytopathol 2015;123:659-68.

11. Ferraz C, Rehfeld C, Krogdahl A, Precht Jensen EM, Rosenberg E, Naz F, et al. Detection of PAX8/PPARG and RET/PTC rearrangements is feasible in routine air-dried fine needle aspiration smears. Thyroid 2012;22:1025-30.

12. Eszlinger M, Krogdahl A, Munz S, Rehfeld C, Precht Jensen EM, Ferraz C, et al. Impact of molecular screening for point mutations and rearrangements in routine air-dried fine-needle aspiration samples of thyroid nodules. Thyroid 2014;24:305-13.

13. Cantara S, Marzocchi C, Fili T, Cardinale S, Forleo R, Castagna MG, et al. Molecular signature of indeterminate thyroid lesions: Current
methods to improve Fine Needle Aspiration Cytology (FNAC) diagnosis. Int J Mol Sci 2017;18:775.
14. Cantara S, Capezzone M, Marchisotta S, Capuano S, Busonero G, Toti P, 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;95:1365-9.
15. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, et al. Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab 2009;94:2092-8.
16. Ohori NP, Singhal R, Nikiforova MN, Yip L, Schoedel KE, Coyne C, et al. BRAF mutation detection in indeterminate thyroid cytology specimens: Underlying cytologic, molecular, and pathologic characteristics of papillary thyroid carcinoma. Cancer Cytopathol 2013;121:197-205.
17. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, et al. Impact of the multi-gene ThyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance cytology. Thyroid 2015;25:1217-23.
18. Su X, Jiang X, Xu X, Wang W, Teng X, Shao A, et al. Diagnostic value of BRAF (V600E)-mutation analysis in fine-needle aspiration of thyroid nodules: A meta-analysis. Onco Targets Ther 2016;9:2495-509.
19. Xing M, Clark D, Guan H, Ji M, Dackiw A, Carson KA, et al. BRAF mutation testing of thyroid fine-needle aspiration biopsy specimens for preoperative risk stratification in papillary thyroid cancer. J Clin Oncol 2009;27:2977-82.
20. Fallahi P, Giannini R, Miccoli P, Antonelli A, Basolo F. Molecular diagnostics of fine needle aspiration for the presurgical screening of thyroid nodules. Curr Genomics 2014;15:171-7.
21. Nabhan F, Porter K, Lupo MA, Randolph GW, Patel KN, Kloos RT. Heterogeneity in positive predictive value of RAS mutations in cytologically indeterminate thyroid nodules. Thyroid 2018;28:729-38.
22. Censi S, Cavedon E, Bertazza L, Galuppin F, Watutantrige-Fernando S, De Lazzari P, et al. Frequency and significance of Ras, Tert Promoter, and Braf Mutations in cytologically indeterminate thyroid nodules: A monocentric case series at a tertiary-level endocrinology unit. Front Endocrinol (Lausanne) 2017;8:273.
23. Gupta N, Dayam AK, Carty SE, Nikiforova MN, Ohori NP, Armstrong M, et al. RAS mutations in thyroid FNA specimens are highly predictive of predominantly low-risk follicular-pattern cancers. J Clin Endocrinol Metab 2013;98:E914-22.
24. Hwang TS, Kim WY, Han HS, Lim SD, Kim WS, Yoo YB, et al. Preoperative RAS mutational analysis is of great value in predicting follicular variant of papillary thyroid carcinoma. Biomed Res Int 2015;2015:697068. doi: 10.1155/2015/697068.
25. Kim SJ, Roh J, Baek JH, Hong SJ, Shong YK, Kim WB, et al. Risk of malignancy according to sub-classification of the atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS) category in the Bethesda system for reporting thyroid cytopathology. Cytopathology 2017;28:65-73.
26. Bellevicine C, Sgariglia R, Migliati I, Viglier E, D’Anna M, Nacchio MA, et al. Different qualifiers of AUS/FLUS thyroid FNA have distinct BRAF, RAS, RET/PTC, and PAX8/PPARγ alterations. Cancer Cytopathol 2018;126:317-25.