Malays J Pathol 2023; 45(3) : 375 – 390

ORIGINAL ARTICLE

A mutation panel comprising *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} replicated retrospective histopathological examination findings in differentiating benign goitre from malignant papillary thyroid cancer in a cohort of Malaysian patients

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Abstract

Thyroid malignancy status is usually confirmed through histopathological examination (HPE) following thyroidectomy. In Malaysia, the application of molecular markers in pre-operative diagnosis of thyroid cancer remains unexplored. In this study, *BRAF* and *NRAS* gene mutation panel was assessed, and the results were compared with retrospective HPE findings. Malaysian patients with benign goitre (BTG: n=33) and papillary thyroid cancer (PTC: n=25; PTCa: n=20, PTCb: n=5) were recruited at Universiti Malaya Medical Centre from September 2019 to December 2022. PCR-direct DNA sequencing of *BRAF*^{V600}, *NRAS*^{G12}, *NRAS*^{G13}, and *NRAS*^{Q61} was conducted on DNA extracted from the patients' thyroid tissue specimens following thyroidectomy and HPE. *BRAF*^{V600E} and *NRAS*^{Q61R} mutations showed absolute PTC-specificity with PTC-sensitivity of 32% and 28%, respectively. *NRAS*^{Q61H} demonstrated lower PTC-specificity (94%) but higher PTC-sensitivity (72%) compared to the *BRAF*^{V600E} and *NRAS*^{Q61R} mutations. Although the *NRAS*^{G12} and *NRAS*^{G13} variants were absent in this study, a novel *NRAS*^{V14D} mutation was detected in a PTCa patient. Unlike PTCb, coexistence of *BRAF*^{V600E} and *NRAS*^{Q61} variants was commonly observed among the PTCa patients. Notably, all PTCb patients had *NRAS*^{Q61H} mutation with one patient carried both the *NRAS*^{Q61H} and *BRAF*^{V600E} mutations. Association analysis revealed potential link between gender, *BRAF*^{V600E} mutation and lymph node metastasis. In conclusion, mutation panel comprising *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} did not discriminate the two PTC subtypes but replicated the retrospective HPE findings in differentiating BTG from PTC. The application of this mutation panel in pre-operative diagnosis of thyroid nodules requires further validation in a larger sample size, preferably incorporating fine-needle aspirate biopsies.

Keywords: papillary thyroid cancer; benign goitre; *BRAF*; *NRAS*; histopathological examination; fine-needle aspiration cytology

INTRODUCTION

Thyroid nodules are prevalent, with approximately 7% being palpable.[1] While the majority of these nodules are benign, malignancies can be detected in up to 6.5% of cases.[2] Fine-needle aspiration cytology (FNAC) is the most commonly used and cost-effective method for determining which patients with thyroid nodules require surgical intervention.[3,4] The cytology findings are classified according to the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) framework. Although it is highly specific for identifying thyroid malignancy, cytology examination has a lower sensitivity, with 30%

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of nodules being indeterminate that requires further confirmation through post-operative histopathological examination (HPE).[5-7] HPE results revealed that approximately 40% of the indeterminate nodules are actually malignant.[8] As a result, patients with benign goitre (BTG) may unnecessarily undergo thyroidectomy. To reinforce the diagnostic accuracy of FNAC, a potential strategy is the combinational use of genetic markers.[9]

The most common type of thyroid cancer is papillary thyroid cancer (PTC), accounting for approximately 80% of all thyroid cancer cases.[10] Two subtypes of PTC, namely PTCa and PTCb were recently proposed based on variations in cytomorphological background and the underlying molecular mechanism of the malignancy.[11,12] PTC is often associated with oncogenic activation of the mitogen-activated protein kinase (MAPK) due to genetic changes. The most commonly observed genetic alterations associated with PTC include *RET/PTC* chromosomal rearrangement, as well as point mutations in the *BRAF* and *RAS* proto-oncogenes.[13] Mutations in at least one of these genes were detected in over 70% of PTC cases, with the *BRAF*^{V600E} mutation being the most prevalent.[13-16] The *BRAF* gene encodes the serine/threonine protein kinase B-Raf. Amino acid substitution of valine (V) to glutamate (E) at position 600 results in the constitutively active B-Raf^{V600E} protein.[17] This *BRAF*^{V600E} mutation upregulates the MAPK signalling pathway in the absence of external stimuli. Mutations in three members of the *RAS* gene family (*HRAS*, *NRAS*, and *KRAS*) have also been reported in thyroid cancer.[18] The most common *RAS* mutations in thyroid tumours were detected in the *NRAS* gene, of which codons 12, 13, and 61 are the mutation hotspots, with the *NRAS*^{Q61} point mutation being the most common.[10,19,20] These *RAS* mutations affect the GTPase activity of the RAS proteins by modifying the binding affinity of RAS to GTP.[21] Substitution of the wild-type Gln-61 with other amino acid residues such as Arg (*NRAS*^{Q61R}), Lys (*NRAS*^{Q61K}), and His (*NRAS*^{Q61H}), has been associated with various types of cancers, including the thyroid.[22,23] The high prevalence of *BRAF* and *RAS* mutations in thyroid malignancies has made the two mutations candidates for molecular markers in the diagnosis of thyroid nodules.[24-27] The combination of FNAC and pre-operative *BRAF*^{V600E} mutation analysis has been shown to increase diagnostic sensitivity from 75.7% to 92.3% and diagnostic accuracy from 78.7% to 90.6%, compared to FNAC alone.[26] However, little is known about the prevalence and clinical significance of *BRAF* and *NRAS* mutations in Malaysian PTC patients. Therefore, the potential use of these mutations as biomarkers in Malaysian patients with thyroid nodules could not be gauged.

To address this gap in knowledge, this retrospective study was conducted to examine the sensitivity and specificity of *BRAF* and *NRAS* point mutations in Malaysian patients with BTG and PTC tumours. The study aimed to determine whether the mutational analysis results can replicate the findings of HPE. The present study also aimed to identify and compare the prevalence of *BRAF* and *NRAS* point mutations between the two PTC subtypes. The findings from this study may contribute to the development of personalised and effective approaches for PTC diagnosis. These approaches can be utilised alongside or as alternatives to current diagnostic procedures, treatments, and management strategies for PTC in Malaysia.

MATERIALS AND METHODS

Study design and subjects

This pilot study included patients with palpable thyroid nodules who were admitted to Universiti Malaya Medical Centre (UMMC) between September 2019 and December 2022. The study protocol was approved by the Medical Research Ethics Committee of UMMC (MREC ID NO: 2019619-7540) and conducted in accordance with the International Conference on Harmonisation of Good Clinical Practice (ICH GCP) guidelines and the Declaration of Helsinki. Prior to the study, written informed consent was obtained from all patients.

In the project workflow as outlined in Figure 1, thyroid tissue specimens obtained from thyroidectomies were immediately submerged in Allprotect tissue reagent (Qiagen, Hilden, Germany) at 4 °C overnight and subsequently stored at –80 °C. After the malignancy status of the thyroid nodules was confirmed through HPE, the patients were grouped into BTG (n = 33) and PTC (n = 25). PTC patients were further classified into (i) PTC without BTG cytomorphological background (PTCa, n = 20) and (ii) PTC with BTG cytomorphological background (PTCb, n = 5).

Genomic DNA extraction from thyroid tissue specimens

Genomic DNA (gDNA) was extracted from the

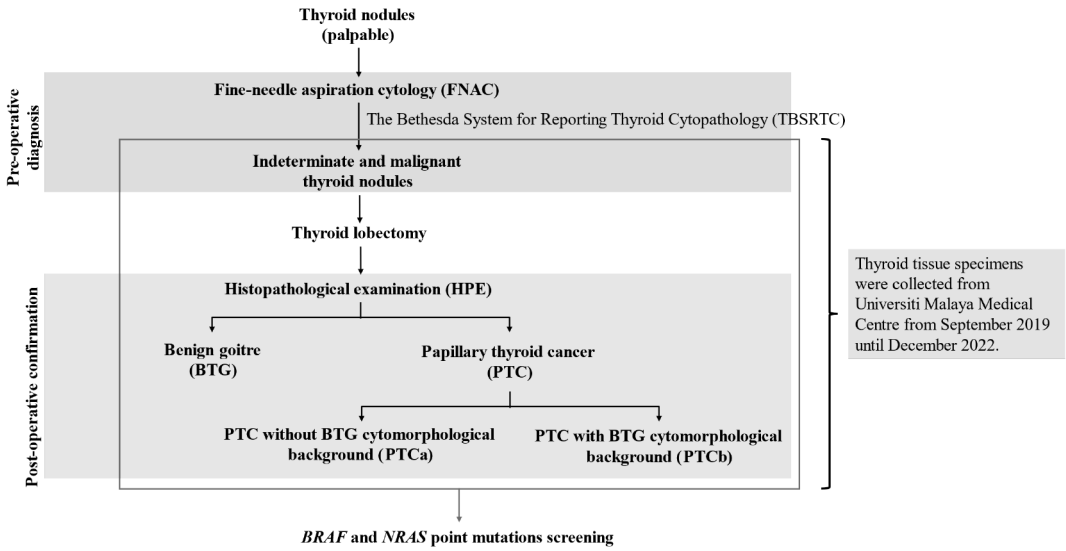


FIG. 1. Overview of the project workflow.

tissue samples using Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted gDNA were determined using Thermo Scientific NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

Polymerase chain reaction (PCR)-direct DNA sequencing

Primer pairs specifically targeting *BRAF*^{V600}, *NRAS*^{G12}, and *NRAS*^{G13} mutations were designed using Primer3 (https://primer3.ut.ee/), while primers for *NRAS*^{Q61} mutation screening were obtained from Campennì *et al*. (2015). The details of the primers are presented in Table 1. PCR was carried out following the protocol outlined in Lee *et al*. (2016). Subsequently, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Sequencing of the PCR products was performed using the Applied Biosystems 3730XL DNA Analyzer (Thermo Fisher Scientific, Massachusetts, USA). The obtained Sanger sequencing electropherograms were then analysed, and the sequencing results were compared to the reference gene sequence using Basic Local Alignment Search Tool

TABLE 1: Sequences of the primers used for mutations screening

Gene	Targeted mutations	Primer	Annealing temperature (°C)	Product size (bp)
BRAF	*BRAF*^{V600}	Forward 5' CCTCAATTCTTACCATCCAC 3'	52	199
		Reverse 5' CTCTTCATAATGCTTGCTCTGATAG 3'		
NRAS	*NRAS*^{G12}	Forward 5' CCAGAAGTGTGAGGCCGATA 3'	51	248
	NRAS^{G13}	Reverse 5' CTGGATTGTCAGTGCGCTTT 3'		
NRAS	*NRAS*^{Q61}	Forward 5' TCTTACAGAAAACAAGTGGT 3'	44	174
		Reverse 5' GTAGAGGTTAATATCCGCAA 3'		

(BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) of National Center for Biotechnology Information (NCBI). The results of mutational analysis were then compared among BTG, PTC, PTCa, and PTCb patients. Mutation(s) which was not reported in the Database for Single Nucleotide Polymorphisms (dbSNP, https://www.ncbi.nlm.nih.gov/snp/) and the Catalogue of Somatic Mutations in Cancer (COSMIC, https://cancer.sanger.ac.uk/cosmic) databases are considered novel.[28]

In-silico functional analysis of a novel mutation

The functional impact of the novel mutation identified in the present study was predicted using three *in-silico* functional prediction tools, namely Polymorphism Phenotyping v2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/), and MutationTaster (https://www.mutationtaster.org/).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8, unless otherwise stated. The diagnostic performance of the mutations for PTC was calculated using the Wilson/Brown method to determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Comparisons of categorical variables were performed using Spearman's rank correlation coefficient analysis and Fisher's exact test. In addition, the genotype and allele frequencies of the variants among different disease groups were analysed using Fisher's exact probability test in Genepop software. The statistical significance of the results was determined with a significance level of *p*-values less than 0.05.

RESULTS

$BRAF^{V600}$, $NRAS^{G12}$, $NRAS^{G13}$, and $NRAS^{Q61}$ mutations in our cohort of patients

Figure 2 shows a representative Sanger sequencing electropherograms of PCR products targeting $BRAF^{V600E}$, $NRAS^{Q61R}$, and $NRAS^{Q61H}$ mutations. Findings from the mutational screening in the BTG and PTC patients are summarised in Table 2(a) and Table 2(b), respectively. $BRAF^{V600E}$ was absent in the BTG patients. Among the 25 PTC patients, eight of them (32%) carried the $BRAF^{V600E}$ mutation, where seven of them belonged to the PTCa group (35%).

Although none of the patients had the $NRAS^{G12}$ and $NRAS^{G13}$ variants, one of the PTC patients (PTC14) had a novel mutation that resulted in amino acid change from valine (Val) to asparagine (Asp) at the 14th amino acid residue ($NRAS^{V14D}$) (Figure 3(a)). *In silico* functional analysis using PolyPhen-2, SIFT, and MutationTaster predicted that the $NRAS^{V14D}$ mutation was functionally deleterious.

Two $NRAS^{Q61}$ variants namely $NRAS^{Q61R}$ and $NRAS^{Q61H}$, were identified in this study. $NRAS^{Q61R}$ was detected in 28% of the PTC patients (six PTCa and one PTCb patients), while 72% of the PTC patients harboured the $NRAS^{Q61H}$ mutation (13 PTCa patients and five PTCb patients).

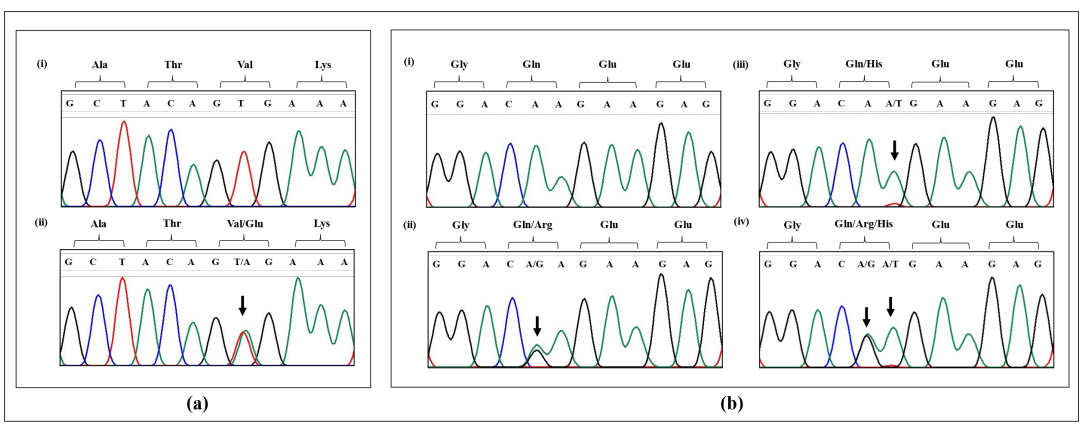


FIG. 2. Representative PCR-Sanger sequencing electropherograms of wild-type and mutant *BRAF* (a) and *NRAS* (b). (a) homozygous T/T (wild type) in PTC10 (i) and heterozygous T/A for $BRAF^{V600E}$ mutation in PTC5 (ii). (b) wild-type $NRAS^{Q61}$ in PTC11 (i), heterozygous A/G for $NRAS^{Q61R}$ mutation in PTC16 (ii), heterozygous A/T for $NRAS^{Q61H}$ mutation in PTC20 (iii), and both $NRAS^{Q61R}$ and $NRAS^{Q61H}$ mutations in PTC22. The mutation is indicated with an arrow.

TABLE 2: Detection of *BRAF* and *NRAS* mutations in (a) BTG and (b) PTC patients.

(a) *BRAF* and *NRAS* mutations in BTG patients.

Patient ID	Age	Gender	Ethnicity	$BRAF^{V600E}$	$NRAS^{G12}$	$NRAS^{G13}$	$^{\#}NRAS^{V14D}$	$NRAS^{Q61R}$	$NRAS^{Q61H}$
BTG1	50	Female	Indian	-	-	-	-	-	-
BTG2	57	Female	Malay	-	-	-	-	-	-
BTG3	80	Female	Chinese	-	-	-	-	-	-
BTG4	58	Female	Chinese	-	-	-	-	-	-
BTG5	58	Female	Malay	-	-	-	-	-	-
BTG6	78	Female	Indian	-	-	-	-	-	-
BTG7	50	Female	Chinese	-	-	-	-	-	+
BTG8	63	Male	Chinese	-	-	-	-	-	-
BTG9	66	Female	Malay	-	-	-	-	-	-
BTG10	61	Female	Malay	-	-	-	-	-	-
BTG11	62	Female	Indian	-	-	-	-	-	-
BTG12	45	Female	Indian	-	-	-	-	-	-
BTG13	38	Female	Malay	-	-	-	-	-	-
BTG14	32	Female	Malay	-	-	-	-	-	-
BTG15	39	Female	Malay	-	-	-	-	-	-
BTG16	57	Male	Malay	-	-	-	-	-	-
BTG17	78	Male	Malay	-	-	-	-	-	-
BTG18	25	Female	Indian	-	-	-	-	-	-
BTG19	43	Female	Malay	-	-	-	-	-	-
BTG20	41	Female	Chinese	-	-	-	-	-	-
BTG21	57	Female	Malay	-	-	-	-	-	-
BTG22	65	Female	Indian	-	-	-	-	-	-
BTG23	49	Female	Indian	-	-	-	-	-	-
BTG24	33	Female	Malay	-	-	-	-	-	-
BTG25	50	Female	Indian	-	-	-	-	-	-
BTG26	76	Female	Malay	-	-	-	-	-	+
BTG27	66	Female	Malay	-	-	-	-	-	-
BTG28	37	Female	Malay	-	-	-	-	-	-
BTG29	63	Female	Indian	-	-	-	-	-	-
BTG30	78	Female	Malay	-	-	-	-	-	-
BTG31	64	Female	Malay	-	-	-	-	-	-
BTG32	72	Female	Indian	-	-	-	-	-	-
BTG33	42	Female	Malay	-	-	-	-	-	-
Total				**0 (0)**	**0 (0)**	**0 (0)**	**0 (0)**	**0 (0)**	**2 (6%)**

(b) *BRAF* and *NRAS* mutations in PTC patients

Patient ID	Age	Gender	Ethnicity	*BRAF*^{V600E}	*NRAS*^{G12}	*NRAS*^{G13}	$^{\#}$*NRAS*^{V14D}	*NRAS*^{Q61R}	*NRAS*^{Q61H}	Lymph nodes metastasis	Additional note (Thyroid disorders or cancer history)
PTC1	51	Female	Indian	+	-	-	-	-	+	-	Family history of thyroid disorders
PTC2	21	Female	Malay	-	-	-	-	-	-	-	
PTC3	44	Female	Malay	-	-	-	-	-	-	-	
PTC4	78	Male	Malay	+	-	-	-	+	+	+	
PTC5	39	Male	Indian	+	-	-	-	+	+	-	
PTC6	55	Female	Chinese	+	-	-	-	+	+	-	
PTC7	39	Female	Indian	-	-	-	-	-	+	-	
PTC8*	42	Female	Malay	-	-	-	-	-	+	-	Endometrioid adenocarcinoma
PTC9	30	Female	Malay	-	-	-	-	-	+	+	
PTC10	59	Female	Chinese	-	-	-	-	-	-	-	
PTC11	46	Female	Malay	-	-	-	-	-	-	-	
PTC12*	57	Female	Chinese	-	-	-	-	-	+	-	
PTC13*	34	Female	Malay	-	-	-	-	+	+	-	
PTC14	67	Female	Malay	-	-	-	+	-	+	-	
PTC15	63	Female	Malay	-	-	-	-	-	-	-	
PTC16	32	Male	Malay	+	-	-	-	+	-	-	
PTC17*	58	Female	Malay	-	-	-	-	-	+	-	Lung adenocarcinoma, no distant metastasis
PTC18	57	Male	Malay	+	-	-	-	-	+	+	
PTC19	42	Male	Chinese	-	-	-	-	-	+	-	
PTC20	59	Female	Indian	-	-	-	-	-	+	-	
PTC21	48	Female	Malay	-	-	-	-	+	+	-	
PTC22	51	Female	Malay	-	-	-	-	+	+	-	
PTC23*	47	Female	Chinese	+	-	-	-	-	+	-	Developed goitre since 16 years old. Left breast cancer at 27 years old. Right invasive breast carcinoma in January 2022. Diagnosed with PTC on March 2022 Family history of goitre
PTC24	28	Female	Malay	-	-	-	-	-	-	-	
PTC25	56	Female	Malay	+	-	-	-	-	+	-	
Total				**8 (32%)**	**0 (0)**	**0 (0)**	**1 (4%)**	**7 (28%)**	**18 (72%)**	**3 (12%)**	

(+) indicates the presence while (-) indicates the absence of mutation or metastasised lymph node(s).
(*) indicates PTCb patients, while those without any indication are specifically categorised as PTCa patients.
($^{\#}$) indicates a novel mutation.
BTG: Benign goitre
PTC: Papillary thyroid cancer
PTCa: Papillary thyroid cancer without benign goitre (BTG) cytomorphological background
PTCb: Papillary thyroid cancer with benign goitre (BTG) cytomorphological background

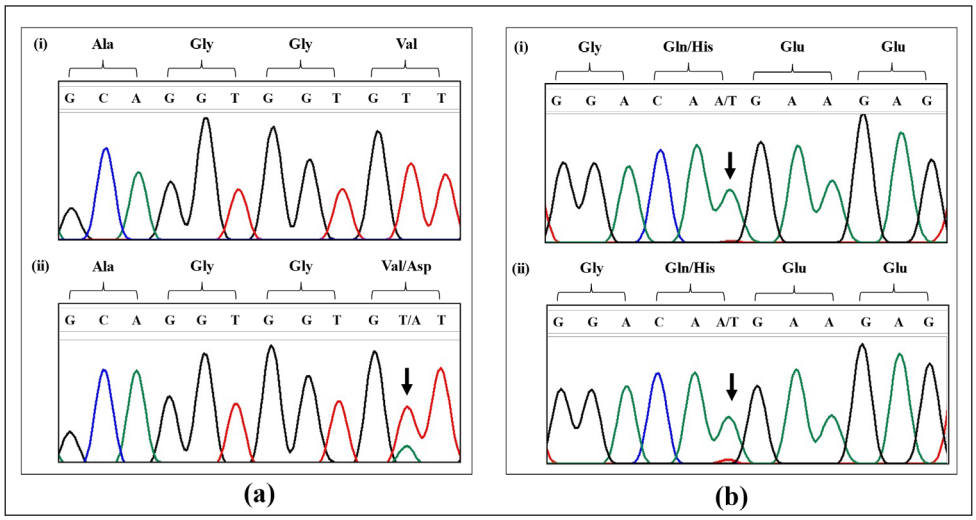


FIG. 3. PCR-Sanger sequencing electropherograms of a novel *NRAS*^{V14D} mutation (a) and *NRAS*^{Q61H} mutation (b). (a) homozygous T/T (wild type) in PTC10 (i) and heterozygous T/A for *NRAS*^{V14D} mutation in PTC14 (ii). (b) heterozygous A/T for *NRAS*^{Q61H} mutation in BTG7 (i) and BTG26 (ii). The mutation is indicated with an arrow.

Additionally, two BTG patients (BTG7 and BTG26) were found to have the *NRAS*^{Q61H} mutation (Figure 3(b)). Noteworthy that all the PTC patients carrying the *BRAF*^{V600E} mutation were found to have either *NRAS*^{Q61R}, *NRAS*^{Q61H}, or both *NRAS*^{Q61R} and *NRAS*^{Q61H} mutations. Among the PTC patients, three patients in the PTCa group (PTC4, PTC9, and PTC18) had lymph node metastasis.

High prevalence of NRAS^{Q61H} mutation in PTCb patients

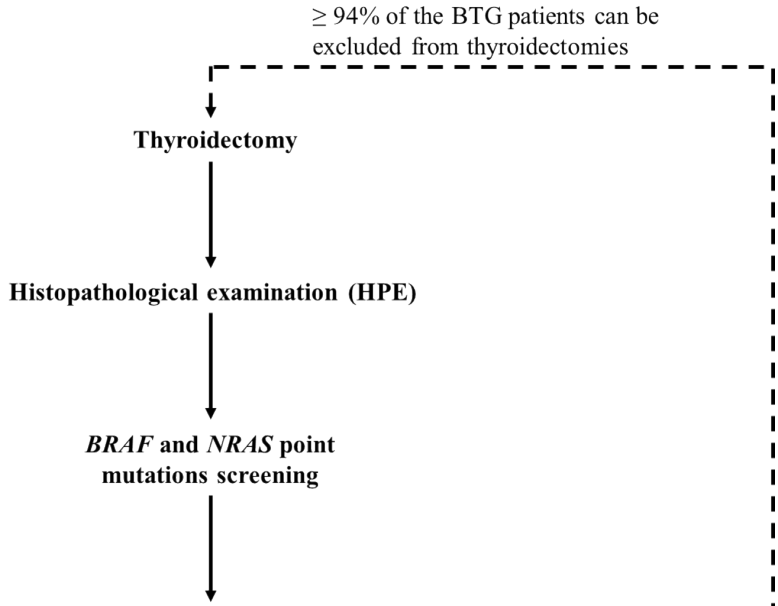
Based on the findings shown in Table 2(b), *NRAS*^{Q61H} mutation was detected in all the five PTCb patients. Among the five, one patient (PTC23) was a compound heterozygote for the *NRAS*^{Q61H} and *BRAF*^{V600E} mutations. PTC23 had a history of prolonged goitre since the age of 16, and she was diagnosed with left breast cancer at the age of 27. She was also diagnosed with right invasive breast cancer and was diagnosed with PTC two months after.

Concordance between BRAF^{V600E}, NRAS^{Q61R}, and NRAS^{Q61H} mutations and HPE findings

Figure 4 summarises the mutations that were detected in this cohort of BTG and PTC patients, and their potential impacts (as evaluated retrospectively) on pre-operative decision making. Among the 33 BTG patients and 25 PTC patients included in this study, a total of 21 individuals were identified as carrying at least one mutation. This indicated that the use of molecular markers in this study could reduce thyroid lobectomies by approximately 64% (37 out of 58 cases). Specifically, at least 94% (31 out of 33) of BTG patients could be accurately excluded from surgery based on the mutation screening results: *BRAF*^{V600E} (100%), *NRAS*^{Q61R} (100%), and *NRAS*^{Q61H} (94%). However, when relying solely on the results of mutation screening, approximately 24% (6 out of 25) of PTC patients may be falsely excluded from further evaluation because none of the targeted mutations were identified in their thyroid tissue specimens.

Table 3 shows the concordance between the *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutational analysis and HPE results in our cohort of patients. Both the *BRAF*^{V600E} and *NRAS*^{Q61R} mutations displayed specificity and PPV of 100% for PTC. On the other hand, the *NRAS*^{Q61H} mutation, despite having the highest sensitivity (72%) [95% CI (52.42%, 85.72%)] among the studied mutations, had a specificity and PPV for PTC of 94% [95% CI (80.39%, 98.92%)] and 90% [95% CI (69.90%, 98.22%)], respectively. The *NRAS*^{Q61H} mutation was found to have the highest NPV among all mutations [82%, 95% CI (66.58%, 90.78%)]. However, the use of more than one mutation in combination did not result in an improvement to the performance, such as NPV, as compared to the use of a single mutation.

PTCb: Papillary thyroid cancer with benign goitre (BTG) cytomorphological background



Groups	*n*	*BRAF*^{V600E} (%)	*NRAS*^{Q61R} (%)	*NRAS*^{Q61H} (%)	At least one mutation (%)
BTG	33	0 (0)	0 (0)	2 (6%)	2 (6%)
PTC	25	8 (32%)	7 (28%)	18 (72%)	19 (76%)
Total	58	8 (14%)	7 (12%)	20 (34%)	21 (36%)

FIG. 4. Mutational analysis and potential influence on pre-operative decision-making. BTG, benign goitre; PTC, papillary thyroid cancer; *n*, number of patients

Association between the mutations and clinicopathologic characteristics of PTC

Figure 5 shows a correlogram linking data from the mutational analysis with clinical characteristics of the PTC patients. The prevalence of *BRAF*^{V600E} mutation (r = 0.514, p = 0.009) and lymph node metastasis (r = 0.431, p = 0.032) demonstrated a positive correlation with the gender of the patients. Multivariate analysis further unravelled significant difference of *BRAF*^{V600E} mutation rate between male and female PTC patients in this preliminary study [odds ratio (OR) (95% CI) = 16 (1.51, 204.1)] (p = 0.0235) (Table 4).

TABLE 3: Concordance between the *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutational analysis and histopathological examination (HPE) results

	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Positive predictive value, % (95% CI)	Negative predictive value, % (95% CI)
BRAF^{V600E}	32 (17.21, 51.59)	100 (80.64, 100)	100 (67.56, 100)	66 (52.15, 77.56)
NRAS^{Q61R}	28 (14.28, 47.58)	100 (89.57, 100)	100 (64.57, 100)	65 (50.99, 76.37)
NRAS^{Q61H}	72 (52.42, 85.72)	94 (80.39, 98.92)	90 (69.90, 98.22)	82 (66.58, 90.78)
BRAF^{V600E} + *NRAS*^{Q61R}	16 (6.40, 34.65)	100 (80.64, 100)	100 (51.01, 100)	61 (47.79, 72.96)
BRAF^{V600E} + *NRAS*^{Q61H}	28 (14.28, 47.58)	100 (80.64, 100)	100 (64.57, 100)	65 (50.99, 76.37)
NRAS^{Q61R}+ *NRAS*^{Q61H}	24 (11.50, 43.43)	100 (80.64, 100)	100 (60.97, 100)	63 (49.87, 75.20)
BRAF^{V600E} + *NRAS*^{Q61R}+ *NRAS*^{Q61H}	12 (4.17, 29.96)	100 (80.64, 100)	100 (43.85, 100)	60 (46.81, 71.88)

Sensitivity measures the ability of the mutation test to correctly identify individuals with the disease or condition within the disease group.
Specificity measures the ability of the mutation test to correctly exclude individuals without the disease or condition within the disease group.
95% confidence intervals (CIs) are presented as (lower limit, upper limit) in brackets.

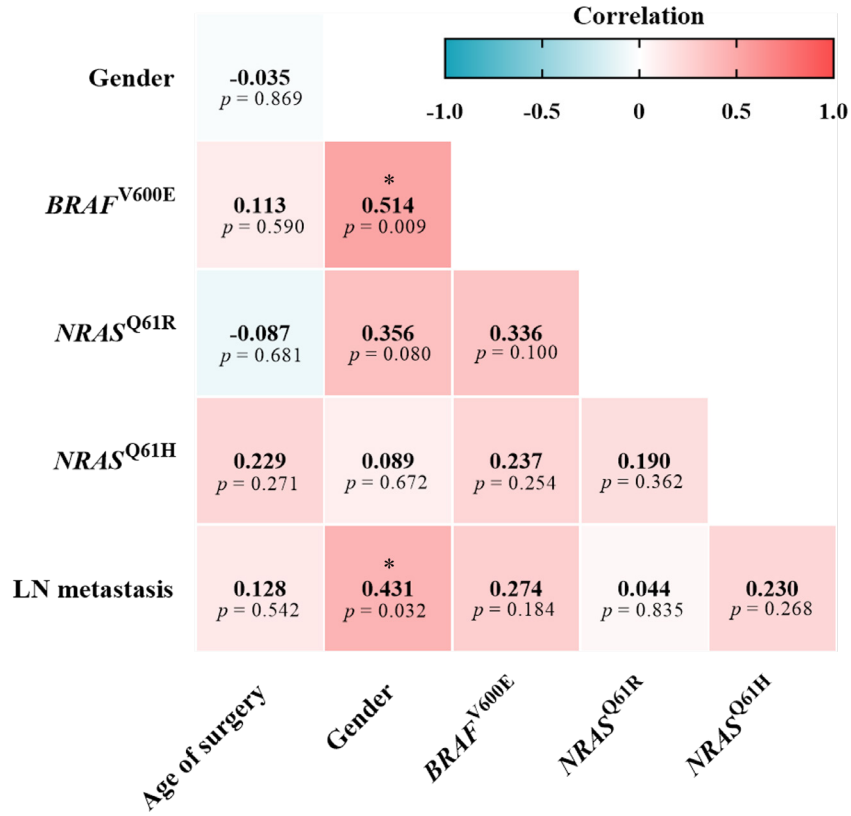


FIG. 5. Correlogram showing the association between molecular analysis and clinical characteristics of the PTC patients. LN, lymph node. (*) indicates significant difference at $p < 0.05$.

Genotypic and allelic frequencies of $BRAF^{V600E}$, $NRAS^{Q61R}$, and $NRAS^{Q61H}$ mutations in BTG and PTC patients

Table 5 displays the genotypic and allelic frequencies of $BRAF^{V600E}$, $NRAS^{Q61R}$, and $NRAS^{Q61H}$ between different disease groups. The frequencies of $BRAF^{V600E}$, $NRAS^{Q61R}$, and $NRAS^{Q61H}$ genotypes and alleles were significantly different between the BTG and PTC groups ($p < 0.05$) (Table 5(a)). Additionally, significant differences were observed in the genotypic and allelic frequencies of the $BRAF^{V600E}$ mutation among the BTG, PTCa, and PTCb groups. However, only the pairwise comparison of the BTG and PTCa groups showed a significant difference (Table 5(b)(i)). The genotypic and allelic frequency differences for the $NRAS^{Q61R}$ variant were significant only between the BTG and PTCa groups (Table 5(b)(ii)), while $NRAS^{Q61H}$ mutation was able to distinguish between the BTG and PTCa groups and the BTG and PTCb groups. None of the four variants could differentiate between the PTCa and PTCb subtypes ($p \geq 0.05$).

DISCUSSION

FNAC is a fundamental diagnostic tool to discriminate malignant from benign thyroid nodules in order to reduce unnecessary thyroidectomies.[29] However, the status of around 30% of the thyroid nodules are cytologically indeterminate, of which only around one-third of the indeterminate nodules were confirmed to be malignant through HPE.[30,31] In the past decades, numerous studies have demonstrated promising results in reinforcing the FNAC accuracy through its combinational use with molecular markers such as a panel of *BRAF* and *NRAS* mutations.[7,32] The applicability of this panel of mutations as a molecular marker in a Malaysian context remains uncertain due to the absence of mutation prevalence and related data.

In the present study, $BRAF^{V600E}$ mutation was found to be PTC-specific, replicating findings from other studies.[33,34] However, the occurrence of $BRAF^{V600E}$ mutation in PTC patients was found to be lower (32%) in our patient cohort, compared to the reported rates

TABLE 4: Association between mutations and clinicopathological characteristics of the patients

Parameters	*BRAF*^{V600E}		*NRAS*^{Q61R}		*NRAS*^{Q61H}	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Age (years)						
> 40	1.250 (0.229, 7.674)	> 0.9999	0.381 (0.055, 2.093)	0.3554	2.625 (0.478, 18.090)	0.3554
> 50	2.381 (0.438, 11.000)	0.4110	0.750 (0.155, 3.754)	> 0.9999	3.125 (0.539, 18.130)	0.3783
> 60	1.071 (0.065, 10.420)	> 0.9999	1.333 (0.080, 13.000)	> 0.9999	0.750 (0.077, 12.520)	> 0.9999
Gender						
Male (n = 5)	16.000 (1.510, 204.100)	0.0235*	6.000 (0.885, 39.120)	0.1130	1.714 (0.177, 24.100)	> 0.9999
Female (n = 20)						
Lymph node metastasis						
Yes	5.333 (0.505, 81.090)	0.2313	1.333 (0.080, 13.000)	> 0.9999	∞ (0.339, ∞)	0.5343
No						

OR: Odds ratio
95% confidence intervals (CIs) are presented as (lower limit, upper limit) in brackets.
(*) indicates significant difference at $p < 0.05$.

in European and American populations (40% to 45%), Middle Eastern populations (52%), and PTC patients from East Asian countries (71% to 76%).[35-37] Additionally, the *BRAF*^{V600E} detection rate in the PTC patients of this study was lower compared to the Southeast Asian countries like Thailand (61%), Vietnam (83%), and Singapore (65%), but similar to Indonesia (36%) and the Philippines (45%).[35] The lower detection rate may be contributed by the genetic heterogeneity of the multi-ethnic population in Malaysia.[38] The relatively lower incidence of *BRAF*^{V600E} in the Malaysian population with PTC, compared to Singapore, a neighbouring multi-ethnic country, could potentially be attributed to the predominant Malay ethnicity in Malaysia (constituting over 60% of the population, with 64% of PTC patients included in this study being Malays), as opposed to Singapore, where the Chinese population exceeds 75% (with 20% of PTC patients included in this study being Chinese). This discrepancy may also account for the detection rate being comparatively similar to that reported in Indonesia (36%) due to the close genetic relationship between the Malay and Indonesian populations.[39] Furthermore, dissimilar frequencies of genotypic and karyotypic variations in genes have been discerned among different ethnicities within the Malaysian populace.[40] However, the relatively small sample size of the present study unfortunately limits the comparison of mutation frequencies between different ethnicities. Consequently, further investigations into the prevalence of these mutations in PTC patients of diverse ethnicities in Malaysia are warranted and could pave the way for more personalised disease management strategies.

The most frequently reported point mutations in *NRAS* genes are of the *NRAS*^{Q61} type, with *NRAS*^{Q61R} being the prevailing variant.[41,42] However, in this study, the prevalent variant was the *NRAS*^{Q61H} (72%) instead of the *NRAS*^{Q61R} (28%), thus contradicting other studies that reported the dominance of *NRAS*^{Q61R} in thyroid neoplasms. *NRAS*^{Q61} mutations lead to constitutive activation of NRAS protein due to disruption of the GTP/GDP switch mechanism.[21] Consequently, the persistently active NRAS protein signals downstream pathways independently, contributing to uncontrolled cell proliferation, survival, and potentially promoting the development of cancer. Additionally, the occurrence of *NRAS* gene mutations was found to be higher in PTC patients

TABLE 5: Genotypic frequency and allelic frequency of *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutations in different disease groups.

(a) Genotypic frequency and allelic frequency of BRAF^{V600E}, NRAS^{Q61R}, and NRAS^{Q61H} mutations in BTG and PTC groups.

Variants	Genotype	Number of patients (%)				*p*-value	Allele	Total haplotypes (%)				*p*-value
		BTG (n = 33)		PTC (n = 25)				BTG		PTC		
BRAF^{V600E}	T|T (wild type)	33	(100)	17	(68)	0.00051*	T	66	(100)	42	(100)	0.00079*
	T|A (heterozygous mutant)	0	0	8	(32)		A	0	0	8	0	
	A|A (homozygous mutant)	0	0	0	0							
NRAS^{Q61R}	A|A (wild type)	33	(100)	18	(72)	0.00143*	A	66	(100)	43	(86)	0.00201*
	A|G (heterozygous mutant)	0	0	7	(28)		G	0	0	7	(14)	
	G|G (homozygous mutant)	0	0	0	0							
NRAS^{Q61H}	A|A (wild type)	31	(94)	7	(28)	< 0.00001*	A	64	(97)	32	(64)	0.00003*
	A|T (heterozygous mutant)	2	(6)	18	(72)		T	2	(3)	18	(36)	
	T|T (homozygous mutant)	0	0	0	0							

(b) Genotypic frequency and allelic frequency of *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutations in BTG, PTCa, and PTCb groups.

(i) Genotypic frequency

Variants	Genotype	Number of patients (%)						*p*-value	Pair-wise omparison	*p*-value
		BTG (n = 33)		PTCa (n = 20)		PTCb (n = 5)				
BRAF^{V600E}	T|T (wild type)	33	(100)	13	(65)	4	(80)		BTG vs PTCa	0.00049*
	T|A (heterozygous mutant)	0	0	7	(35)	1	(20)	0.00032*	BTG vs PTCb	0.13526
	A|A (homozygous mutant)	0	0	0	0	0	0		PTCa vs PTCb	0.64098
NRAS^{Q61R}	A|A (wild type)	33	(100)	14	(70)	4	(80)		BTG vs PTCa	0.00227*
	A|G (heterozygous mutant)	0	0	6	(30)	1	(20)	0.00131*	BTG vs PTCb	0.13154
	G|G (homozygous mutant)	0	0	0	0	0	0		PTCa vs PTCb	1.00000
NRAS^{Q61H}	A|A (wild type)	31	(94)	7	(35)	0	0		BTG vs PTCa	< 0.00001*
	A|T (heterozygous mutant)	2	(6)	13	(65)	5	(100)	< 0.00001*	BTG vs PTCb	0.00007*
	T|T (homozygous mutant)	0	0	0	0	0	0		PTCa vs PTCb	0.27233

(ii) Allelic frequency

Variants	Allele	Total haplotypes (%)						*p*-value	Pair-wise comparison	*p*-value
		BTG (n=33)		PTCa (n=20)		PTCb (n=5)				
BRAF^{V600E}	T	66	(100)	33	(82.5)	9	(90)		BTG vs PTCa	0.00084*
	A	0	0	7	(17.5)	1	(10)	0.00162*	BTG vs PTCb	0.13488
									PTCa vs PTCb	1.00000
NRAS^{Q61R}	A	66	(100)	34	(85)	9	(90)		BTG vs PTCa	0.00299*
	G	0	0	6	(15)	1	(10)	0.00210*	BTG vs PTCb	0.13140
									PTCa vs PTCb	1.00000
NRAS^{Q61H}	A	64	(97)	27	(68)	5	(50)		BTG vs PTCa	0.00002*
	T	2	(3)	13	(32)	5	(50)	< 0.00001*	BTG vs PTCb	0.00032*
									PTCa vs PTCb	0.45787

BTG: Benign goitre
PTCa: Papillary thyroid cancer without BTG cytomorphological background
PTCb: Papillary thyroid cancer with BTG cytomorphological background
(*) indicates significant difference at $p < 0.05$.

in this study compared to what was previously reported.[43,44] The disparity in the prevalence of this mutation suggests the possibility of distinct underlying molecular mechanisms in the development of PTC within our patient cohort. Although neither *NRAS*^{G12} nor *NRAS*^{G13} variant was identified in this study, the primer pairs targeting those mutations revealed the presence of a novel variant, *NRAS*^{V14D}. This variant, which was detected in one PTC patient (PTC14), was predicted to be functionally deleterious when analysed *in-silico*. To confirm the *in-silico* findings, further biological functional analysis would need to be carried out to study its potential impact to the *NRAS* protein product and the oncogenic activation of the signalling pathway.

Findings from this study indicate that mutational screening can safely exclude at least 94% of patients diagnosed with BTG from undergoing thyroidectomy for malignancy confirmation through HPE. Regarding patients with PTC, 19 PTC patients (76%) included in this study were found to harbour at least one mutation, while six of them did not exhibit any of the *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutations. This suggests that 24% of PTC patients might be falsely excluded from thyroidectomies based on the mutation screening results. It is particularly important to note that this exclusion was independent of the cytology findings obtained from FNAC. For routine cases in UMMC, patients with FNAC findings categorised as Bethesda III, IV, V, and VI were usually considered for hemi- or total thyroidectomies. Notably, approximately 30% of thyroid nodules were classified as indeterminate,[30,31] and among these indeterminate cases, less than 40% were confirmed to be malignant based on HPE diagnosis.[31] However, in this study, patients were only included after their malignancy status were confirmed through HPE results, focusing specifically on BTG patients and those diagnosed with PTC. Hence, the incorporation of molecular marker detection alongside FNAC findings for thyroid nodules could enhance the pre-operative diagnostic sensitivity and specificity of these nodules. A recent study combining cytology findings with *BRAF*^{V600E} mutation analysis has resulted in a significantly higher sensitivity of 96% and specificity of 94.3% in diagnosing pre-operative thyroid nodules than either FNAC or *BRAF*^{V600E} mutation alone.[45] Moreover, the establishment of a panel that combines multiple gene mutations has been widely utilised in the diagnosis of thyroid nodules.[46-49] Therefore, the

performance of combining multiple mutations in reproducing the HPE findings of our patient cohorts was also analysed. The combination of multiple mutations did not demonstrate an improvement in the NPV compared to the individual use of a single mutation, which aligns with the findings of a previous study.[27] However, the performance of the combination of multiple mutations with cytology findings was shown to have an increased diagnosis accuracy (from 60% to 76%) and higher NPV (from 35% to 48%).[27] Therefore, a prospective study incorporating fine-needle aspirate biopsies mutational analysis and combining them with FNAC cytology findings holds promise for offering a more comprehensive assessment and understanding of the diagnostic efficacy associated with these mutations.

It is noteworthy to mention that all patients who tested positive for *BRAF*^{V600E} in the current study also exhibited concurrent *NRAS*^{Q61R}, *NRAS*^{Q61H} or both *NRAS*^{Q61R} and *NRAS*^{Q61H} mutations. The majority of these patients were PTCa, with seven out of eight PTC patients with coexisting *BRAF*^{V600E} and *NRAS*^{Q61} mutations being PTCa patients. While the coexistence of *BRAF*^{V600E} and *NRAS*^{Q61} mutations has been previously reported in melanomas, it remains less well-documented in PTC.[50] Interestingly, the *NRAS*^{Q61H} mutation was detected in all PTCb patients, while *BRAF*^{V600E} mutation was only detected in one PTCb patient (PTC23). PTC23 had a history of goitre since the age of 16 years old before she was finally diagnosed with PTC in March 2022 at the age of 47 years old. She was diagnosed with left breast cancer at 27 years old followed by right invasive breast cancer at the age of 47 years old. As compared to *NRAS*^{Q61R} that was associated with spontaneous melanoma tumourigenesis, *NRAS*^{Q61H} mutation has been linked to slower progression of melanoma.[51] Furthermore, previous study has suggested a slower progression of PTCb from BTG.[11] Hence, it is hypothesised that *NRAS*^{Q61H} mutation may have contributed towards the development of PTCb from benign goitre. In addition to *NRAS*^{Q61H} mutation, the coexisting *BRAF*^{V600E} mutation might enhance the aggressiveness and metastatic potential of PTCb. *BRAF*^{V600E} mutation is associated with more aggressive PTC phenotypes and poorer prognosis.[52] Moreover, cases of breast metastasis in PTC have been reported.[53,54] The presence of the *NRAS*^{Q61H} mutation in the two BTG patients (BTG7 and BTG26) may have put them at risk of developing PTCb. Additionally, BTG7 was found to have thyroid goitre for the past six years. The potential transformation of BTG to PTC has been previously discussed,[11,55] and the identification of the *NRAS*^{Q61H} mutation in BTG patients in this study may be associated with the BTG-to-PTCb transformation. However, further investigation is needed to validate this hypothesis in BTG patients with the *NRAS*^{Q61H} mutation.

In the context of correlating clinicopathological characteristics and mutation profiles, moderate positive correlations were observed between gender and the *BRAF*^{V600E} mutation. *BRAF*^{V600E} was identified in 80% of the male PTC patients and 20% of the female PTC patients. The odds ratio of 16 observed in this patient cohort indicates a significantly higher likelihood of developing PTC in male patients with the *BRAF* mutation. *BRAF*^{V600E} was found to be associated with male PTC patients.[56] In addition, male gender has been identified as a risk factor for mortality in PTC patients with the *BRAF*^{V600E} mutation.[57] However, the *BRAF*^{V600E} mutation was found to be dominant in female colorectal cancer and non-small cell lung cancer patients.[58,59] An association was found between gender and lymph node metastasis in this study. However, due to the small number of PTC patients with lymph node metastasis (only three cases), further study with a larger sample size is necessary to confirm the current findings.

The genotypic and allelic frequencies for *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutations showed significant differences between the BTG and PTC groups, as well as among the BTG, PTCa, and PTCb groups. These findings suggested heterozygous mutant genotype of *BRAF*^{V600E} (T|A), *NRAS*^{Q61R} (A|G), and *NRAS*^{Q61H} (A|T) have the potential to differentiate between BTG and PTC patients. Considering the presence of only heterozygous mutation in the current study, it is suggested that the "A" allele in the *BRAF*^{V600E} mutation, the allele "G" in the *NRAS*^{Q61R} mutation and the allele "T" in the *NRAS*^{Q61H} mutation are pathogenic alleles that can functionally affect the encoded proteins. On the other hand, pairwise comparisons of the genotypic and allelic frequencies among the BTG, PTCa, and PTCb groups indicated significant differences in *BRAF*^{V600E} and *NRAS*^{Q61R} mutations between the BTG and PTCa groups only. Significant differences in genotypic and allelic frequencies of the *NRAS*^{Q61H} mutation were identified between the BTG and PTCa groups, as well as between the BTG and PTCb groups. However, further validation using a larger sample

size is required due to the relatively small sample size of the PTCb group.

Taken together, the findings from the *BRAF*^{V600E} and *NRAS*^{Q61R} mutational analysis were able to replicate the retrospective HPE diagnosis. Moreover, the panel of *BRAF*^{V600E}, *NRAS*^{Q61R}, and *NRAS*^{Q61H} mutations was able to accurately differentiate BTG from PTC patients but was unable to distinguish between the two PTC subtypes. The sole use of the mutation panel could exclude 94% of the BTG patients from unnecessary thyroidectomy to confirm malignancy status through HPE but it may also produce false negative diagnosis of 24% of the PTC patients. To potentially apply the panel as PTC biomarkers in Malaysia, validation in a larger sample size (allowing for further categorisation into different ethnic groups) and the incorporation of fine-needle aspirate biopsies are required. Further *in-vitro* investigations are necessary to evaluate the potential role of *NRAS*^{Q61H} mutation in BTG-to-PTCb transformation and reaffirm the potential association between gender with *BRAF*^{V600E} mutation prevalence and lymph node metastasis in PTC patients.

Funding Statement: This study was funded by the Ministry of Higher Education Malaysia [FRGS/1/2018/SKK08/UM/02/16]; and the Universiti Malaya [RMF1116-2021, RMF0795-2021, and RMF1025-2021]. The funders had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

Authors' contributions: EZH participated in the study design, performed the experiments, analysed, and interpreted the data, drafted the manuscript, prepared figures, and tables. MMAJ performed the experiments, analysed, and interpreted the data, and reviewed manuscript drafts. AAA analysed the data, and reviewed manuscript drafts. NKL performed surgeries, involved in specimen collection process, and contributed the clinical data of the patients and reviewed manuscript drafts. SMJ conceived and designed the experiments, analysed, and interpreted the data, reviewed drafts, and critically revised the final manuscript. All authors read and approved the final manuscript.

Conflict of interests: The authors declared no conflict of interest.

REFERENCES

1. Cappelli C, Castellano M, Pirola I, *et al*. Thyroid nodule shape suggests malignancy. Eur J Endocrinol. 2006;155(1):27-31.
2. Tamhane S, Gharib H. Thyroid nodule update on diagnosis and management. Clin Diabetes Endocrinol. 2016;2:17.
3. Gharib H, Papini E, Garber JR, *et al*. American association of Clinical Endocrinologists, American college of endocrinology, and Associazione Medici Endocrinologi medical guidelines for clinical practice for the diagnosis and management of thyroid nodules - 2016 update. Endocr Pract. 2016;22(5):622-39.
4. Gharib H, Papini E, Valcavi R, *et al*. American Association of Clinical Endocrinologists and Associazione Medici Endocrinologi Medical Guidelines for Clinical Practice for the Diagnosis and Management of Thyroid Nodules. Endocr Pract. 2006;12(1):63-102.
5. Paulson VA, Rudzinski ER, Hawkins DS. Thyroid cancer in the pediatric population. Genes. 2019;10(9):723.
6. Wang H, Mehrad M, Ely KA, *et al*. Incidence and malignancy rates of indeterminate pediatric thyroid nodules. Cancer Cytopathol. 2019;127(4):231-9.
7. Nikiforov YE, Steward DL, Robinson-Smith TM, *et al*. Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab. 2009;94(6):2092-8.
8. Capezzone M, Cantara S, Di Santo A, *et al*. The Combination of Sonographic Features and the Seven-Gene Panel May be Useful in the Management of Thyroid Nodules With Indeterminate Cytology. Front Endocrinol. 2021;12:613727.
9. Li X, Li E, Du J, *et al*. *BRAF* mutation analysis by ARMS-PCR refines thyroid nodule management. Clin Endocrinol. 2019;91(6):834-41.
10. Abdullah MI, Junit SM, Ng KL, *et al*. Papillary thyroid cancer: Genetic alterations and molecular biomarker investigations. Int J Med Sci. 2019;16(3):450-60.
11. Eng ZH, Abdullah MI, Ng KL, *et al*. Whole-exome sequencing and bioinformatic analyses revealed differences in gene mutation profiles in papillary thyroid cancer patients with and without benign thyroid goitre background. Front Endocrinol. 2023;13:1039494.
12. Abdullah MI, Lee CC, Mat Junit S, *et al*. Tissue and serum samples of patients with papillary thyroid cancer with and without benign background demonstrate different altered expression of proteins. PeerJ. 2016;4:e2450.
13. Nikiforova MN, Nikiforov YE. Thyroid cancer. In: Molecular Pathology in Clinical Practice. 2nd ed. Springer International Publishing; 2016. p.471-85.
14. Nam JK, Jung CK, Song BJ, *et al*. Is the BRAF^{V600E} mutation useful as a predictor of preoperative risk in papillary thyroid cancer? Am J Surg. 2012;203(4):436-41.
15. Silver JA, Bogatchenko M, Pusztaszeri M, *et al*. *BRAF V600E* mutation is associated with aggressive

features in papillary thyroid carcinomas ≤ 1.5 cm. J Otolaryngol Head Neck Surg. 2021;50(1):63.
16. Nikiforov YE. Thyroid carcinoma: molecular pathways and therapeutic targets. Mod Pathol. 2008;21(Suppl 2):S37□S43.
17. Angell TE, Lechner MG, Jang JK, *et al*. BRAF^{V600E} in Papillary Thyroid Carcinoma Is Associated with Increased Programmed Death Ligand 1 Expression and Suppressive Immune Cell Infiltration. Thyroid. 2014;24(9):1385-93.
18. Moura MM, Cavaco BM, Leite V. *RAS* proto-oncogene in medullary thyroid carcinoma. Endocr Relat Cancer. 2015;22(5):R235-R252.
19. Campennì A, Giovinazzo S, Curtò L, *et al*. Thyroid hemiagenesis, Graves' disease and differentiated thyroid cancer: a very rare association: case report and review of literature. Hormones. 2015;14(3):451-8.
20. Saha S, Joshi A, Noronha V, *et al*. *NRAS* mutation in differentiated thyroid cancer. Cancer Research, Statistics, and Treatment. 2022;5(4):722-7.
21. Hobbs GA, Der CJ, Rossman KL. RAS isoforms and mutations in cancer at a glance. J Cell Sci. 2016;129(7):1287-92.
22. An JH, Song KH, Kim SK, *et al*. RAS mutations in indeterminate thyroid nodules are predictive of the follicular variant of papillary thyroid carcinoma. Clin Endocrinol. 2015;82(5):760-6.
23. Bonhomme B, Godbert Y, Perot G, *et al*. Molecular Pathology of Anaplastic Thyroid Carcinomas: A Retrospective Study of 144 Cases. Thyroid. 2017;27(5):682-92.
24. Salvatore G, Giannini R, Faviana P, *et al*. Analysis of BRAF point mutation and RET/PTC rearrangement refines the fine-needle aspiration diagnosis of papillary thyroid carcinoma. J Clin Endocrinol Metab. 2004;89(10):5175-80.
25. Rossi ED, Martini M, Capodimonti S, *et al*. *BRAF* (V600E) mutation analysis on liquid-based cytology-processed aspiration biopsies predicts bilaterality and lymph node involvement in papillary thyroid microcarcinoma. Cancer Cytopathol. 2013;121(6):291-7.
26. Zhao CK, Zheng JY, Sun LP, *et al*. BRAF^{V600E} mutation analysis in fine-needle aspiration cytology specimens for diagnosis of thyroid nodules: The influence of false-positive and false-negative results. Cancer Med. 2019;8(12):5577-89.
27. Perdana AB, Putri RI, Rachmawati R, *et al*. Clinical Utility of BRAF, NRAS, and TERT Promoter Mutation in Preoperative Thyroid Fine-Needle Aspiration Biopsy: A Diagnostic Study From Dharmais Cancer Hospital. APJCP. 2020;21(11):3267-77.
28. Tate JG, Bamford S, Jubb HC, *et al*. COSMIC: the Catalogue Of Somatic Mutations In Cancer. Nucleic Acids Res. 2019;47(D1):D941-D947.
29. Karimi-Yazdi A, Motiee-Langroudi M, Saedi B, *et al*. Diagnostic Value of Fine-Needle Aspiration in Head and Neck Lymphoma: A Cross-Sectional Study. Indian J Otolaryngol Head Neck Surg. 2014;66(1):277-80.
30. Alshaikh S, Harb Z, Aljufairi E, *et al*. Classification of thyroid fine-needle aspiration cytology into Bethesda categories: An institutional experience and review of the literature. Cytojournal. 2018;15:4.
31. Yaprak Bayrak B, Eruyar AT. Malignancy rates for Bethesda III and IV thyroid nodules: a retrospective study of the correlation between fine-needle aspiration cytology and histopathology. BMC Endocr Disord. 2020;20(1):48.
32. Moses W, Weng J, Sansano I, *et al*. Molecular testing for somatic mutations improves the accuracy of thyroid fine-needle aspiration biopsy. World J Surg. 2010;34(11):2589-94.
33. Nikiforova MN, Kimura ET, Gandhi M, *et al*. BRAF Mutations in Thyroid Tumors Are Restricted to Papillary Carcinomas and Anaplastic or Poorly Differentiated Carcinomas Arising from Papillary Carcinomas. J Clin Endocrinol Metab. 2003;88(11):5399-404.
34. Cohen Y, Xing M, Mambo E, *et al*. BRAF Mutation in Papillary Thyroid Carcinoma. J Natl Cancer Inst. 2003;95(8):625-7.
35. Rashid FA, Munkhdelger J, Fukuoka J, *et al*. Prevalence of *BRAF^{V600E}* mutation in Asian series of papillary thyroid carcinoma-a contemporary systematic review. Gland Surg. 2020;9(5):1878-900.
36. Bychkov A. Prevalence of *BRAF*^{V600E} mutation in Asian patients with thyroid cancer. Malays J Pathol. 2017;19(1):95-6.
37. Abubaker J, Jehan Z, Bavi P, *et al*. Clinicopathological Analysis of Papillary Thyroid Cancer with *PIK3CA* Alterations in a Middle Eastern Population. J Clin Endocrinol Metab. 2008;93(2):611-8.
38. Nayra S A, Vivian R, JosÉ SebastiÃO Dos S, *et al*. Impact of Ethnicity on Somatic Mutation Rates of Pancreatic Adenocarcinoma. In Vivo. 2018;32(6):1527-31.
39. Hatin WI, Nur-Shafawati AR, Zahri M-K, *et al*. Population Genetic Structure of Peninsular Malaysia Malay Sub-Ethnic Groups. PLoS One. 2011;6(4):e18312.
40. Kevin-Tey WF, Wen WX, Bee PC, *et al*. KIR genotype and haplotype frequencies in the multiethnic population of Malaysia. Hum Immunol. 2023;84(3):172-85.
41. Gupta N, Dasyam AK, Carty SE, *et al*. *RAS* mutations in thyroid FNA specimens are highly predictive of predominantly low-risk follicular-pattern cancers. J Clin Endocrinol Metab. 2013;98(5):E914-E922.
42. Nikiforova MN, Lynch RA, Biddinger PW, *et al*. *RAS* Point Mutations and PAX8-PPARγ Rearrangement in Thyroid Tumors: Evidence for Distinct Molecular Pathways in Thyroid Follicular Carcinoma. J Clin Endocrinol Metab. 2003;88(5):2318-26.
43. Melo M, Gaspar da Rocha A, Batista R, *et al*. *TERT*, *BRAF*, and *NRAS* in Primary Thyroid Cancer and Metastatic Disease. J Clin Endocrinol Metab. 2017;102(6):1898-907.
44. Tobiás B, Halászlaki C, Balla B, *et al*. Genetic alterations in Hungarian patients with papillary thyroid cancer. Pathol Oncol Res. 2016;22(1):27-33.
45. Du J, Han R, Chen C, *et al*. Diagnostic Efficacy of Ultrasound, Cytology, and BRAF^{V600E} Mutation Analysis and Their Combined Use in Thyroid Nodule Screening for Papillary Thyroid Microcarcinoma. Front Oncol. 2022;11:746776.

46. Khatami R, Metter C, Heimann A, *et al*. Accuracy of targeted next generation sequencing panel (TNGS) in indeterminate thyroid fine needle aspiration (FNA) specimens with surgical follow up. Laboratory Investigation. 2018;98:152-3.
47. Nikiforova MN, Mercurio S, Wald AI, *et al*. Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. Cancer. 2018;124(8):1682-90.
48. Chin PD, Zhu CY, Sajed DP, et al. Correlation of ThyroSeq Results with Surgical Histopathology in Cytologically Indeterminate Thyroid Nodules. Endocr Pathol. 2020;31(4):377-84.
49. Livhits MJ, Kuo EJ, Leung AM, *et al*. Gene Expression Classifier vs Targeted Next-Generation Sequencing in the Management of Indeterminate Thyroid Nodules. J Clin Endocrinol Metab. 2018;103(6):2261-8.
50. Raaijmakers MIG, Widmer DS, Narechania A, *et al*. Co-existence of *BRAF* and *NRAS* driver mutations in the same melanoma cells results in heterogeneity of targeted therapy resistance. Oncotarget. 2016;7(47):77163-74.
51. Murphy BM, Terrell EM, Chirasani VR, *et al*. Enhanced BRAF engagement by NRAS mutants capable of promoting melanoma initiation. Nat Commun. 2022;13(1):3153.
52. Costa AM, Herrero A, Fresno MF, *et al*. *BRAF* mutation associated with other genetic events identifies a subset of aggressive papillary thyroid carcinoma. Clin Endocrinol. 2008;68(4):618-34.
53. Yu F, Ma J, Huo K, *et al*. Association between breast cancer and thyroid cancer: a descriptive study. Transl Cancer Res. 2017;6(2):393-401.
54. Worapongpaiboon R, Vongsaisuwon M. Breast metastasis of papillary thyroid carcinoma. BMJ Case Rep. 2022;15(7):e251081.
55. Eng ZH, Abdul Aziz A, Ng KL, *et al*. Changes in antioxidant status and DNA repair capacity are corroborated with molecular alterations in malignant thyroid tissue of patients with papillary thyroid cancer. Front Mol Biosci. 2023;10:1237548.
56. Li C, Lee KC, Schneider EB, *et al*. *BRAF* V600E mutation and Its Association with Clinicopathological Features of Papillary Thyroid Cancer: A Meta-Analysis. J Clin Endocrinol Metab. 2012;97(12):4559-70.
57. Wang F, Zhao S, Shen X, *et al*. *BRAF* V600E Confers Male Sex Disease-Specific Mortality Risk in Patients With Papillary Thyroid Cancer. J Clin Oncol. 2018;36(27):2787-95.
58. Chen D, Huang JF, Liu K, *et al*. $BRAF^{V600E}$ Mutation and Its Association with Clinicopathological Features of Colorectal Cancer: A Systematic Review and Meta-Analysis. PLoS One. 2014;9(3):e90607.
59. Cui G, Liu D, Li W, *et al*. A meta-analysis of the association between BRAF mutation and nonsmall cell lung cancer. Medicine. 2017;96(14):e6552.