RESEARCH ARTICLE

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Lymph Node Metastasis in Papillary Thyroid Carcinoma, A Study of *BRAF* V600E and TERT Promoter Mutations

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Abstract

Objective: This study was designed to determine the role of *BRAF* V600E and TERT mutations in the incidence of neck lymph node (LN) metastasis in patients with papillary thyroid carcinoma (PTC). **Methods:** This was a cross-sectional study, involving PTC patients at Dr. Cipto Mangunkusumo Hospital, Jakarta. Data were obtained retrospectively based on medical records, except for *BRAF* V600E and TERT promoter mutations. Tumor tissue specimens of PTC's patients were transferred to the Integrated Laboratory of Faculty of Medicine, Universitas Indonesia. *BRAF* gene multiplication was performed with KOD One PCR Master Mix (Toyobo KMM-201), while TERT gene multiplication was performed with PCR Master Mix. Data analysis was performed with SPSS version 20. The data were analyzed using univariate and bivariate analysis with the Chi-Square test. **Result:** 42 PTC patients were included in the study; 19 (45%) had *BRAF* mutation, 20 (48%) had TERT mutation, and 20 (48%) had LN metastases. *BRAF* V600E mutation was associated with LN metastasis [p<0.001, OR = 25.33 (95% CI 4.92 – 130.34)], while TERT mutation was not. Patients with *BRAF*+ and TERT- mutations were 18.00 times (95% CI 2.01 - 161.05) more likely to develop LN metastasis than patients with *BRAF*- and TERT-. Furthermore, the presence of TERT mutation along with *BRAF* mutation increased the risk to 60.00 (95% CI 4.72 – 763.04) higher than patients with *BRAF*- and TERT-. **Conclusion:** *BRAF* mutation was associated with LN metastasis in PTC patients, but not TERT mutations. However, the presence of TERT mutation in PTC's patients with *BRAF* mutation increased the risk of LN metastasis.

Keywords: Papillary thyroid cancer- lymph node metastasis- mutations- *BRAF*- TERT

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Introduction

One of the most frequent endocrine malignant tumors is thyroid carcinoma, and its yearly incidence is steadily rising. Thyroid carcinoma is ranked ninth among all cancers with the highest incidence in Indonesia, per Pathological Based Registration in Indonesia. According to data, papillary thyroid carcinomas (PTC) account for 80–85% of all thyroid carcinomas [1,2]. PTC is present in 83% of thyroid carcinomas, based on data from Dharmais National Cancer Center Hospital in Indonesia [3]. PTCs generally have a favorable prognosis, with the ten-year survival rate reaching 80 - 95% in middle-aged patients. However, neck lymph node (LN) metastases can increase PTC patients' morbidity since it's associated with increasing recurrence [4].

Identifying neck LN metastases remains a challenge, particularly when no metastasis is discovered. Until now, molecular diagnostic tests have been crucial in the treatment of cancer, especially thyroid cancer. Numerous studies have examined genetic mutations and chromosomal rearrangements and shown that they are useful markers for thyroid cancer diagnostic tools. Most of these genetic changes occur in the Mitogen-Activate protein kinase (MAPK) pathway, including RET/PTC rearrangement (20-40%), *BRAF* mutations (45%) and RAS (10-20%) [5,6]. Aside from that, there are several TERT markers which have been researched in the last five years and show an important role in the pathogenesis of thyroid cancer, including the C228T mutation, C250T mutation, and rs2853669 in the telomerase reverse transcriptase (TERT) gene [7].

The genetic markers that have been studied so far have their respective advantages and disadvantages. As a result, many researchers have tried to combine and compare the *BRAF* V600E and TERT markers. The sensitivity of

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the TERT promoter mutation examination is 7-10%, but when combined with the *BRAF* mutation examination the sensitivity increases to 38% with a specificity of 100% [8], which shows that the sensitivity value of the TERT promoter mutation examination will increase when combined with the *BRAF* mutation. Therefore, the aim of this study was to determine the role of *BRAF* V600E and TERT mutations in the incidence of neck LN metastasis in PTC patients.

Materials and Methods

Study design

This study used a cross-sectional design and included PTC patients from Dr. Cipto Mangunkusumo Hospital in Jakarta, Indonesia. Data regarding patients' characteristics were gathered retrospectively from medical records. For *BRAF* V600E mutation, this work employed secondary data from a prior study by Harahap et al. (2023), while TERT promoter data were obtained directly utilizing tissue specimens held in the Anatomical Pathology Division, Department of Surgery, Dr. Cipto Mangunkusumo Hospital. This research was conducted in June - July 2023. Approval was obtained from the Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia - Dr. Cipto Mangunkusumo Hospital (authorization number: KET-783/UN2.F1/ETIK/PPM.00.02.2023).

Study population

This study's inclusion criteria include 1) papillary thyroid cancer (PTC) patients who were receiving treatment at Dr. Cipto Mangunkusumo Hospital's Surgical Oncology Clinic from 2020-2022, 2) PTC patients who agreed to participate in the study and, 3) PTC patients with sufficient medical records. Tissue samples that were no longer adequate for assessing the presence of *BRAF* and TERT mutations were excluded from the study.

Based on the prevalence of *BRAF* and TERT mutations in lymph node metastasis from previous studies [9,10], the sample size for this study was required to be at least 19 persons for each group (patients with and without LN metastasis). Samples were selected using the consecutive sampling method.

Clinical data collection

DNA extraction

DNA was extracted using QIAamp DNA FFPE Tissue Kit for 3-8 pieces with FFPE thickness of 5-10 micrometers.

Detection of BRAF V600E mutation

The *BRAF* gene multiplication was carried out using KOD One Polymerase Chain Reaction (PCR) Master Mix (Toyobo KMM-201). The part of the *BRAF* gene that was amplified was exon 15. 100 mg of DNA template was added into a PCR tube containing 50 µL ddH20, 25 µl KOD, and 0.3 µM for each *BRAF*V600E forward primer and reverse primer sample. The primers used were 5'-TCATAATGCTTGCTCTGATAGGA-3' (forward) and 5'-GGCCAAAAATTT-AATCAGTGGA-3' (reverse). The following steps were taken during the amplification reaction: (i) denaturation for 15 seconds at 95°C, (ii) annealing for 30 seconds at 57°C, (iii) elongation for 30 seconds at 72°C, and (iv) holding at 4°C. 2 µL of PCR results were electrophoresed with 1% Tris-borate-EDTA (TBE) agarose. If a band appears then *BRAF* amplification is proven to be present [11].

Detection of TERT promoter mutation

The TERT gene multiplication stage was carried out using Polymerase Chain Reaction (PCR) Master Mix (2X MyTaq HS Red Mix, forward primer, reverse primer, and Nuclear-free water). The primers used were 5'-GCTGGAAGGTGAAGGGGCA-3' (forward) and 5'-GGAAGCGCG GCCCAGAC-3' (reverse). The master mix was homogenized and 23 µL of it was separated into a 0.2 mL PCR tube. 2 µL of DNA template was inserted into the PCR which already contained the Master mix. The amplification reaction was carried out in the following sequence: (i) initial PCR activation for 5 minutes at 95°C, (ii) denaturation for 15 seconds at 95°C, (iii) annealing for 30 seconds at 64°C 40 times, (iv) extension for 30 seconds at 72°C, (v) final extension for 3 minutes at 72°C, and (vi) leave at 4°C before unloading the machine. Following this, 2 µL of PCR results were electrophoresed. If a band appears then TERT amplification is proven to be present.

Statistical analysis

Every variable was categorical, so the data were represented in frequencies and percentages (%). To assess the relationship between variables, bivariate analysis was done using the Chi-Square test if eligible or using the Fisher's Exact test as an alternative. Data analysis was performed with SPSS version 20. The results were expressed as p-values, odds ratio (OR), and 95% confidence interval (CI). If the p-value for each test was less than 0.05, the analysis was regarded as significant. All statistical tests were two-sided.

Results

Characteristics of study subjects

In this study, 42 PTC patients were included (Table 1). A total of 29 patients (69.0%) were under the age of 55. Most patients in this study were female. Only three patients (7.1%) were classified as stage III-IV, while the rest were classified as stage I-II. Organ metastases were found in all individuals with stages III-IV. Tall cell (50.0%) was the most common cancer cell type among patients in this study. This study found that 19 (45%) PTC patients had *BRAF* mutation, while 20 patients (48%) had TERT mutation.

Association between BRAF V600E mutation and lymph node metastasis

BRAF V600E mutation was detected in 16 (84.2%) of the individuals who had LN metastasis. On the other hand, among individuals who did not have LN metastasis, *BRAF* mutation was found in 17.4% of them. There is a statistically significant difference in *BRAF* mutation proportion between patients with and without LN metastasis ($p < 0.001$). Patients with LN metastasis were

Table 1. Characteristics of Study Subjects

Variable	Category	n (%)
Age	≥55 years old	13 (31.0)
	<55 years old	29 (69.0)
Sex	Male	15 (35.7)
	Female	27 (64.3)
Nutritional status (BMI)	Under weight	21 (50.0)
	Normal weight	21 (50.0)
Lesion size (USG)	≥2 cm	34 (81.0)
	<2 cm	8 (19.0)
Variant	Tall cell	21 (50.0)
	Classic	8 (19.0)
	Follicular	10 (23.8)
	Oncocytic	3 (7.1)
Stage	High stage (III-IV)	3 (7.1)
	Low stage (I-II)	39 (92.9)
Extrathyroidal extension	Yes	11 (26.2)
	No	31 (73.8)
Organ metastases	Yes	3 (7.1)
	No	39 (92.9)

25.33 times more likely to have a *BRAF* mutation (95% CI 4.92-130.34) (Table 2).

Association between TERT rs2853669 mutation and lymph node metastasis

Among 20 patients with TERT mutation, 12 of them were discovered to have LN metastasis. Nevertheless, we found 8 patients with LN metastasis who did not have TERT mutation. Instead, among patients without LN metastasis, most individuals (60%) had TERT mutation. However, there was no significant difference of TERT mutation incidence between PTC patients with and without LN metastasis (p = 0.126) (Table 3).

Association between combined BRAF V600E and TERT mutations and lymph node metastasis

PTC patients who had both *BRAF* and TERT mutations at the same time were 60.00 times (95% CI 4.72 - 763.04) more likely to have LN metastasis compared to those who did not have any mutations (Table 4). Patients with *BRAF* mutation but without TERT mutation also had a higher risk of having LN metastasis compared to those without any mutations.

Discussion

Our findings revealed that there was an association between *BRAF* V600E mutation and LN metastasis in individuals with PTC. Patients with *BRAF* V600E mutation were more likely to develop LN metastasis.

Table 2. Association between *BRAF* V600E Mutation and Lymph Node Metastasis

BRAF V600E mutation	Lymph node metastasis				Total	P-value	OR (95% CI)
	Yes	%	No	%			
Yes	16	84.2	3	15.8	19	< 0.001	25.33
No	4	17.4	19	82.6	23		(4.92–130.34)
Total	20	47.6	22	52.4	42		

OR, odds ratio; CI, confidence interval

Table 3. Association between TERT rs2853669 Mutation and Lymph Node Metastasis

TERT rs2853669 mutation	Lymph node metastasis				Total	P-value	OR (95% CI)
	Yes	%	No	%			
Yes	12	60	8	40	20	0.126	2.63
No	8	36.4	14	63.6	22		(0.75–9.13)
Total	20	47.6	22	52.4	42		

OR, odds ratio; CI, confidence interval

Table 4. Association between Combined *BRAF* V600E and TERT Mutations and Lymph Node Metastasis

Profile	Lymph node metastasis				Total	P-value	OR (95% CI)
	Yes	%	No	%			
BRAF + TERT +	10	90.9	1	9.1	11	<0.01	60 (4.72–763.04)
BRAF + TERT –	6	75	2	25	8	0.017	18 (2.01–161.05)
BRAF – TERT +	2	22.2	7	77.8	9	0.941	1.71 (0.20–15.02)
BRAF – TERT –	2	14.3	12	85.7	14	Ref	-

ref, reference; OR, odds ratio; CI, confidence interval

TERT rs2853669 mutation, on the other hand, was not linked to LN metastasis in PTC patients.

Of 42 patients in this study, 29 patients (69.0%) were under the age of 55. According to data, PTC is more common in middle-aged persons with an average age of 50 years [12]. PTC occurs more frequently in individuals aged >40 years in the Asian and Pacific population in the United States, except for the Japanese population, which generally experiences the disease at 70 years or older [13].

In this study, more women than men experienced PTC, with a total of 27 patients (64.3%). Data have shown that PTC occurs predominantly in women, with a 3 to 1 female-to-male ratio [12]. This result also aligns with a prior study by Remer et al. that reported that PTC more frequently occurs in women with a ratio of 4:1 [14]. Reproductive factors related to the fluctuation of sexual hormones during the menstruation cycle and pregnancy, a history of infertility, and a history of breast cancer are hypothesized to be the causes of why PTC occurs more in women [15,16].

USG examination revealed that 34 of the 42 patients in this study had lesions that were ≥2 cm. According to the Eighth Edition of American Joint Committee on Cancer (AJCC) staging system, many of the patients in this study had tumors that were T2 or larger. The probability of lymph node metastasis was shown to be four times higher in tumors larger than 20 mm, compared to smaller tumors (OR = 4.082) [17]. The results of this study are in line with the meta-analysis written by Mao et al. which concluded that the risk factors for lymph node metastasis in PTC patients were tumor size > 1 cm, age < 45 years, extrathyroidal extension, capsular invasion, male sex, and tumor location in the upper 1/3 of the lobe [18].

The tall cell form of PTC was seen in half of the patients in this study (50%), followed by follicular (23.8%), classic (19.0%), and oncocytic (7.2%). Another study conducted in Indonesia discovered that the follicular type is the most frequent PTC among younger people [19]. In this study, a higher proportion of LN metastasis was found in the tall cell variant, compared to follicular and oncocytic variants. This is supported by a previous narrative review, which stated that the aggressive variants of PTC included tall cell, diffuse sclerosing, columnar cell, solid, and Hobnail. These types were associated with high rates of recurrence and metastasis and lower chance of survival [20].

PTC was diagnosed in 39 of the 42 individuals in this study at an early stage (stages I-II). Extrathyroidal involvement was observed in 11 individuals (26.2%) in this research, indicating that 11 of 42 patients were classed as T3b or higher. Although tumor size ≥2 cm was about 81.0% in this study, there were only 3 patients (7.1%) in the high stage category (III-IV), all of whom had organ metastases. The findings of this study are consistent with the findings of previous studies, who discovered that PTC was more commonly detected at stage I [21,22].

This study found that in PTC patients, *BRAF* V600E mutation was linked to lymph node metastasis. In terms of patients' characteristics, *BRAF* V600E mutation did not significantly correlate with age, sex, extrathyroidal extension, nutritional status, and organ metastases. There was no significant difference in the occurrence of *BRAF* mutation between the tall cell variant and the other variants, such as the classic, follicular, and oncocytic types. According to a prior study, the presence of *BRAF* V600E mutation was significantly associated with higher mortality in PTC patients [23]. This finding is similar to that of Sahin et al. (2020), who discovered that the *BRAF* V600E mutation was significantly associated with patients who had one or more LN metastases compared to individuals who did not have mutations. According to the multivariate analysis, nodule size, microcalcifications, and *BRAF* V600E mutations were all independently related with LN metastasis [24]. In addition, a study in Indonesia by Harahap et al. (2023) concluded that *BRAF* V600E was associated with LN metastasis in PTC patients. They reported a significant association between *BRAF* V600E mutation with advanced clinical stage, tall cell variant of PTC, lymphovascular invasion, non-encapsulated morphology, and extrathyroidal extension.

The findings in this study differ from a previous study of Chinese patients, which reported that the *BRAF* V600E mutation only plays a role as an initiator of tumorigenesis in PTC and is not involved in LN metastasis [25]. Another study found that *BRAF* V600E mutation was not an independent risk factor for LN metastasis in aggressive and follicular variants of PTC [26]. *BRAF* V600E mutation was only associated with regional lymph node metastasis in the classic form of PTC. In PTC cases, *BRAF* mutation causes a shift in MAPK pathway signaling. As a result, cell activities such as transcription programs, cell growth, and cell proliferation will be increased [27].

In this study, there was no significant association between TERT mutation at the marker rs2853669 with the occurrence of LN metastasis in PTC (p=0,126). This result is in line with a previous study where it was reported that rs2853669 polymorphism had no significant effect on the increased transcriptional activity in follicular thyroid carcinoma [28]. Similarly, Vidinov et al. (2021) discovered no significant connection between TERT rs2853669 mutation and LN metastasis in PTC. This study only discovered that there was a link between TERT rs2853669 mutation and smaller tumor sizes in PTC [29].

In contrast, a previous study in Japanese patients found that there was a significant relationship between the coexistence of TERT rs2853669 and C288T mutations and larger tumor sizes ($p > 0.01$) and a higher TERT promoter activity compared to single C288T or rs2853669 mutations [7,30]. In addition, the activity of TERT promoter in rs2853669 mutation was discovered to be higher than in C250T mutation. This difference could be influenced by the fact that in this study, we didn't examine TERT mutation at the markers C288T and C250T. Moreover, the study by Hirokawa et al. (2020) didn't analyze the relationship between rs2853669 mutation and LN metastasis and only addressed the link between rs2853669 mutation and tumor sizes.

This study also investigated the relationship between combined *BRAF* V600E and TERT promoter rs2853669 mutations and the development of LN metastasis. Patients with both *BRAF* and TERT mutations had the highest probability of having LN metastasis compared to patients

with *BRAF* mutation only or patients without both mutations (p=0.009, OR 60.00 [95% CI 4.72–763.043]). This finding is consistent with the findings of a previous meta-analysis which discovered that *BRAF* V600E and TERT promoter co-mutation was strongly related with poor patient prognosis, including LN metastasis, extrathyroidal extension, and advanced stage of the carcinoma [31].

This is the first study at Dr. Cipto Mangunkusumo Hospital to investigate the TERT rs2853669 mutation and its association with the occurrence of LN metastases. Furthermore, this work builds on a recent study by Harahap et al. (2023), which investigated the link between *BRAF* V600E mutation and clinicopathological characteristics of PTC. A prior study by Perdana et al. linked *BRAF* mutation to the chance of thyroid nodule malignancy based on cytology results, however it used cytological samples from a fine-needle aspiration biopsy (FNAB) [32].

However, this study had limitations. First, this work used secondary data from a previous study, and the samples weren't chosen using a random sampling method, thus they do not represent the population of PTC patients, limiting the study's generalizability. Second, this study was conducted using a retrospective method, where research data were taken from medical records, so we couldn't control the bias of other variables that may influence the results. Another limitation is that we only studied TERT rs2853669 mutation, and did not include C228T dan C250T mutations because there was a change in the NCBI database where only rs2853669 was listed as a TERT mutation agent [33]. In fact, components of C228T dan C250T mutations are most frequently used to study telomerase activity and tumor aggressivity, according to several previous works [6,34,35].

In conclusion, this study found that *BRAF* V600E mutation was significantly associated with the occurrence of LN metastasis in papillary thyroid carcinoma. TERT rs2853669 mutation, on the other hand, had no significant association with LN metastasis in PTC. Our finding also revealed that the presence of TERT mutation along with *BRAF* mutation in PTC patients increased the likelihood of LN metastasis. Further research is needed featuring primary data, random sampling, or a cohort design to provide better results and more accurately predict the relationship between *BRAF* V600E and TERT rs2853669 mutations and the probability of LN metastasis in PTC. Additionally, more study with TERT rs2853669 and C228T markers is required to determine the relationship between *BRAF* V600E and TERT promoter co-mutation and LN metastasis in PTC.

Author Contribution Statement

A.R.S., D.K., and B.A. designed the study. A.R.S. and D.K. carried the experiment and performed the measurement. A.S.H. and A.R.S. contributed to sample preparation. A.R.S. and N.C.S. performed statistical analysis. All authors discussed the results and contributed to the final manuscript.

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This study received no external funding.

Approval

This work was approved by the Faculty of Medicine Universitas Indonesia as a student thesis to obtain a subspecialist degree in surgical oncology.

Ethical Declaration

This study was approved by the Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia - Dr. Cipto Mangunkusumo Hospital (HREC-FMUI/CMH) with the authorization number KET-783/UN2.F1/ETIK/PPM.00.02.2023 (date of approval: 12 June 2023).

Data Availability

The datasets are not publicly available due to ethical restrictions but are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

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