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RESEARCH ARTICLE

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VE1 immunohistochemistry is an adjunct tool for detection of $BRAF^{V600E}$ mutation: Validation in thyroid cancer patients

Faiza A. Rashid[1] | **Sobia Tabassum**[1] | **Mosin S. Khan**[2] | **Hifzur R. Ansari**[3] | **Muhammad Asif**[4] | **Ahmareen K. Sheikh**[5] | **Syed Sameer Aga**[6]

[1]Department of Biological Sciences, International Islamic University, Islamabad, Pakistan

[2]Government Medical College Srinagar and Associated SMHS and Superspeciality Hospital, Srinagar, India

[3]King Abdullah International Medical Research Center, King Saud bin Abdulaziz University for Health Sciences, Ministry of National Guard Health Affairs, Jeddah, Saudi Arabia

[4]Department of Histopathology, Armed Forces Institute of Pathology, central Military Hospital, Rawalpindi, Pakistan

[5]Department of Pathology, Pakistan Institute of Medical Sciences, Islamabad, Pakistan

[6]Basic Medical Sciences, College of Medicine- Jeddah, King Saud bin Abdulaziz University for Health Sciences, King Abdullah International Medical Research Center, Ministry of National Guard Health Affairs, Jeddah, Kingdom of Saudi Arabia

Correspondence

Sobia Tabassum, Department of Biological Sciences, International Islamic University, H-10 Campus, Islamabad, Pakistan.
Email: sobia.tabasum@iiu.edu.pk

Syed Sameer Aga, Basic Medical Sciences, College of Medicine- Jeddah, King Saud bin Abdulaziz University for Health Sciences, King Abdullah International Medical Research Center, Ministry of National Guard Health Affairs, Jeddah, Kingdom of Saudi Arabia.
Email: agas@ksau-hs.edu.sa; agasy@ngha.med.sa

Abstract

Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy among other endocrine tumors, and $BRAF^{V600E}$ is a frequent genetic mutation occurring in the disease. Although different molecular techniques, most importantly sequencing has been widely recognized as a gold standard but molecular diagnosis remains an expensive, laborious, and time-intensive process. Recently, immunohistochemistry (IHC) with anti-BRAF V600E (VE1) antibody has increased practical utility and implemented clinically for the detection of $BRAF^{V600E}$ mutation. Therefore, the study aimed to evaluate diagnostic accuracy of VE1 IHC for detecting the $BRAF^{V600E}$ mutation frequency and clinical implementation in diagnostic laboratories. In this study, 72 formalin fixed paraffin-embedded tissues (FFPE) were used to determine the $BRAF^{V600E}$ mutation status using IHC and Sanger sequencing. The mutation was found in 29% and 28% cases using IHC and Sanger sequencing, respectively. Furthermore, the results showed 100% sensitivity, 98.07% specificity, 95.2% positive predictive value, and 100% negative predictive value. Notably, significant associations were found between $BRAF^{V600E}$ status and tumor stage, tumor focality, and extrathyroidal extensions, respectively. VE1 IHC was found to be a highly sensitive, specific, and diagnostically accurate method in this cohort. Therefore, $BRAF^{V600E}$ detection through IHC has been considered as the best tailored technique for routine pathology laboratories.

KEYWORDS

$BRAF^{V600E}$, diagnostic accuracy, papillary thyroid carcinoma, sanger sequencing, VE1 immunohistochemistry

[Correction added on 18 December 2020, after first online publication: Affiliation number 6 and figures legends for Figure 2 and 3 has been corrected.]

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1 | INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most frequent type of thyroid cancer (TC) that accounts for greater than 80% of thyroid malignancies.[1,2] Recently, molecular target therapies based on specific oncogenic genetic aberrations have yielded promising results for the treatment of PTC. Enormous efforts have uncovered aberrations involved in the development and progression of PTC.[3] *BRAF* mutations are the most common oncogenic driver mutations correlated with thyroid cancer.[4] Among all *BRAF* mutations identified, *BRAF*^{V600E} accounts for more than 90% of those mutations. It encodes a serine threonine protein kinase, belonged to mitogen activated protein kinase signaling (MAPK) pathway.[5] The missense mutation is present in exon 15 of gene and located at chromosome 7q34. *BRAF* T>A transversion (thymine to adenine) at nucleotide position 1799 (c.T1799>A) results in substitution of valine (V) into glutamic acid (E) at codon 600.[6,7]

The rate of *BRAF*^{V600E} mutation in PTC patients mostly depends on the target population and clinicopathological characteristics, including gender, age, tumor stage, tumor focality, lymphovascular invasions, and extrathyroidal extensions.[8] The mutation is found in approximately 50% of PTC cases among western series descended from the USA.[9,10] Overall, the prevalence rate varies in different countries, primarily Asian populations have a higher *BRAF* rate than Western countries.[11] However, the prevalence is more heterogeneous in Asian populations, spanning from 28.2% to 90% mutation.[12-14]

In routine molecular pathology laboratories, different molecular methodologies such as Sanger sequencing, allele-specific PCR (AS-PCR), droplet digital PCR (ddPCR) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are employed for the detection of *BRAF*^{V600E} mutation[7,15-18]. Although different techniques have different performance rates and sensitivities, the technique performs to detect the mutation may have significant correlation with prevalance of *BRAF*^{V600E}. Sanger sequencing has been widely acknowledged as a gold standard to determine the *BRAF*^{V600E} mutation, but molecular examination remains laborious, time intensive and an expensive process.[19-21] Additionally, molecular methods require established molecular pathology laboratories, to determine *BRAF*^{V600E} mutation which however is not always possible in resource constraint settings.[22,23]

Over the past 5 to 10 years, the clinical utility of IHC with mutation-specific antibodies has increased considerably and implemented clinically for the detection of mutation.[24,25] Several studies have been reported on the performance of VE1 IHC technique to detect the *BRAF*^{V600E} mutation, and most of these reports showed exceptional concordance between this method and molecular genotyping, thus IHC can be used as an alternative method to Sanger sequencing.[1,26,27] The IHC technique consumes less time to perform multiple tests as compared to molecular methods, consequently decreasing the turnaround time.[8]

The foremost purpose of this study was to evaluate concordance rate between VE1 IHC and Sanger sequencing to determine the *BRAF*^{V600E} mutation in our cohort, in correlation to various clinicopathological characteristics of PTC patients.

2 | MATERIALS AND METHODS

2.1 | Study design

This was a hospital-based retrospective study conducted by the Department of Biological Sciences, International Islamic University, Islamabad and Department of General Surgery, Pakistan Institute of Medical Sciences (PIMS), Islamabad, Pakistan from 2016 to 2019. The present study was approved by Institution Review Board (IRB) of University and Ethical Review Board (ERB) of Pakistan Institute of Medical Sciences (PIMS), Islamabad. Consent form was signed and obtained from each patient.

2.2 | Sample size and sample collection

We evaluated the clinicopathological information of one hundred (n = 100) TC patients who underwent total/hemi-thyroidectomy at PIMS Islamabad, Pakistan. Surgically operated and histologically confirmed classic PTC and follicular variants of PTC patients were involved in the study. A total of 72 consecutive PTC patients who accomplished inclusion criteria were registered for this study, and their formalin fixed tissue blocks were used for DNA extraction and IHC (Figure 1). All hematoxylin and eosin slides (H&E) of enrolled cases were reviewed independently by two experienced histopathologists, and cases were classified according to diagnostic standards and terminologies of World health Organization.[28]

2.3 | Preparation of tissue samples and DNA isolation

Thin-section slides of FFPE tissue blocks were prepared at the time of diagnosis. The area containing tumor cells on H&E slides was marked by histopathologists, and unstained slides were deparaffinized, depending on the size of selected tumor area. Then, slides were soaked twice in xylene (each time 15 minutes) and subsequently in 96% alcohol for 5 minutes. Slides were once again soaked in distilled water. Tissues were scraped with the disposable needle and transferred to Eppendorf tubes. Total DNA was isolated using FFPE tissue extraction kit ''QIAamp DNA'' (Syngen, Wroclaw, Poland). Quality of isolated DNA was measured using NanoDrop spectrophotometer.

2.4 | *BRAF*^{V600E} mutation analysis

BRAF^{V600E} mutation analysis was done for all 72 cases. After DNA isolation, exon 15 of *BRAF* gene genomic was amplified using primers; Forward 5′-GCTTGCGCTGATAGAATAATGAG -3′, Reverse 5′-GATACTCAGCACGATCCTTGG-3′ (Sigma Aldrich) giving rise

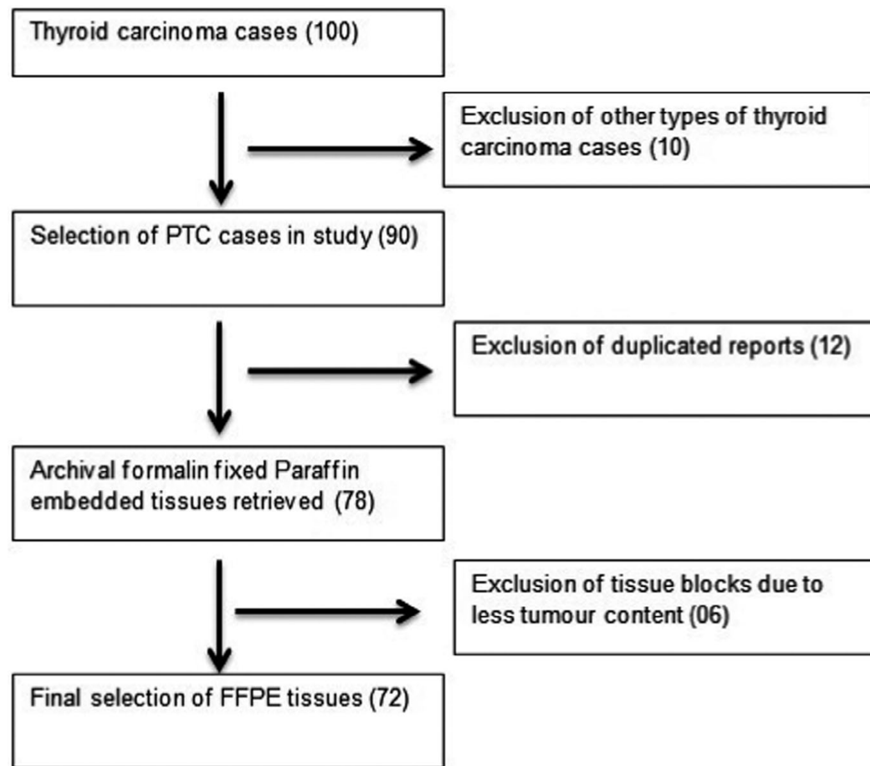

FIGURE 1 Flowchart showing the inclusions and exclusions of study subjects for final analysis

to 224bp amplicon. Sanger sequencing of the amplified product was performed using automated DNA sequencer (ABI sequencer, Applied Biosystems).

2.5 | Detection of *BRAF*^{V600E} mutation using IHC

IHC was performed on 5 μm thick sections of FFPE tissue blocks. Automated Ventanna BenchMark immunostainer was used for analysis. Tissue samples were incubated with the clone VE1 (mouse monoclonal antibody) at 38°C for 5 minutes. VE1 immunoreactivity was visualized using optiview IHC DAB kit (Ventanna Medical Systems). The samples were counterstained with bluing reagent and hematoxylin for 4 minutes. Normal thyroid tissue was used as a negative control tissue. The IHC scoring was independently done by two pathologists by considering H-scoring system, who were unaware to the *BRAF* sequencing status. H-score is a semi quantitative scoring system that is obtained by both intensity of positive cells (0, no staining; 1+, weak; 2+, moderate; 3+, strong) and proportion (0%–100%, increase in 5% increments), as previously explicated.[8,29]

2.6 | Statistical analysis

SPSS statistical software package version 23.0 (SPSS, Chicago, IL) was used to perform Descriptive statistics, chi-square, and Student's *t* test. $P \le 0.05$ was considered as significant. Formulas used for calculating diagnostic accuracy parameters were adopted, as described previously.[30]

3 | RESULTS

3.1 | Clinicopathological features of PTC patients

The demographic and clinicopathological characteristics of PTC cases are described in Table 1. In the present study, 72 PTC tissues were analyzed and their clinicopathological characteristics were noted. Average age of patients at the time of diagnosis was 46 ± 14 years (range, 32 to 60 years). Most of the PTC patients were females (75%) as compared to males. Moreover, younger age patients were more predisposed to PTC than elders in this cohort. Cancer staging was in accordance to the guidelines of American Joint Committee on Cancer (AJCC), 8th edition.[31] Histopathological investigation of PTC tissues showed capsular, lymphovascular invasions, and extrathyroidal extensions in 57%, 58%, and 54% of cases, respectively (Table 1).

3.2 | *BRAF*^{V600E} mutational analysis using IHC and correlation with clinicopathological features PTC patients

The representative pictures showing diffuse and focal staining on performing VE1 IHC are depicted in Figure 2. In terms of

TABLE 1 Demographic and Clinicopathological features of PTC patients

Characteristics	Patients, N = 72 (%)
Age at diagnosis (years), mean ± SD	46 ± 14
Age in years	
Age < 55 years	54 (75)
Age ≥ 55 years	18 (25)
Gender	
Male, n (%)	22 (30.6)
Female, n (%)	50 (69.4)
Staging, Age < 55 years	
Stage I	21 (29.2)
Stage II	33 (45.8)
Staging, Age ≥ 55 years	
Stage I + II	7 (9.7)
Stage III & above	11 (15.3)
Tumor focality	
Multifocal	32 (44.4)
Unifocal	40 (55.6)
Capsular invasion	
Absent	41 (56.9)
Present	31 (43.1)
Lymphovascular invasion	
Absent	42 (58.3)
Present	30 (41.7)
Extrathyroidal extension	
Absent	39 (54.2)
Present	43 (45.8)

Note: Data are represented in percentages (%), except age in mean + SD.

TABLE 2 Correlation of *BRAF*^{V600E} mutation status with clinicopathological features of PTC patients

Characteristics	VE1 IHC, (n = 72)		
	Positive 21 (29.0%)	Negative 51 (71.0%)	*P* value
Age			
<55 years	15 (27.8)	39 (72.2)	0.653
≥55 years	06 (33.3)	12 (66.7)	
Gender			
Male	09 (41)	13 (59)	0.1
Female	12 (24)	38 (76)	
Tumor staging, <55 years			
Stage I	09 (5.8)	12 (15.2)	0.048
Stage II	06 (9.2)	27 (23.8)	
Tumor staging, ≥55 years			
Stage I + II	02 (2.3)	05 (4.7)	0.05
Stage III + above	04 (3)	07 (7.3)	
Tumor focality			
Unifocal	08 (20)	32 (80)	0.05
Multifocal	13 (41)	19 (59)	
Lymphovascular invasion			
Present	15 (35.7)	27 (64.3)	0.19
Absent	06 (20)	24 (80)	
Capsular Invasion			
Present	15 (37)	26 (63)	0.1
Absent	06 (19)	25 (81)	
Extrathyroidal extension			
Present	16 (41)	23 (59)	0.01
Absent	05 (15.2)	28 (84.8)	

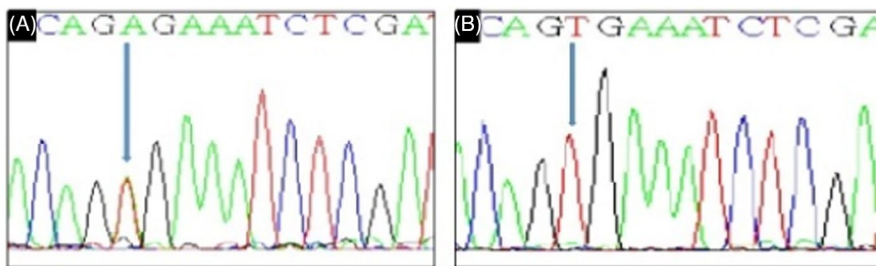

FIGURE 2 Mutation Sequencing: A, Partial electropherogram (forward) of mutant in exon 15 of the BRAF gene codon 600 (T→A;transversion); B, Partial electropherogram (forward) of an adjacent normal

IHC, *BRAF*^{V600E} mutation was present in 29.0% (21 of 72) of enrolled PTC patients and significantly associated with tumor stage, tumor focality and extrathyroidal extension ($P \le 0.05$). However, *BRAF*^{V600E} mutation was not significantly associated with patient age and gender ($P > 0.05$). Capsular and lymphovascular invasions were also not significantly correlated to *BRAF*^{V600E} positive cases ($P > 0.05$) (Table 2).

3.3 | Evaluation of concordance between VE1 IHC and sequencing

The partial electropherograms showing T to A transversion in case of *BRAF*^{V600E} mutation, as depicted in Figure 3. In this study, the *BRAF*^{V600E} mutation was positive in 21/72 cases (29%) using IHC while *BRAF*^{V600E} mutation was positive in 20/72 cases (28%) using Sanger sequencing. When the results of both methods were compared, 20

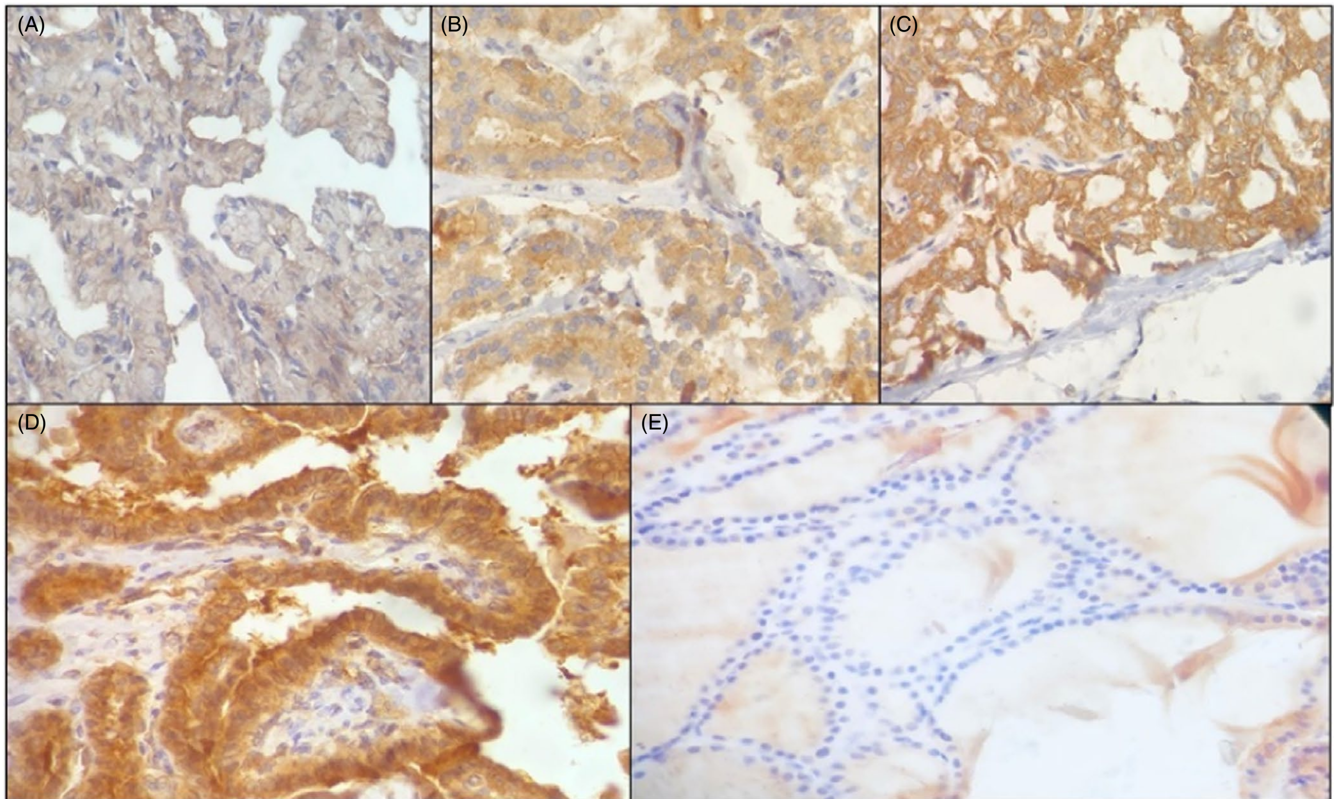

FIGURE 3 Representative images of BRAFV600E mutant VE1 immunostaining. PTC is shown with diffuse cytoplasmic staining A, 0, negative; B, 1+, weak; C, 2+, moderate; D, 3+, strong; E, normal thyroid tissue

TABLE 3 Comparison of IHC and sequencing for determining *BRAF*^{V600E} mutation status

IHC	Sequencing Positive	Sequencing Negative	Total	Concordance rate
Positive				
1+	2	1	3	2/3 (67%)
2+	8	0	8	8/8 (100%)
3+	10	0	10	10/10 (100%)
Negative				
0	0	51	51	51/51 (100%)
Total	20	52	72	71/72 (98.6%)

cases found true positive, while 51 cases were true negative. There were no false negative results. However, one case with positive *BRAF*^{V600E} mutation using VE1 IHC was negative using sequencing (false positive). Overall, the rate of concordance was 98.6% between IHC and sequencing (Table 3). We found sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of VE1 IHC as 100%, 98.07%, 95.2%, and 100%, respectively.

4 | DISCUSSION

The *BRAF*^{V600E} mutation is a well-known diagnostic and prognostic marker for PTC among other subtypes of thyroid carcinoma, and an important target for the *BRAF*^{V600E} specific inhibitors. At present time, usefulness of IHC for the *BRAF*^{V600E} mutation analysis is under immense discussion among scientists. In this study, we confirmed practical utility of IHC method for the *BRAF*^{V600E} mutation detection and analyzed concordance with Sanger sequencing, a gold standard in this study. Moreover, the *BRAF*^{V600E} mutation was associated with indicators of poor prognosis factors in PTC.[22,32]In this study, the *BRAF*^{V600E} mutation rate was found to be 29.0% which is comparable, if not exact, to prior studies.[8,13,21] The possible explanations of difference in the *BRAF*^{V600E} mutation rate relate to the selection bias in samples and most importantly due to heterogeneity in ethnic characteristics.[7,17,33] PTC patients demonstrated significant correlations of the *BRAF*^{V600E} mutation determined by IHC with adverse prognostic factors such as tumor stage, tumor focality, and extrathyroidal extension. Extrathyroidal extension has been known as an important prognostic factor related to recurrence and disease persistence.[22] In contrast to this study, some previous studies reported lack of association of *BRAF*^{V600E} mutation determined by IHC and adverse clinical characteristics such as extrathyroidal extensions.[33,34] The bias in clinical outcome may be due to heterogeneity in patients demographic data, the size of study samples, and histological subtypes of PTC tissues obtained for analysis.[35] Thyroid cancer is the only cancer found in young patients particularly in females due to hormonal effects. But in the current study, significant difference ($P > 0.05$) in the *BRAF*^{V600E} rate was not detected in terms of age and gender, which was inconsistent with previous study.[36]

VE1 IHC indicated excellent analytic performance and the high concordance with Sanger sequencing for the detection of mutation.

The high sensitivity and specificity of results were determined, with no false negative and only one false positive case. The reason of false positive result may be due to sample contamination or antigen cross reactivity.[35,36] In this study, the VE1 IHC method was able to detect low tumor cellularity, high tumor heterogeneity, and low mutant allele frequency. Additionally, to the best of our knowledge, decalcification does not obstruct with the results of IHC test. However, prior decalcification of samples is not appropriate for Sanger sequencing. Several reports suggested that VE1 immunostaining successfully detected *BRAF*^{V600E} mutation when applied to small sized tissue samples such as fine needle aspirates and core biopsy samples before surgery.[37-39]

In former studies, different molecular methods such as real-time PCR, sequencing and SNaPshot PCR have been employed as gold standards to compare with the results of VE1 immunostaining. However, some of these methods reported more discordant cases when compared to VE1 IHC which could either be due to difference in IHC protocol used or sensitivity of techniques.[22,35] Interestingly, most of the studies addressed discordant cases either by re-performing IHC and genotyping or by employing of more sensitive molecular methods.[19,35,40]

There are various limitations in the current study. Firstly, different histological types of thyroid carcinoma, including tall cell variant PTC, anaplastic TC and microcarcinomas, were not included in the study which could be a reason for bias in clinical correlation analysis. Secondly, high quality FFPE tissue samples were acquired for our study, which however cannot always be possible in clinical study. Most of the PTC samples for diagnostic testing were obtained from core needle biopsy (CNB) and fine-needle aspiration (FNA) with low tumor content. These types of samples may not be suitable for Sanger sequencing, and hence, diagnostic validity parameters including sensitivity and specificity may bias the results. However, several studies have highlighted the superior performance of highly sensitive ddPCR, to detect mutation from FNA and low-abundance DNA mutation samples.[41] Thirdly, mutations with less fractional abundance (from 5% to 10%) were reported as negative in our clinical settings because it could not be detected by Sanger sequencing, while only ≥10% fractional abundance would have been reported as positive. Fourthly, due to limited resource settings, single type of molecular test may increase the risk of false positive and false negative results. Therefore, more sensitive and combination of molecular techniques are required to validate discordant cases. Lastly, this is a single-center-based study with a small series of patients; hence, large sample size is warranted to confirm the clinical utility of IHC in *BRAF*^{V600E} testing.

5 | CONCLUSIONS

In our cohort, IHC using VE1 antibody was found to be strongly concordant with the Sanger sequencing. Taking everything into consideration, BRAF IHC can be consider as an initial or alternative tool for *BRAF*^{V600E} mutation analysis. Thus, due to high diagnostic accuracy, this technique probably is used instead of Sequencing for clinical implementation in routine diagnostic laboratories.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conceptualization, FR; Methodology, FR, MK, MA, and HA; Validation, ST; Formal Analysis, FR and MK; Investigation, FR; Resources, SSA and TK; Writing—Original Draft Preparation, FR and ST; Writing—Review and Editing, MK, SSA and HA; Supervision, ST and SSA; Project Administration, ST.

ORCID

Syed Sameer Aga https://orcid.org/0000-0002-8186-1149

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