Original paper

Utility of *BRAF* immunohistochemistry for the detection of *BRAF* mutations in papillary thyroid carcinoma. Analysis of detailed histological subtypes

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The *BRAF* mutation has been observed in 32–73% of papillary thyroid carcinomas. Despite the superiority of genetic tests for detecting *BRAF* mutations, they have inherent disadvantages. The objective of this study was to analyze the specificity and sensitivity of *BRAF* immunohistochemistry (IHC) that has been used in recent years and may serve as an alternative to genetic testing, in papillary thyroid carcinoma.
A total of 103 cases of papillary thyroid carcinoma were analyzed for *BRAF V600E* mutation by real-time polymerase chain reaction (PCR) and IHC.
The results demonstrated that the *BRAF V600E* mutation was identified in 55 (53.3%) of 103 cases by real-time PCR, while *BRAF* IHC positivity was observed in 65 (63.1%) of 103 cases. The study found that the specificity of *BRAF* IHC was 79.2%, while the sensitivity was 100%. The real-time PCR analysis identified 10 cases that were positive for *BRAF* IHC and exhibited wild-type *BRAF*.
BRAF IHC can be utilized as a screening test and as a partial alternative to genetic tests. This is due to its low cost, straightforward routine application, moderate to high specificity, and high sensitivity.

Key words: *BRAF*, mutation, thyroid, papillary, carcinoma, immunohistochemistry.

Introduction

Papillary thyroid carcinoma (PTC) is the most prevalent form of thyroid malignancy, accounting for approximately 80% of all thyroid carcinomas [1]. While the majority of PTCs exhibit an excellent prognosis, a small proportion may demonstrate aggressive behavior [2]. The *BRAF* mutation has been identified as a significant contributor to the pathogenesis of PTC, with an incidence rate of 32–73% in PTC cases [3, 4]. A variety of techniques have been employed to detect *BRAF* mutations, including Sanger sequencing, pyrosequencing, real-time polymerase chain reaction (PCR), and next-generation sequencing [5]. Despite their esteemed status as the gold standard, PCR-based methods are not without drawbacks. These include high cost, the need for specific infrastructure, and difficulty in evaluation [6]. Conversely, *BRAF V600E* immunohistochemistry (IHC), which has recently been introduced, is straightforward and rapid to perform; however, its utility is constrained by its uncertain sensitivity and specificity. The objective of this study was to compare the *BRAF V600E* IHC and *BRAF V600E* mutation analysis by real-time PCR to ascertain the specificity and sensitivity of *BRAF V600E* IHC in papillary thyroid carcinomas.

Material and methods

Patient selection

A total of 103 PTC cases diagnosed in 2015–2022 were included in the study. Data on the demographic characteristics and clinicopathologic features of the

Table I. Median/mean age and dimensions

Age (mean ±SD/median (min–max)	46.3 ±14.8	45 (17–80)
Dimension (mean ±SD/median (min–max)	2.1 ±1.4	1.8 (0.2–7)

max – maximum, min – minimum, SD – standard deviation

atients were obtained from medical records. Two pathologists with expertise in thyroid pathology conducted a comprehensive evaluation of the tumors, including the assessment of histologic subtype, lymph node metastasis, and extrathyroidal spread. The distribution of histologic subtypes is presented in Table I. The cases were classified into three categories based on the presence of a tall cell component, a classic component without a tall cell component, or neither. The cases with both tall and classic subtypes were included in the tall cell group.

Molecular analysis of *BRAF V600E* mutation

Deparaffinization

Depending on the size of the tissue, 5–10 5-micron-thick sections were taken from the formalin-fixed paraffin-embedded tissue (FFPE) and placed in a microcentrifuge tube. Then, 500 μl of xylene was added. The tube was vortexed for 10 seconds and left at 15–30°C for five minutes. Next, 500 μl of absolute ethanol was added, and the tube was vortexed for 10 seconds. The tube was left at 15–30°C for five minutes. Then, the tube was centrifuged at 16,000 g for two minutes. The supernatant was removed. One milliliter of ethanol was added, and the tube was vortexed for 10 seconds. The supernatant was removed again. The tube was left open and incubated at 56°C for 10 minutes. Then, 180 ml of DNA tissue lysis buffer (DNA TLB) and 70 ml of proteinase K (PK) were added.

DNA isolation

For DNA isolation, the sample/DNA TLB/PK mixture was vortexed for 30 seconds. The tubes were then incubated at 56°C for 60 minutes, after which they were vortexed for 10 seconds. Then, the tubes were left at 90°C for 60 minutes. Then, 200 μl of DNA paraffin binding buffer was added to the tubes, which were left at 15–30°C for 10 minutes. Then, 100 μl of isopropanol was added, and the mixture was transferred to filtration/collection tubes. The filtration/collection tubes were then centrifuged at 8000 × g for one minute. Next, 500 μl of DNA wash buffer I was added to the tubes. After centrifuging again at 8000 × g for one minute, 500 μl of wash buffer II was added. After one final centrifugation, 100 ml of DNA elution buffer (DNA EB) was added to prepare the genetic material for measurement.

Genetic analysis

The cobas 4800 *BRAF V600* mutation test kit (Roche Diagnostics, Indianapolis, IN, USA) was employed for genomic analysis. The kit was used to screen for *V600E* and select *non-V600E* mutations, including *V600D, V600E2*, and *V600K*. The analysis was conducted using the LightCycler 480 system (Roche Diagnostics, Indianapolis, IN, USA), which employs real-time PCR with 1% specificity.

BRAF immunohistochemical analysis

Four-micron-thick sections were obtained from the FFPE blocks. The tissues included in the study were fixed in 10% neutral buffered formalin for at least 24–48 hours. No macrodissection was performed on the tissues. The sections were affixed to adhesive slides and incubated in an oven at 60°C for two hours. The samples were deparaffinized at 75°C for four minutes using the EZ Prep method on the Benchmark XT platform (Ventana Medical Systems, Inc., Tucson, AZ, USA). Subsequently, the antibody was added to the sections and incubated at 37°C for 40 minutes. Subsequently, the sections were washed and incubated with diaminobenzidine for eight minutes at 37°C (OptiView detection kit, Ventana). Subsequently, the sections were counterstained with Harris hematoxylin for a period of two minutes. Subsequently, *anti-BRAF V600E* IHC (VE1 clone, Ventana Medical Systems, Roche Diagnostics) was applied to the prepared sections. Previously genetically proven *BRAF V600E* mutant thyroid papillary carcinoma and colorectal carcinoma sections were used as positive controls.

The stained sections were then examined under a light microscope. The scoring was performed in accordance with the following cytoplasmic staining criteria:

- 10% or less weak staining or no staining at all: 0 (Figure 1A),
- weak staining in more than 10% of cells: 1+ (Figure 1B),
- moderate staining in more than 10% of cells: 2+ (Figure 1C),
- strong staining in more than 10% of cells: 3+ (Figure 1D).

A score of 1 or more was considered positive, while a score of 0 was considered negative [7–10].

Statistical analysis

The data were analyzed using SPSS 22.0. The χ^2 test was used to calculate the specificity and sensitivity of *BRAF V600E* IHC, while the independent-samples *t*-test/Mann-Whitney *U* test was used to compare numerical variables. The *p*-value was considered significant if it was less than 0.05. The small

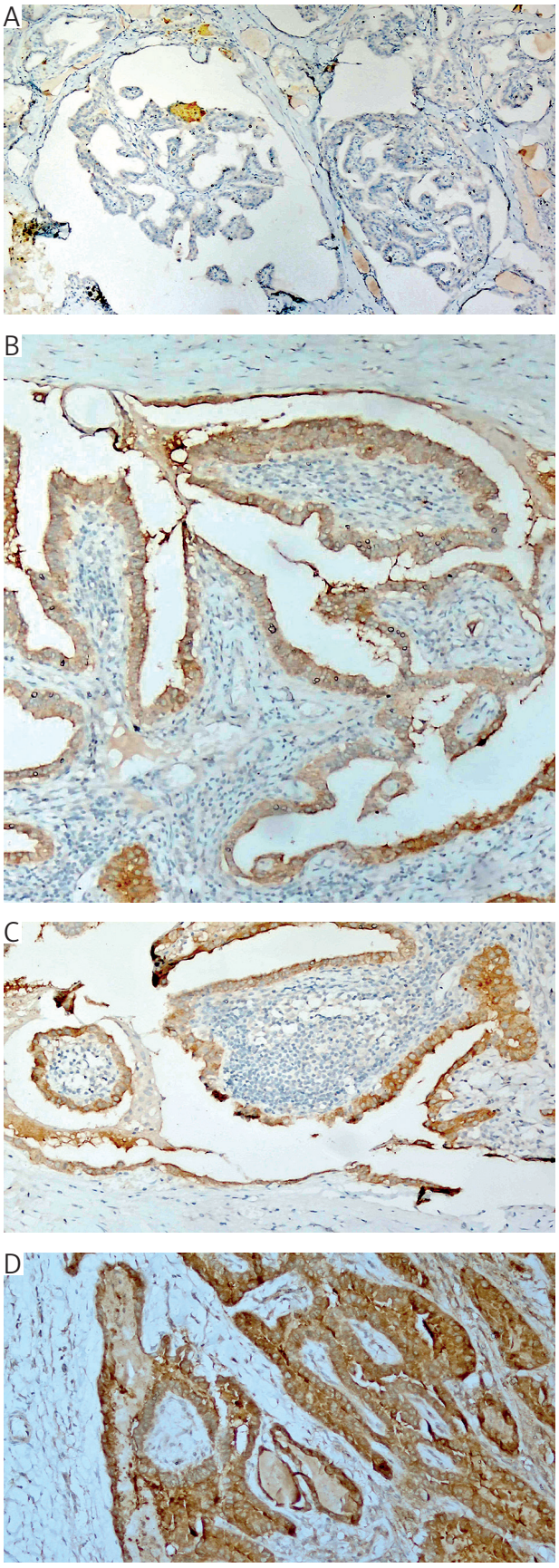

Figure 1. *BRAF V600E* immunohistochemistry (IHC) scoring. **A**) Negative *BRAF* immunostain. **B**) 1+ positive *BRAF* IHC. **C**) 2+ *BRAF* IHC. **D**) 3+ *BRAF* IHC

number of some histological subtypes limited statistical analysis.

Results

Of the patients, 75 (73%) were female and 28 (27%) were male. Table I presents a summary of the general age and tumor size data for the patients.

The *BRAF V600E* mutation was detected in 55 out of 103 (53.3%) cases by real-time PCR. Of the 103 cases, 48 exhibited wild-type *BRAF*, while 65 (63.1%), including 10 of the aforementioned cases, demonstrated positive *BRAF* IHC. Thirty-eight cases were negative by *BRAF* IHC.

The presence of a *BRAF* mutation was not associated with tumor size, whereas increasing age was significantly associated with tumor size (Table II).

Eight cases demonstrated extrathyroidal spread. The *BRAF* mutation was not associated with gender, lymph node metastasis, or extrathyroidal spread (Table III).

No significant correlation between age and size was found when cases were divided by histological subtypes. The number of subtypes, *BRAF* mutation status, *BRAF* IHC staining status, and number of lymph node metastases are presented in Table IV. Furthermore, a statistical analysis was conducted on the histologic subtypes, which were divided into three groups: those containing a tall cell component, those containing a classic component without a tall cell component, and those classified as "others". No significant association was observed between lymph node metastasis and the three histologic subtype groups (37% vs. 47% vs. 44%, respectively). However, tumors with tall cell and classic components exhibited a significant *BRAF V600E* mutation. The first group exhibited the highest frequency of *BRAF* IHC expression (83%), followed by the second group (51%), and the third group without both (22%) ($p < 0.05$).

Similarly, the first group demonstrated the highest frequency of *BRAF V600E* IHC expression (87%), followed by the second group (64%), and the third group without both (28%) ($p < 0.05$).

A comparison of *BRAF* IHC data with the real-time PCR method revealed that the sensitivity and specificity of *BRAF* IHC were 100% and 79.2%, respectively. The positive predictive value (PPV) of the *BRAF* IHC test was determined to be 100%, while the negative predictive value (NPV) was calculated to be 84.6%.

Upon examination of the 10 tumors that had been identified as negative for *BRAF* mutation by real-time PCR but positive by *BRAF* IHC, it was found that they were all microcarcinomas with a mean size of 0.7 cm (0.2–0.9 cm) (Figures 2A, B). No significant dimensional discrepancy was observed between cases

Table II. *BRAF* status and age/dimension association

Parameters	No mutation		*BRAF* mutant/mutation		*p*-value
	Mean ±SD	Median (Q1–Q3)	Mean ±SD	Median (Q1–Q3)	
Age	42.2 ±14.5	43 (32.5–51)	49.9 ±14.2	53 (40–60)	0.007
Dimension	2.3 ±1.6	2 (1.0–3.5)	1.9 ±1.3	1.8 (1.1–2.5)	0.493

SD – standard deviation

Table III. *BRAF* mutation and clinicopathological features

Parameters	No mutation	*BRAF* mutant/mutation	*p*-value
	n (%)	*n* (%)	
Sex			
Male	13 (27.0)	15 (27.2)	0.983
Woman	35 (72.9)	40 (72.7)	
Lymph node			
Negative	31 (64.5)	25 (45.4)	0.052
Positive	17 (35.4)	30 (54.5)	
Capsule			
Negative	29 (60.4)	27 (49.0)	0.250
Positive	19 (39.5)	28 (50.9)	
Variant			
Tall	4 (8.3)	20 (36.3)	0.001*
Classic	30 (62.0)	31 (56.3)	
Other	14 (29.1)	4 (7.2)	

Table IV. Histological subtypes and *BRAF* mutation status

Histologic subtype	Number	*BRAF*m	*BRAF* (+) IHC	Lymph node	Extrathyroidal spread
Classic + tall	14	12	13	7	3
Tall	9	7	7	2	0
Follicular + tall	1	1	1	0	0
Tall cell total, *n* (%)	24	20 (83)	21 (87)	9 (37)	3
Classic	40	24	30	17	4
Classic + follicular	11	3	5	3	0
Classic + oncocytic	5	3	3	4	1
Classic + solid	5	1	1	5	0
Classic total, *n* (%)	61	31 (51)	39 (64)	29 (47)	5
Encapsulated classic	11	3	4	3	0
Infiltrative follicular	2	1	1	2	0
Solid/trabecular	2	0	0	0	0
Oncocytic	2	0	0	2	0
Columnar	1	0	0	1	0
Other total, *n* (%)	18	4 (22)	5 (28)	8 (44)	0
Total	103	55	65	46	8

BRAFm – BRAF mutant/mutation, IHC – immunohistochemistry, N – number

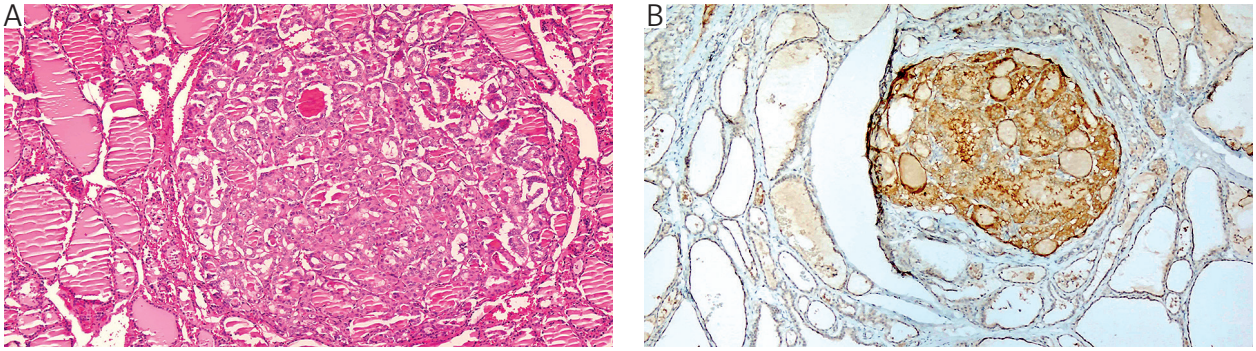

Figure 2. Papillary microcarcinoma, follicular variant that has wild type *BRAF* in real-time polymerase chain reaction. **A**) HE stain. **B**) 3+ positivity in *BRAF* immunohistochemistry for the same tumor

exhibiting concordance or discordance between PCR and IHC results.

Of the 10 cases, one exhibited tall cell features, eight displayed classic patterns, and one presented with other histologic subtypes (six pure classic, two classic with infiltrative follicular, one classic with tall cell, and one encapsulated classic variant).

Discussion

The *BRAF* mutation is a clinical factor that can be exploited as a therapeutic target with prognostic significance across a range of malignancies [11, 12]. Consequently, the safe and expeditious analysis of *BRAF* mutations is paramount for the implementation of personalized treatment regimens.

The VE1 clone of *BRAF V600E*, a mutation-specific monoclonal antibody, has recently been employed in such analyses and has been reported to exhibit high sensitivity and specificity, with minimal cross-reactivity with wild-type and other mutant *BRAF* forms [7].

In the present study, a *BRAF* mutation was identified in 53% of PTC cases, a rate that aligns with analogous studies in the literature, which reported rates of 27–90% [13–16]. While earlier research has indicated a link between *BRAF* mutations and tumor size, lymph node metastasis, and extrathyroidal spread, the present study did not reveal a substantial correlation. The relevant literature will be made available for review.

After the cases were divided by histologic subtypes, they were analyzed into three groups: those with a tall cell component, those with a classic component without a tall cell component, and others. Tumors exhibiting a tall-cell and a classic component demonstrated *BRAF* mutation rates of 83–51%, respectively. *BRAF* mutations were significantly more prevalent in tumors with a tall cell classic component than in tumors of other histologic subtypes (22%). In analogous studies reported in the extant/existing literature, the *BRAF* mutation was observed more frequently in tumors exhibiting both tall cell and classic components [9, 17].

In the present study, the sensitivity, specificity, PPV, and NPV of VE1 IHC for the *BRAF V600E* mutation were 100%, 79.2%, 100%, and 84.6%, respectively, with real-time PCR as the gold standard. The literature contains numerous studies that report the specificity and sensitivity of *BRAF* IHC as 100% in four studies [7, 10, 18, 19]. It has also been documented that the method under discussion is superior to specific molecular techniques [20]. However, when real-time PCR was selected as the gold standard, *BRAF* IHC was reported to have a sensitivity of 84–100%, specificity of 58–100%, PPV of 82–100%, and NPV of 65–100% [21]. In the studies mentioned above, false-negative and false-positive results have been reported to be associated with histologic subtype [9], the type of molecular method employed [8, 22–26], and errors in IHC evaluation (cut-off criteria) [23, 24, 27, 28]. In the present study, 10 cases were identified as negative by real-time PCR but showed *BRAF* IHC expression. The preponderance of the classic component in the majority of these cases, coupled with the observation that they were all below 1 cm (with a mean size of 0.7 cm), supports the notion that both subtype and size may contribute to false negativity or false positivity. In previous studies, cases that were negative by molecular testing and positive by IHC were found to be effective when the presence of frozen material [29] and small tumor size [30] were taken into account. Furthermore, Bullock et al. have indicated that a low neoplastic-to-nonneoplastic ratio is an effective indicator in such cases, and that the presence of benign thyroid tissue, stromal tissue, and inflammatory cells may result in false-negative molecular results [22]. In a separate study, cases exhibiting molecular negativity and IHC positivity had a mean size of 2–5 mm. Furthermore, all cases demonstrated moderate to severe desmoplasia, which could have led to a low concentration of mutant DNA [29].

As demonstrated in our study, *BRAF* IHC exhibited excellent concordance with the literature in

identifying wild-type *BRAF* [9]. When the merits and limitations of *BRAF* IHC are considered collectively, it is regarded as a primary approach due to its cost-effectiveness and simplicity of implementation. The lack of inter-observer reproducibility, due to the number of researchers involved in the study and the small number of some histological subtypes, is a factor limiting the study's findings.

A comprehensive evaluation of the results of this study, in conjunction with the existing literature, revealed that the *BRAF V600E* IHC VE1 clone exhibited high sensitivity and a high rate of mutation exclusion in negative results. The relatively low specificity may indicate the need for genetic confirmation when selecting targeted therapy in cases with positive *BRAF* IHC.

Conclusions

BRAF IHC offers numerous advantages. The process does not necessitate DNA extraction, exhibits no false negatives due to DNA quality, is more cost-effective than PCR-based methods, is faster, and requires neither well-trained technicians nor specific infrastructure. Furthermore, the evaluation is not complex, and IHC is already a routine practice for pathologists [31].

Disclosures

1.Institutional review board statement: Not applicable.
2.Assistance with the article: None.
3.Financial support and sponsorship: None.
4.Conflicts of interest: None.

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