Comparison of Immunohistochemistry and Direct Sanger Sequencing for Detection of the *BRAF*[V600E] Mutation in Thyroid Neoplasm

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Background: The *BRAF*[V600E] mutation is the most common genetic alteration identified in papillary thyroid carcinoma (PTC). Because of its costs effectiveness and sensitivity, direct Sanger sequencing has several limitations. The aim of this study was to evaluate the efficiency of immunohistochemistry (IHC) as an alternative method to detect the *BRAF*[V600E] mutation in preoperative and postoperative tissue samples.

Methods: We evaluated 71 patients who underwent thyroid surgery with the result of direct sequencing of the *BRAF*[V600E] mutation. IHC staining of the *BRAF*[V600E] mutation was performed in 49 preoperative and 23 postoperative thyroid specimens.

Results: Sixty-two patients (87.3%) had PTC, and of these, *BRAF*[V600E] was confirmed by direct sequencing in 57 patients (91.9%). In 23 postoperative tissue samples, the *BRAF*[V600E] mutation was detected in 16 samples (70%) by direct sequencing and 18 samples (78%) by IHC. In 24 fine needle aspiration (FNA) samples, *BRAF*[V600E] was detected in 18 samples (75%) by direct sequencing and 16 samples (67%) by IHC. In 25 core needle biopsy (CNB) samples, the *BRAF*[V600E] mutation was detected in 15 samples (60%) by direct sequencing and 16 samples (64%) by IHC. The sensitivity and specificity of IHC for detecting the *BRAF*[V600E] mutation were 77.8% and 66.7% in FNA samples and 99.3% and 80.0% in CNB samples.

Conclusion: IHC could be an alternative method to direct Sanger sequencing for *BRAF*[V600E] mutation detection both in postoperative and preoperative samples. However, application of IHC to detect the *BRAF*[V600E] mutation in FNA samples is of limited value compared with direct sequencing.

Keywords: Immunohistochemistry; Sanger sequencing; BRAF; Mutation; Thyroid neoplasms

INTRODUCTION

The *BRAF*[V600E] mutation is the most common mutation detected in papillary thyroid carcinoma (PTC). In Korea, PTCs harboring *BRAF*[V600E] were reported in up to 80% of the population [1-3]. The *BRAF* oncogene encodes the human gene for B-type Raf kinase. *BRAF*[V600E] is a T1799A point mutation in exon 15, resulting in the substitution of valine to glutamate at residue 600 (V600E). It activates the RAS/RAF/MAPK signaling pathway by disrupting hydrophobic interactions between residues in both the activation loop and the ATP-binding site [4]. *BRAF*[V600E] blocks apoptosis and regulates proliferation and invasiveness [5]. In addition, *BRAF*[V600E] is associated with aggressive clinical behavior and a higher risk of recurrence of PTC [6-8] and may help in detecting malignancy in some thyroid nodules [9,10]. The *BRAF*[V600E] mutation is one of the most stressed molecular markers for its clinical role in thyroid cancer.

The gold standard method for molecular diagnosis of the *BRAF*[V600E] mutation is direct Sanger sequencing, which is highly reliable and specific [11]. However, the sensitivity of Sanger sequencing was reported to identify only 7% to 20% of mutated alleles in a background of wild-type alleles [11,12]. The tumor content of malignant cells is reported to influence the sensitivity of Sanger sequencing [13]. Over the last decade, several alternatives to Sanger sequencing for detecting the *BRAF*[V600E] mutation have been reported with different sensitivities and specificities [11,14-16].

Recently, immunohistochemistry (IHC) using a BRAF[V600E]-specific antibody (VE1) has been used to detect the *BRAF*[V600E] mutation [17]. IHC is a widely available and low-cost technique. Previous studies suggest that IHC is highly sensitive for detecting the *BRAF*[V600E] mutation [18-20].

In the present study, the efficiency of BRAF[V600E] IHC as an alternative method to direct Sanger sequencing was evaluated in preoperative and postoperative thyroid tissue samples.

METHODS

Tissue samples and study design
Seventy-one patients who underwent thyroid surgery between January 2011 and January 2013 in the Asan Medical Center, Seoul, Korea, were included in this retrospective study. Results on the *BRAF* mutation status using direct Sanger sequencing were available. Diagnostic preoperative tissue samples were obtained by either fine needle aspiration (FNA) or core needle biopsy (CNB). Postoperative formalin-fixed, paraffin-embedded (FFPE) samples were obtained after thyroid surgery. IHC staining for the *BRAF*[V600E] mutation was performed in preoperative and postoperative thyroid specimens. The results of *BRAF*[V600E] IHC were compared with those of direct Sanger sequencing. The study protocol was approved by the Institutional Review Board of the Asan Medical Center (AMC 2016-0821). Informed consent was obtained from all patients for *BRAF*[V600E] mutation analysis.

Cytological and histopathological diagnosis
All thyroid specimens were reviewed by an experienced endocrine pathologist (D.E.S.). FNA cytology results were categorized into six categories using the Bethesda system [21]. Preoperative CNB specimens were evaluated based on the criteria proposed by the Korean endocrine pathology thyroid CNB study group [22]. Surgical specimens were reviewed and diagnosed based on the World Health Organization classification criteria [23].

Ultrasonography-guided fine needle aspiration and core needle biopsy procedures
Neck ultrasound (US) examinations were performed using either an iU22 unit (Philips Healthcare, Bothell, WA, USA) or an EUB-7500 unit (Hitachi Medical Systems, Tokyo, Japan) equipped with a linear high-frequency probe (5 to 14 MHz), as previously reported [24]. The scanning protocol included both transverse and longitudinal real-time imaging. All US examinations and FNA/CNB procedures were performed by experienced radiologists. FNA procedures were performed under US guidance using a 23-gauge needle connected to a 10 mL syringe [25]. US-guided CNB procedures were performed using an 18-gauge, 1.1 or 1.6 cm excursion, double-action, spring-activated needle (TSK Ace-cut, Create Medic, Yokohama, Japan) [26,27].

Immunohistochemistry for the *BRAF*[V600E] mutation
IHC for the *BRAF*[V600E] mutation was performed from 49 preoperative FNA cell blocks or CNB specimens and all postoperative thyroidectomy specimens using mouse anti-BRAF V600E (clone VE1, 1:4 dilution, Ventana Medical Systems, Tucson, AZ, USA) and the OptiView DAB IHC Detection Kit (Roche, Tucson, AZ, USA) on a Ventana BenchMark XT autoimmunostainer (Ventana Medical Systems) [28]. Whole tissue sections (4 μm) were transferred to poly-L-lysine-coated adhesive slides and dried at room temperature for 10 minutes. Antigen retrieval was performed using the heat-induced epitope retrieval method,

followed by 20 minutes at 65°C in an incubator. After incubation in CC1 solution (Roche) for 32 minutes, primary antibody was added and incubated for 16 minutes. A haptenated secondary antibody was added and incubated for a further 10 minutes. Alpha-hapten-horseradish peroxidase was added to the sections. After incubation for 10 minutes, diaminobenzidine was added and incubated for 10 minutes. Negative controls were performed by omitting the primary antibody. Positive controls were melanoma tissue.

Slides were evaluated by an experienced pathologist (D.E.S.) who was blinded to the *BRAF* mutational status. Diffuse homogeneous cytoplasmic staining in all tumor cells was considered as positive. Non-specific staining of colloids and equivocally weak or focal cytoplasmic staining was considered as negative.

Direct Sanger sequencing for *BRAF*
Sanger sequencing was performed as previously described [27]. Briefly, exon 15 of the *BRAF* gene was amplified by polymerase chain reaction (PCR) using the primers 5′-TGCTT-GCTCTGATAGGAAAATG-3′ (forward) and 5′-CTGATGGG ACCCACTCC AT-3′ (reverse). The amplification protocol comprised initial denaturation at 94°C for 2 minutes; 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 40 seconds; followed by a final extension at 68°C for 7 minutes using KOD FX Taq DNA polymerase (Toyobo, Osaka, Japan). PCR products were electrophoresed on 2.5% (wt/vol) agarose gels and subsequently sequenced using the above forward primer and Big Dye Terminator (ABI Systems, Applied Biosystems, Foster City, CA, USA). DNA sequences and the *BRAF* mutation were determined using an ABI-PRISM 3100 automatic sequencer (Applied Biosystems).

Statistical analysis
Statistical analyses were performed using R version 3.0 and the R libraries (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). Categorical variables are presented as numbers with percentages. McNemar's chi-square test was used to evaluate the performance of two tests. All *P* values were two-sided and *P* values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics of study subjects and tissue samples
Sixty-two of 71 patients (87.3%) had PTC including one patient with a follicular variant of PTC, and of these, the *BRAF*[V600E] mutation was detected in 57 patients (91.9%) by direct sequencing. Four patients (5.6%) had follicular thyroid carcinoma, and an additional four patients (5.6%) had nodular hyperplasia. Wild-type *BRAF* was confirmed in 14 tumors (19.7%).

Comparison of direct Sanger sequencing and IHC results for the *BRAF*[V600E] mutation using postoperative samples
Table 1 shows the results of direct Sanger sequencing and IHC staining for the *BRAF*[V600E] mutation in 23 postoperative FFPE samples. The *BRAF*[V600E] mutation was detected in 16 samples (70%) using direct sequencing. IHC for *BRAF*[V600E] was positive in 18 FFPE samples (78%). Of these samples, two showed discrepant results between the two methods. IHC results were positive, whereas the results of direct sequencing showed wild-type *BRAF* (Fig. 1A, B). The sensitivity and specificity of IHC for detecting the *BRAF*[V600E] mutation in postoperative samples were 100% and 71.4%. There was no significant difference in detecting the *BRAF*[V600E] mutation between two tests in postoperative samples (*P*=0.480).

Comparison of direct Sanger sequencing and IHC results for the *BRAF*[V600E] mutation using preoperative samples
Table 2 shows the results of direct Sanger sequencing and IHC staining for the *BRAF*[V600E] mutation in 49 preoperative samples including 24 FNA samples and 25 CNB samples. The *BRAF*[V600E] mutation was detected in 33 samples (67%) using direct sequencing and 32 samples (65%) using IHC. In preoperative samples, inconsistent results between the two methods were detected in nine patients (18.4%). Of these, four were positive by IHC and negative by direct sequencing. Five patients were negative with IHC and positive with direct sequencing methods. The sensitivity and specificity of IHC for detecting the *BRAF*[V600E] mutation in preoperative samples were 84.9% and 75.0%. No difference was found between two tests in detecting

Table 1. Comparison of the Results of Direct Sanger Sequencing and IHC for *BRAF*[V600E] in Postoperative Samples

BRAF[V600E] IHC	Sanger sequencing		Total
	Wild-type	*BRAF*[V600E]	
Negative	5 (71)	0	5 (22)
Positive	2 (29)	16 (100)	18 (78)
Total	7 (30)	16 (70)	23 (100)

Values are expressed as number (%).
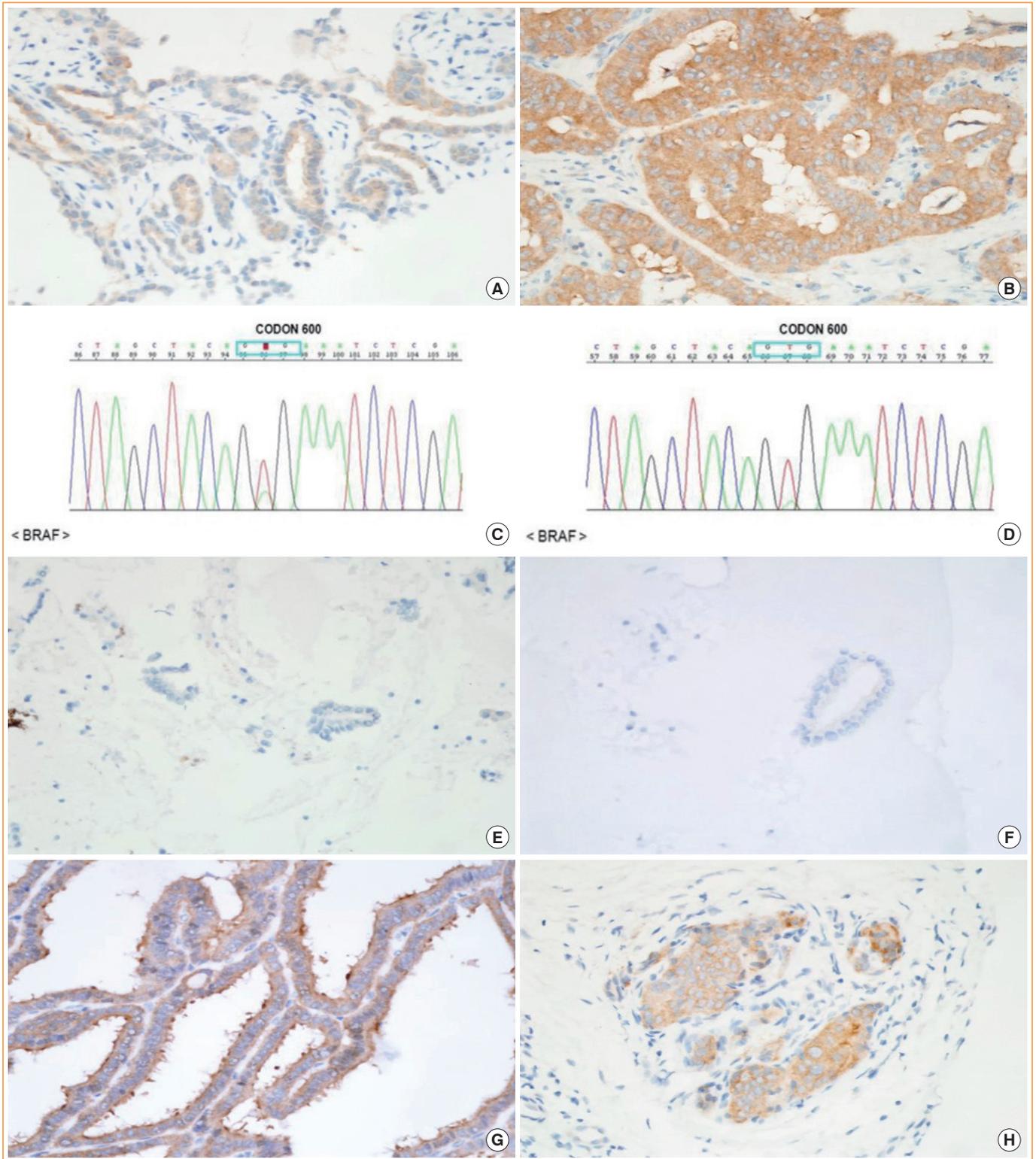IHC, immunohistochemistry.

Fig. 1. Detection of the *BRAF*[V600E] mutation in different thyroid tissue specimens with inconsistent results between direct Sanger sequencing and immunohistochemistry (IHC) (A-F). Two postoperative thyroidectomy specimens were positive by immunohistochemistry for the *BRAF*[V600E] mutation (A, B, ×400), but negative by direct Sanger sequencing. Two preoperative fine needle aspiration cell block specimens showed discrepant results with positive direct Sanger sequencing (C, D) results and negative IHC results (E, F, ×400). Two preoperative core needle biopsy specimens were positive by IHC for the *BRAF*[V600E] mutation (G, H, ×400), but negative by direct Sanger sequencing.

Table 2. Comparison of the Results of Direct Sanger Sequencing and IHC for $BRAF^{V600E}$ in Preoperative Samples

$BRAF^{V600E}$ IHC	Sanger sequencing		Total
	Wild-type	$BRAF^{V600E}$	
Total preoperative samples			
Negative	12 (75)	5 (15)	17 (35)
Positive	4 (25)	28 (85)	32 (65)
Total	16 (33)	33 (67)	49 (100)
FNA specimens			
Negative	4 (67)	4 (22)	8 (33)
Positive	2 (23)	14 (78)	16 (67)
Total	6 (25)	18 (75)	24 (100)
CNB specimens			
Negative	8 (80)	1 (7)	9 (36)
Positive	2 (20)	14 (93)	16 (64)
Total	10 (40)	15 (60)	25 (100)

Values are expressed as number (%).
IHC, immunohistochemistry; FNA, fine needle aspiration; CNB, core needle biopsy.

the $BRAF^{V600E}$ mutation in preoperative samples ($P=0.999$).

In 24 FNA specimens, the $BRAF^{V600E}$ mutation was detected in 18 samples (75%) by direct sequencing and 16 samples (67%) by IHC. The results of the two methods were discordant in six of 24 samples (25%). Two samples were positive for the $BRAF^{V600E}$ mutation by IHC, but direct sequencing results showed wild-type $BRAF$. The $BRAF^{V600E}$ mutation was detected in four samples using direct sequencing, but these were negative using IHC. $BRAF^{V600E}$ mutations from two samples using direct sequencing are shown in Fig. 1C, D. However, results of IHC were negative in the two corresponding FNA cell blocks as shown in Fig. 1E, F. The sensitivity and specificity of IHC for detecting the $BRAF^{V600E}$ mutation in FNA samples were 77.8% and 66.7%. There was no statistical significant difference in detecting the $BRAF^{V600E}$ mutation between two tests in FNA samples ($P=0.683$).

In 25 CNB specimens, the $BRAF^{V600E}$ mutation was detected in 15 samples (60%) by direct sequencing and 16 samples (64%) by IHC. The results of direct sequencing and IHC differed in three of 25 samples (12%). The $BRAF^{V600E}$ mutation was detected in one sample by direct sequencing, but was negative in IHC. Two samples were positive for the $BRAF^{V600E}$ mutation using IHC, but were not detected by direct sequencing (Fig. 1G, H). The sensitivity and specificity of IHC for detecting the $BRAF^{V600E}$ mutation in CNB samples were 99.3% and 80.0%.

There was no significant difference in detecting the $BRAF^{V600E}$ mutation between two tests in CNB samples ($P=0.999$).

DISCUSSION

The presence of the $BRAF^{V600E}$ mutation is considered an intermediate risk factor in the recently revised American Thyroid Association guideline for thyroid nodules and differentiated thyroid cancer [29]. This indicates that the presence of $BRAF^{V600E}$ is generally considered as a risk factor for poor prognosis or aggressive behavior of thyroid cancer. $BRAF^{V600E}$ mutation analysis is difficult to perform in every thyroid cancer patient, because of the issue of cost-effectiveness.

The efficiency of IHC, a low-cost method, for detecting the $BRAF^{V600E}$ mutation was evaluated as an alternative technique to direct Sanger sequencing in different preoperative and postoperative thyroid tissue samples. The sensitivity of IHC staining for detecting the $BRAF^{V600E}$ mutation was found to be superior to that of direct Sanger sequencing in postoperative tissue samples. In preoperative tissue samples, IHC staining performed better in CNB samples.

The $BRAF^{V600E}$ mutation was found in 92% of PTC patients in the present study. This result is consistent with findings of previous studies demonstrating a relatively high prevalence of the $BRAF^{V600E}$ mutation in Korean PTC patients [1,30]. This implies that detection of the $BRAF^{V600E}$ mutation could play a role in the diagnosis or management of PTC patients, especially in Korea.

According to our data, the concordance between direct Sanger sequencing and IHC for detecting $BRAF^{V600E}$ was superior in postoperative tissue samples compared with preoperative tissue samples. Discordance between the two methods in postoperative tissue samples was found in only two patients, who were wild-type by direct Sanger sequencing and positive using IHC. In preoperative tissue samples, discordant results between the two methods were found in nine samples, of which four were wild-type by direct Sanger sequencing and positive using IHC, and the remaining five were *vice versa*. These results imply that IHC is more suitable for postoperative tissue samples than preoperative tissue samples.

Preoperative tissue samples were obtained by FNA and CNB. Data showed that CNB samples were more suitable for IHC staining than FNA samples, even though there were no statistical differences between Sanger sequencing and IHC staining methods on both tissue samples. According to previous studies, IHC on FNA samples shows relatively less specificity and sensitivity than on FFPE samples [18,31,32]. However, Crescenzi

et al. [33] reported that IHC performed on thyroid CNB samples perfectly matched genetic analysis of BRAF status. These data support the use of IHC on CNB samples to detect *BRAF*^V600E at preoperative evaluation of thyroid nodules.

Five tissue samples were positive by direct Sanger sequencing and negative by IHC, with four FNA samples and one CNB sample. All of these samples were collected preoperatively. These findings may be due to loss of antigen expression of the mutation. For example, tissue ischemic areas surrounding necrotic areas or damaged tissue have reduced BRAF^V600E protein expression, which may be the cause of the false-negative IHC results, particularly in small biopsies such as FNA [18,34].

As shown in Supplemental Table S1, the FNA sample from subject number 1 showed negative results by direct Sanger sequencing and positive results by IHC. In this patient, subsequent reverse transcriptase-PCR revealed the *BRAF*^V600E mutation. This implies that methods for detecting the *BRAF*^V600E mutation may affect the results of mutation analysis. Combination of IHC and molecular approaches has been suggested for better detection of the *BRAF*^V600E mutation [35]. However, the cost-effectiveness of using these approaches requires consideration.

In conclusion, IHC could be an alternative approach to direct Sanger sequencing for detecting the *BRAF*^V600E mutation both in postoperative and preoperative tissue samples. However, application of IHC in FNA samples was of limited value compared with direct sequencing. Direct Sanger sequencing is the gold standard method for detecting the *BRAF*^V600E mutation and is highly specific. Disadvantages include its relative low sensitivity compared with other tests [11,35] and high costs. IHC on appropriate tissue samples is an alternative and superior approach in detecting the *BRAF*^V600E mutation because of its improved performance and low cost.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Conception or design: Y.K.S., W.B.K., W.G.K., D.E.S. Acquisition of data: H.S.O., H.K., P.S., M.K., J.C. Analysis or interpretation of data: H.S.O., H.K., M.J.J., T.Y.K., J.C., W.G.K. Drafting the work or revising: H.S.O., H.K., W.G.K., D.E.S. Final approval of the manuscript: Y.K.S., W.B.K., W.G.K., D.E.S.

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