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ORIGINAL RESEARCH

Profile of *BRAF*V600E, *BRAF*K601E, *NRAS*, *HRAS*, and *KRAS* Mutational Status, and Clinicopathological Characteristics of Papillary Thyroid Carcinoma in Indonesian National Referral Hospital

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Introduction: *BRAF*V600E and *RAS* mutations are the most common gene mutations in papillary thyroid carcinoma (PTC) that may be correlated with its biological behavior. There are still limited data about *BRAF*V600E and *RAS* mutations in Indonesia. This study aims to determine the prevalence of *BRAF*V600E and *RAS* mutations, and their association with clinicopathologic characteristics.

Methods: Patients who had total thyroidectomy from 2019 to 2021 and those who met our study criteria underwent PCR and DNA sequencing analysis for *BRAF*V600E, *BRAF*K601E, exon 2 and 3 of *NRAS, HRAS*, and *KRAS*. Analyses were performed to determine the associations of *BRAF*V600E and *RAS* mutations with clinicopathologic characteristics.

Results: Of 172 PTC patients, *BRAF*V600E mutation was observed in 37.8% of the patients and *RAS* mutations were found in 21.5%. One patient harbored *BRAF*K601E mutation. There was a significant association of *BRAF*V600E with a high-stage (p = 0.033, OR: 3.279; 95% CI: 1.048–10.259), tall-cell variants (p ≤0.001, OR: 41.143; 95% CI: 11.979–141.308), non-encapsulated (p = 0.001, OR: 4.176; 95% CI: 2.008–8.685), lymphovascular invasion (p = 0.043, OR: 1.912; 95% CI: 1.018–3.592), extrathyroidal extension (p = <0.001, OR: 3.983; 95% CI: 1.970–8.054), and lymph node metastasis (p = 0.009, OR: 2.301; 95% CI: 1.224–4.326). Follicular variant (p = 0.001, OR: 7.011; 95% CI: 2.690–18.268), encapsulated (p = 0.017, OR: 2.433; 95% CI: 1.161–5.100), and absent of extrathyroidal extension (p = 0.033, OR: 2.890; 95% CI: 1.052–7.940) were associated with *RAS* mutations.

Conclusion: A significant association between *BRAF*V600E mutation and high clinical stage, tall-cell variants, non-encapsulated morphology, lymphovascular invasion, extrathyroidal extension, and lymph node metastasis in PTC was observed. *RAS* mutations were associated with the follicular variant, encapsulated tumor, and no extrathyroidal extension. *HRAS*-mutated PTC frequently exhibited tumor multifocality.

Keywords: papillary thyroid carcinoma, *BRAF*V600E, *BRAF*K601E, *RAS*, clinicopathological characteristics

Introduction

In recent decades, there has been a dramatic increase in the incidence of thyroid cancer.[1,2] In terms of worldwide and Indonesian cancer incidence, it is currently ranked 7th and 12th, respectively.[3] Papillary thyroid carcinoma (PTC) is a follicular cell-derived tumor attributed to 80–85% of thyroid cancer.[1] Based on its distinctive histopathological

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characteristics, striking variants including tall and columnar cells, oncocytic, solid/trabecular, and those frequently display extrathyroidal extension (ETE), lymph node metastasis (LNM), and distant organ metastasis, are particularly aggressive.[4]

The presence of gene mutation is associated with the behavior and prognosis of the disease. B-Rapidly accelerating fibrosarcoma (*BRAF*V600E) and RAT Sarcoma (*RAS*) mutations are well-published driver mutations in the development of PTC. *BRAF*V600E mutation is typically present in the classic and tall-cell variants, commonly related to a higher level of aggression.[5] Patients with *BRAF* mutation are twice as likely to experience a relapse of their illness and possess greater mortality rates than those without the mutation.[6,7] The human *RAS* gene is divided into Kirsten RAT sarcoma (*KRAS*), neuroblastoma (*NRAS*), and Harvey (*HRAS*), as opposed to *BRAF*V600E mutation, are more prevalent in the follicular variant of PTC.[5] Better prognosis and more indolent disease behavior have been associated with *RAS* mutation.[8,9]

The prevalence of *BRAF*V600E and *RAS* mutations varied between Western and Asian countries, which was believed to be caused by geographic heterogeneity, race, and other risk factors. Americans and Europeans carried *BRAF*V600E mutation in around 35–60% of the patients.[10,11] Meanwhile, the prevalence of *BRAF*V600E mutations was quite high among Asian countries, though the numbers varied.[12] On the other hand, a relatively similar prevalence of *RAS*-positive PTC was found in Europe, the USA, and Asian countries.[13]

Hitherto, studies about PTC and its mutational status are still limited in Indonesia. Yet, most of the studies used immunohistochemistry modality and/or cytology specimens with limited samples.[14–18] Given the dearth of research describing the prevalence of *BRAF* and *RAS* mutations in PTC in Indonesia, this study aimed to assess the prevalence of *BRAF*V600E, *BRAF*K601E, and *RAS* (*NRAS, HRAS*, and *KRAS*) mutations and their association with the clinicopathological profiles of PTC that were related to the tumor behavior and prognosis in Indonesian population.

Subject and Methods

Subject Selection and Evaluation of Histologic Parameters

Every patient who underwent a complete thyroidectomy and was diagnosed with PTC at the Cipto Mangunkusumo Hospital-Faculty of Medicine Universitas Indonesia between 2019 and August 2021 was retrospectively collected. A total of 172 patients were included after eliminating patients with insufficient samples, inaccessible medical records, inappropriate hematoxylin and eosin (H&E) stained slides, and formalin-fixed paraffin-embedded (FFPE) tumor specimens. Medical records were used to acquire clinical information, such as age, gender, and clinical stage. Two certified pathologists from our institution blindly examined the pathological information microscopically, including the tumor size, histological variant, multifocality, nuclear score, LNM, ETE, and lymphovascular invasion (LVI). We excluded PTC with high-grade features (high mitosis index and necrosis) and all follicular variants were invasive. Interobserver agreement was analyzed by using Kappa analysis with nearly perfect agreement results.

*BRAF*V600E, *BRAF*K601E, *HRAS, NRAS*, and *KRAS* Mutational Analysis

DNA Isolation and Purification

Using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA) in the integrated Laboratory of FMUI-CHM, we extracted genomic material from 5-μm thick sections of FFPE tumor tissues. Following the manufacturer's directions, we carried out the subsequent steps of melting the paraffin with xylene, lysing the tissue with Proteinase K, heating, DNA binding, and washing. After 8000 rpm centrifugation with a QIAamp MinElute Column, pure DNA products were obtained (Qiagen, Valencia, CA). Spectrophotometers called NanoDrop TM 2000/2000c were used to measure the final DNA product's quantity (Thermo Fisher Scientific, Waltham, MA). Absorbance measurement was used to evaluate quality. An A260/A280 ratio of 1.8 to 2.0 indicates a high-quality DNA sample.

Polymerase Chain Reaction and DNA Sequencing

BRAF Exon 15 Mutation Analysis

KOD One Polymerase Chain Reaction (PCR) Master Mix was used to perform PCR (Toyobo KMM-201). As shown in Table 1, specific primers were employed to amplify exon 15 of the *BRAF* gene. In the Agilent Surecycler 8800 thermal

Table 1 Primer Pairs for Mutational Analysis

Gene	Exon	Primer Sequence	Size (bp)
BRAF	15	Forward: 5'-TCATAATGCTTGCTCTGATAGGA-3' Reverse: 5'-GGCCAAAAATTTAATCAGTGGA-3'	158
HRAS	2	Forward: 5'- GACGGAATATAAGCTGGTGGTG-3' Reverse: 5'-CCTATCCTGGCTGTGTCCT-3'	178
	3	Forward: 5'-TCCCTGAGCCCTGTCCTCCT-3' Reverse: 5'-GCAAACACACACAGGAAGCC-3'	161
NRAS	2	Forward: 5'-CAATTAACCCTGATTACTGG-3' Reverse: 5'-GGTGGGATCATATTCATCTACA-3'	152
	3	Forward: 5'-TCCCTGCCCCCTTACCCT-3' Reverse: 5'-TTGATGGCAAATACACAGA-3'	173
KRAS	2	Forward: 5'-GTATTTGATAGTGTATTAAC-3' Reverse: 5'-CTCTATTGTTGGATCATATTCG-3'	195
	3	Forward: 5'-CAGACTGTGTTTCTCCCTTCTC-3' Reverse: 5'-ATGATTTAGTATTATTTATGG-3'	181

Abbreviations: bp, base pair; *BRAF*, B-rapidly accelerating fibrosarcoma; *HRAS*, Harvey RAT sarcoma; *NRAS*, neuroblastoma RAT sarcoma; *KRAS*, Kirsten RAT sarcoma.

cycler, PCRs were produced under the following conditions: (i) 94°C denaturation for 2 min, (ii) 40 cycles of 98°C denaturation for 15 sec, 53°C and 55°C annealing for 5 sec, and 68°C elongation for 1 sec, (iii) 68°C elongation for 10 sec, and then (iv) 4°C hold. To verify the quality of the PCR results, the presence of a band using 1% TBE agarose electrophoresis was assessed. DNA sequencing was done by using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA) and ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA). By comparing sequences with the Basic Local Alignment Search Tool (BLAST) and manual reading confirmation, tumors harboring the *BRAF*V600E and *BRAF*K601E mutations were verified.

RAS Mutation Analysis

We used the MyTaq HS Red Mix Kit to perform PCR (Bioline). Exon 2 and exon 3 of the *HRAS, NRAS*, and *KRAS* genes were amplified using certain primers, as shown in Table 1. The following settings were used to achieve PCRs in the Veriti 96-Well Fast Thermal Cycler from Applied Biosystem in Carlsbad, California: (i) 95°C PCR initial activation step for 5 min (ii) 40 cycles of 95°C denaturation for 15 sec, 64°C annealing for 30 sec for exon 2 and 3 *HRAS*, 55°C annealing for 30 sec for exon 2 *NRAS*, 60°C annealing for 30 sec for exon 3 *NRAS*, 54°C annealing for 30 sec for exon 2 *KRAS* and 55°C annealing for 30 sec for exon 3 *KRAS*, and 72°C extension for 30 sec, (iii) 72°C final extension for 3 min, and (iv) 4°C hold before taken out of the machine. The presence of a band was assessed by using 1% TBE agarose electrophoresis to verify the quality of PCR results. DNA sequencing was done by using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA) and ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA). By comparing sequences with the Basic Local Alignment Search Tool (BLAST) and manual confirmation, tumors harboring the *NRAS, HRAS*, and *KRAS* mutation were verified.

Statistical Analysis

All of the research data were processed using Statistical Program for Social Science (SPSS) ver.20. *BRAF* and *RAS* mutational status, patient gender, clinical stage, histological variation, and other categorical data were given as frequencies and percentages. Age and tumor size were presented as median values based on the distribution abnormality of the numerical data. The bivariate analysis employed the Chi-square test or Fisher's exact test to look at the relationship between variables in categorical data. If the p-value for each test was less than 0.05, the analysis was regarded as significant.

Result

We summarized the patients' baseline characteristics in Table 2. This study mostly consisted of patients <55 years old, female, low clinical stage, tumor size <4 cm, multifocal, unencapsulated, without LVI, and without ETE. Distant metastasis was found in the liver, lung, vertebrae, costae, and other bones. The most common histologic variant was the classic variant followed by follicular, tall-cell, solid, and oncocytic variants (Figure 1).

We examined exon 15 of the *BRAF* gene and exons 2 and 3 of all *NRAS, HRAS*, and *KRAS* genes (Table 3). This study revealed that 65 patients were *BRAF*V600E mutants and 37 patients were *RAS* mutants. Almost all *BRAF*V600E mutations were heterozygous and only one homozygous mutant was found. Only one patient harbored *BRAF*K601E mutation. *BRAF* and *RAS* mutations were mutually exclusive. Our result showed that substitution of glutamine-to-arginine at exon 3 *NRAS* mutation (CAA (Gln) > CGA (Arg); Q61R) was the most common type of mutation, followed by glutamine-to-arginine substitution at exon 3 *HRAS* (CAG (Gln) > CGG (Arg)) and glycine-to-arginine substitution at exon 2 *NRAS* (GGT (Gly) > CGT (Arg)) (Figure 2).

Table 2 Baseline Characteristics of the Patients

Clinicopathological Characteristics (N= 172)	Frequencies
Age, years	
Median (min–max)	44 (7–79)
≥55	44 (25.6)
<55	128 (74.4)
Sex	
Male	41 (23.8)
Female	131 (76.2)
Stage	
High stage (III–IV)	14 (8.1)
Low stage (I–II)	158 (91.9)
Tumor size	
Median (min–max), cm	2.5 (0.5–13)
≥4 cm	46 (26.7)
<4 cm	126 (73.3)
Histologic variant	
Follicular	52 (30.2)
Solid	12 (7)
Oncocytic	11 (6.4)
Classic	60 (34.9)
Tall cell	37 (21.5)
Multifocality	
Present	133 (77.3)
Absent	39 (22.7)
Capsule	
Absent	108 (62.8)
Present	64 (37.2)
Lymphovascular invasion	
Present	68 (39.5)
Absent	104 (60.5)
Extrathyroidal extension	
Present	47 (27.3)
Absent	125 (72.7)
Lymph node metastasis	
Present	71 (41.3)
Absent	101 (58.7)
Organ metastasis	
Present	27 (15.7)
Absent	145 (84.3)

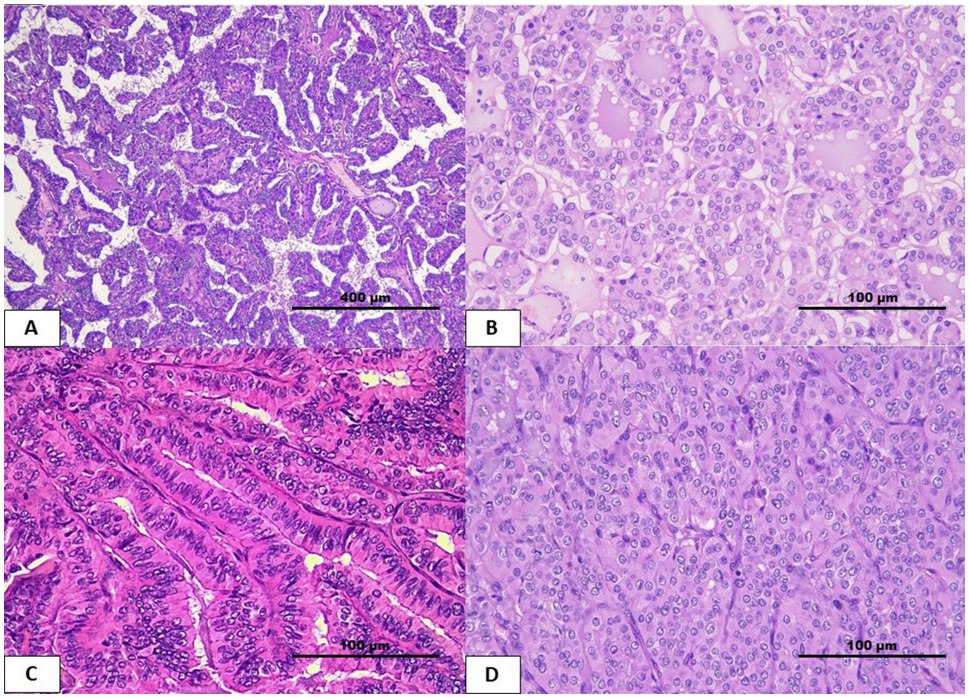

Figure 1 Histopathology variants of papillary thyroid carcinoma (PTC) using H&E staining. (**A**) Classic variant of PTC showed marked papillary structures with fibrovascular core. (**B**) Follicular variant of PTC showed tumor with predominantly follicular pattern lined by typical PTC nuclei. (**C**) Tall-cell variant of PTC is defined by more than 30% of the tumor consisted of tall-cell morphology. (**D**) Solid/trabecular variant of PTC with predominantly solid pattern.

The association between clinicopathological characteristics of PTC and mutational status is displayed in Table 4. There was a significant association of *BRAF*V600E mutation with high clinical stage, tall-cell variant, non-encapsulated tumor, LVI, ETE, and LNM. Whereas only follicular variants, encapsulated tumors, and no ETE were associated with *RAS* mutation.

Table 3 *BRAF* and *RAS* Mutations in PTC

No.	Genes	n (%)	Amino Acid Changes	Histologic Variant (%)
1.	***BRAF* mutations**			
	***BRAF*V600E**	65 (37.8)	65 GTG (Val) > GAG (Glu)	Follicular (10.8), Solid (4.6), Oncocytic (1.5), Classic (33.8), Tall cell (49.3)
	***BRAF*K601E**	1 (100)	1 AAA (Lys) > GAA (Glu)	Follicular (100)
2.	***RAS* mutations**	37 (21.5)		
	NRAS	22 (59.5)		
	Exon 2	6 (27.3)	6 GGT (Gly) > CGT (Arg)	Follicular (50), Classic (50)
	Exon 3	16 (72.7)	15 CAA (Gln) > CGA (Arg)	Follicular (73.3), Classic (20), Solid (6.7)
			1 CAA (Gln) > AAA (Lys)	Solid (100)
	HRAS	14 (37.8)		
	Exon 2	4 (28.6)	1 GGC (Gly) > TGC (Cys)	Follicular (100)
			1 GGT (Gly) > AGT (Ser)	Follicular (100)
			1 GGT (Gly) > CGT (Arg)	Follicular (100)
			1 GGC (Gly) > AGC (Ser)	Classic (100)
	Exon 3	10 (71.4)	9 CAG (Gln) > CGG (Arg)	Follicular (77.8), Solid (22.2)
			1 CAG (Gln) > AAG (Lys)	Follicular (100)
	KRAS	1 (2.7)		
	Exon 2	0 (0)	0	0
	Exon 3	1 (100)	1 CAA (Gln) > CGA (Arg)	Solid (100)

Abbreviations: BRAF, B-Rapidly accelerating fibrosarcoma; RAS, RAT sarcoma; HRAS, Harvey RAT sarcoma; NRAS, Neuroblastoma RAT sarcoma; KRAS, Kirsten RAT sarcoma; Val, valine; Glu, glutamic acid; Lys, lysine; Gly, glycine; Arg, arginine; Gln, glutamine; Cys, cysteine; Ser, serine.

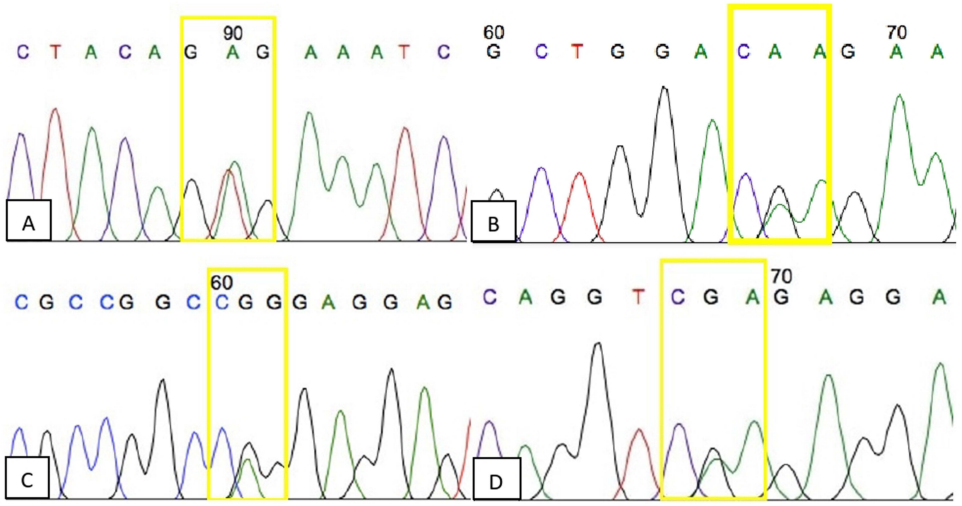

Figure 2 (**A**) *BRAF*V600E mutation in exon 15 showed T to A substitution (GTG/Valine to GAG/Glutamate). (**B**) *NRAS* mutation in exon 3 showed A to G substitution (CAA/Glutamine to CGA/Arginine). (**C**) *HRAS* mutation in exon 3 showed A to G substitution (CAG/Glutamine to CGG/Arginine). (**D**) *KRAS* mutation in exon 3 showed A to G substitution (CAA/Glutamine to CGA/Arginine).

Furthermore, we performed a subgroup analysis to investigate if there are any differences between clinicopathological characteristics and *NRAS-HRAS* mutations after excluding *KRAS-mutated* patients. Except for tumor multifocality, there were no differences in the majority of clinicopathological characteristics between *NRAS* and *HRAS* mutational status (Table 5).

Discussion

RAS/RAF/MEK/ERK (MAPK, mitogen-activated protein kinase) signaling is a crucial route that controls cellular proliferation, differentiation, and survival. *BRAF* and *RAS* genes are well-known proto-oncogenes that contribute to the development of PTC. Missense mutations in codon 600 of exon 15 (V600E) account for most of the activating mutations in the *BRAF* gene. Their association with the behavior of the disease remains controversial despite prior publications concerning the issue.[19,20]

*BRAF*V600E had been known as the most prominent genetic mutation for thyroid carcinoma for decades. However, its worldwide prevalence in PTC ranges widely from 27.3% to 73.4%.[21] A large meta-analysis study from the Asian population also showed a similar prevalence rate for *BRAF*V600E mutation, ranging from 23% to 83% in overall PTC cases.[12] Different sample sizes, multiple tissue sources, various sequence reading techniques, and other geographic considerations including genetic and environmental status may contribute to the significant discrepancies among studies. In the present study, *BRAF*V600E mutation was detected in 37.8% of PTC patients. This result is similar to the previous finding from Southeast Asia by Navarro-Locsin et al,[22] which reported the *BRAF*V600E mutation rate was 38.5%. Similar to our result, there were studies in Indonesia conducted by Heriyanto et al[23] and Perdana AB et al[18] that used molecular methods and reported *BRAF*V600E mutation rates were 40.3% and 31%, respectively. A single-center cohort study in Singapore reported *BRAF* mutation in 56% of the PTC patients.[24] An even higher percentage of *BRAF* mutation was also found in Japanese (82.1%), Vietnamese (83%), and South Korean population (81.3%).[25] Among other regions in Asian continents, *BRAF*V600E mutation was particularly more saturated in Southeast and East Asia.[12] We hypothesize the difference between Indonesia and other Southeast Asian countries is because of geographic conditions including environmental factors. Although still controversial, a high iodine intake has been associated with a higher risk for the occurrence of *BRAF* mutation in PTC.[26] A relatively poor iodine intake in certain provinces in Indonesia may be a plausible explanation for a lower percentage of *BRAF*V600E mutation in this country,[27,28] which needs to be elucidated.

Beside *BRAF*V600E, a mutation that can occur in exon 15 of the *BRAF* gene is *BRAF*K601E. This rare variant showed mutation at nucleotide 1801 in which an A>G transition occurred, producing lysine to glutamic acid

Table 4 Differences Between Clinicopathological Characteristics of PTC and *BRAF*V600E and *RAS* Mutation

Clinicopathological Characteristics	*BRAF*V600E Status		p value	OR	95% CI	*RAS* Status		p value	OR	95% CI
	Mutant (n=65)	Wild-Type (n=107)				Mutant (n=37)	Wild Type (n=135)			
Age, years										
≥55	18 (40.9)	26 (59.1)	0.621[a]	1.193	0.592–2.403	11 (25)	33 (75)	0.514[a]	0.765	0.341–1.714
<55	47 (36.7)	81 (63.3)				26 (20.3)	102 (79.7)			
Sex										
Male	18 (43.9)	23 (56.1)	0.355[a]	1.399	0.686–2.853	10 (24.4)	31 (75.6)	0.607[a]	0.805	0.351–1.844
Female	47 (35.9)	84 (64.1)				27 (20.6)	104 (79.4)			
Stage										
High stage (III–IV)	9 (64.3)	5 (35.7)	0.033[a]	3.279	1.048–10.259	4 (28.6)	10 (71.4)	0.502[a]	0.660	0.195–2.239
Low stage (I–II)	56 (35.4)	102 (64.6)				33 (20.9)	125 (79.1)			
Tumor size. cm										
≥4	18 (39.1)	28 (60.9)	0.827[a]	1.081	0.540–2.162	11 (23.9)	35 (76.1)	0.643[a]	0.827	0.371–1.847
<4	47 (37.3)	79 (62.7)				26 (20.6)	100 (79.4)			
Histological variant										
Follicular	7 (13.5)	45 (86.5)	<0.001[b]	1.000	Ref	25 (48.1)	27 (51.9)	<0.001[b]	7.011	2.690–18.268
Solid	3 (25)	9 (75)		2.143	0.464–9.898	5 (41.7)	7 (58.3)		5.408	1.344–21.769
Oncocytic	1 (9.1)	10 (90.9)		0.643	0.071–5.828	0 (0)	11 (100)		1.132	1.033–1.241
Classic	22 (36.7)	38 (63.3)		3.722	1.434–9.606	7 (11.7)	53 (88.3)		1.000	Ref
Tall cell	32 (86.5)	5 (13.5)		41.143	11.979–141.308	0 (0)	37 (100)		1.132	1.033–1.241
Multifocality										
Present	51 (38.3)	82 (61.7)	0.782[a]	1.111	0.529–2.332	25 (18.8)	108 (81.2)	0.110[a]	0.521	0.232–1.168
Absent	14 (35.9)	25 (64.1)				12 (30.8)	27 (69.2)			
Capsule										
Absent	53 (49.1)	55 (50.9)	0.001[a]	4.176	2.008–8.685	17 (15.7)	91 (84.3)	0.017[a]	2.433	1.161–5.100
Present	12 (18.8)	52 (81.2)				20 (31.3)	44 (68.8)			
Lymphovascular invasion										
Present	32 (47.1)	36 (52.9)	0.043[a]	1.912	1.018–3.592	13 (19.1)	55 (80.9)	0.537[a]	0.788	0.369–1.680
Absent	33 (31.7)	71 (68.3)				24 (23.1)	80 (76.9)			
Extrathyroidal extension										
Present	29 (61.7)	18 (38.3)	<0.001[a]	3.983	1.970–8.054	5 (10.6)	42 (89.4)	0.033[a]	2.890	1.052–7.940
Absent	36 (28.8)	89 (71.2)				32 (25.6)	93 (74.4)			
Lymph node metastasis										
Present	35 (49.3)	36 (50.7)	0.009[a]	2.301	1.224–4.326	13 (18.3)	58 (81.7)	0.392[a]	1.391	0.653–2.962
Absent	30 (29.7)	71 (70.3)				24 (23.8)	77 (76.2)			
Organ metastasis										
Present	13 (48.1)	14 (51.9)	0.227[a]	1.661	0.726–3.800	6 (22.2)	21 (77.8)	0.922[a]	0.952	0.354–2.562
Absent	52 (35.9)	93 (64.1)				31 (21.4)	114 (78.6)			

Notes: [a]Chi-square, [b]Mann–Whitney U.

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Table 5 Differences of Clinicopathological Characteristics of PTC Between *NRAS* and *HRAS* Mutation

Clinicopathological Characteristics	*NRAS*-Mutated (N=22)	*HRAS*-Mutated (N=14)	p-value
Age, years			
≥55	8 (80)	2 (20)	0.149[a]
<55	14 (53.8)	12 (46.2)	
Sex			
Male	4 (44.4)	5 (55.6)	0.236[a]
Female	18 (66.7)	9 (33.3)	
Stage			
High stage (III–IV)	2 (66.7)	1 (33.3)	0.837[a]
Low stage (I–II)	20 (60.6)	13 (39.4)	
Tumor size,cm			
≥4	7 (70)	3 (30)	0.497[a]
<4	15 (57.7)	11 (42.3)	
Histological variant			
Classic	6 (85.7)	1 (14.3)	0.151[a]
Follicular	14 (56)	11 (44)	
Multifocality			
Present	4 (33.3)	8 (66.7)	0.016[a]
Absent	18 (75)	6 (25)	
Capsule invasion			
Present	10 (50)	10 (50)	0.126[a]
Absent	12 (75)	4 (25)	
Lymphovascular invasion			
Present	9 (75)	3 (25)	0.227[a]
Absent	13 (54.2)	11 (45.8)	
Extrathyroidal extension			
Present	4 (100)	0 (0)	0.141[b]
Absent	18 (56.3)	14 (43.7)	
Lymph node metastasis			
Present	8 (66.7)	4 (33.3)	0.629[a]
Absent	14 (58.3)	10 (41.7)	
Other organ metastasis			
Present	4 (80)	1 (20)	0.350[a]
Absent	18 (58.1)	13 (41.9)	

Note: [a]Chi-square, [b]Fisher's exact test.

substitution.[29] In contrast with *BRAF*V600E, *BRAF*K601E-mutated PTC is classified as a relatively low-grade tumor with no aggressive behavior such as ETE and metastases. In this study, we found only one case that harbored the *BRAF*K601E mutation. This particular case featured an encapsulated tumor, a follicular variant, no ETE, low stage, with neither distant nor LNM.

RAS was the second-most frequent genetic mutation discovered in thyroid carcinoma, primarily in follicular-cell-derived malignancies. The prevalence of *RAS* mutation in PTC was varied, ranging from 6.7% to 68.8%.[30–32] Our study identified *RAS* mutation in 21.5% of the PTC patients. This result seems to be similar to a study conducted in India, which reported *RAS* mutation in 20% of the patients.[33] Lower rate of *RAS* mutation was found in Saudi Arabia, in which the rate of *RAS* mutation was 6.1%.[34] Among the three homologous isoforms, *NRAS* was the most frequent mutation displayed in thyroid nodules.[30,34,35] This is similar to our finding where *NRAS* and *HRAS* mutation rates were 59.5% and 37.8%, respectively. Our study also supports evidence from Patel et al[35] where the majority of both *NRAS* and *HRAS* mutations were detected in codon 61. Furthermore, one case of *KRAS* mutation was observed in codon 61.[36] The *KRAS* mutation was higher in follicular adenoma than the *NRAS* mutation, which had a stronger oncogenic potential.[30,35]

In general, PTC has predominantly occurred in women, between the fourth to fifth decades of life.[37–39] This is in line with our observation that 76.2% of PTC patients were female with the median age of diagnosis was 44 years. Despite the lack of association observed, *BRAF*V600E and *RAS* mutation were more prevalent in male and older patients. This pattern is similar to the previous studies.[39–43] One phenomenon of this event could be the neoplastic transition that may begin in older individuals.[44,45]

In this cohort study, most of the patients were diagnosed in stage I and II of the disease. Several studies have explained the association between the clinical stage of PTC and *BRAF*V600E mutation.[21,46] Our finding is consistent with the previous studies in which we found a significant correlation between *BRAF*V600E mutation and high clinical stage (p = 0.033). *RAS* mutation was slightly prevalent (28.6%) in the clinical stage III and IV (p = 0.502).

Consistent with the preceding literature,[43,47] we observed the two most prevalent PTC variants were classic (34.9%) and follicular (30.2%). Interestingly, the number of tall-cell variants found in this study was quite high (21.5%). Our institution is a national referral hospital that is classified as a quaternary-level hospital. Mostly, we received advanced cases that cannot be solved at primary, secondary, and tertiary levels of care. Hence, the samples obtained were usually complicated cases with aggressive behavior.

*BRAF*V600E mutation has long been associated with aggressive variants of PTC.[21,48,49] In the present study, *BRAF*V600E mutation was predominantly found in tall-cell (86.5%), and classic variants (36.7%). We also discovered a significant positive correlation between *BRAF*V600E mutation and histologic variants (p = <0.001), especially classic (OR: 3.722; 95% CI: 1.434–9.606) and tall-cell variants (OR: 41.143; 95% CI: 11.979–141.308). Compared to the *BRAF*V600E mutation, the *RAS* mutation is unlikely to be displayed in the aggressive types of thyroid tumors. It is commonly associated with follicular adenoma, follicular thyroid carcinoma, and follicular variant of PTC.[34,50] This present study supports the previous evidence in which we observed that *RAS* mutation is more prevalent and showed a significant association with the follicular variant of PTC (p = <0.001).

The existence of tumor multifocality and capsules in PTC has been considered as the predictive marker for disease recurrence.[41,51,52] Previous studies concluded that *BRAF*V600E mutation significantly affects tumor multifocality development.[46,53,54] Those findings were incongruities to our present study in which we found no association between multifocality in both *BRAF*V600E and *RAS* mutation. However, *HRAS-mutated* patients were significantly inclined to show multifocality compared to *NRAS-mutated* patients (p = 0.016). We also found a significant association between the presence of tumor capsules in *BRAF*V600E and *RAS* mutation. *BRAF*V600E mutation was significantly more saturated in non-capsulated tumors (p = 0.001), whereas *RAS* mutation was significantly more prevalent in encapsulated tumors (p = 0.017). These findings also corroborate several existing studies.[21,55]

LVI, ETE, and LNM are important prognostic factors in cancer, reflecting the aggressiveness of disease behavior. Supporting previous evidences,[21,46,48,54] a significant association between *BRAF*V600E mutation and the presence of LVI (p = 0.043), ETE (p = <0.001), and LNM (p = 0.009) were displayed in this study. Compared to *the BRAF*V600E mutation, we only found that *RAS* mutation is significantly associated with the absence of LNM (p = 0.033). The distribution of *RAS* mutation, which is more prevalent in encapsulated tumors, might be able to explain this finding.

Several limitations of this study may include a small sample size and limited data related to therapy and survival rate of the patients which may better represent disease aggressiveness. We also did not investigate *TERT* promoter mutation that frequently exists concurrently with *BRAF* or *RAS* mutation. Compared to a single mutation, some studies have reported that co-occurrence between *TERT* promoter mutation with either *BRAF*V600E or *RAS* mutation is associated with an increased PTC aggressiveness highlighted by a higher recurrence rate and decreased disease-free survival rate. It is known that the coexistence of *TERT* and *BRAF*V600E creates a special mechanism that increased the expression of the *TERT* mRNA in PTC.[56,57]

In conclusion, this cohort study consisted of 172 PTC patients with the majority of female, being diagnosed under 55 years, and low clinical stage. *BRAF*V600E mutation was found in 37.8% of the patients. *RAS* mutations were found in 21.5% of the patients. One patient harbored *BRAF*K601E mutation and displayed an indolent morphology. Among *RAS* genes, 59.5% of the patients harbored *NRAS* mutation, 37.8% of the patients harbored *HRAS* mutation, and 2.7% of the patients harbored *KRAS* mutation. There was a significant association between *BRAF*V600E mutation and high clinical stage, tall-cell variants, non-encapsulated morphology, LVI, ETE, and LNM. Only follicular variant, encapsulated

morphology, and absence of ETE were associated with *RAS* mutations. *HRAS*-mutated patients tend to show multifocality.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by The Institutional Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia − Cipto Mangunkusumo Hospital (FMUI-CMH) with the authorization number KET-253/UN2.F1/ETIK/PPM.00.02/2022 (date of approval: 14 March 2022). Our Institutional Review Board's policy states that studies meeting a number of requirements—including those using existing data or documents, pathological specimens, or other diagnostic specimens, in which the documents are managed so that the identity of each subject is protected and cannot be identified—can have the direct informed consent form requirement waived (No.ND-532/UN2.FI/ETIK/PPM.00.02.2022).

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Disclosure

The authors declare no conflict of interest.

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