

The Missing Link between Ki-67 and Papillary Thyroid Carcinoma

Shahrin Niza Abdullah Suhaimi¹, Mohamad Aznan Shuhaili², Nurismah Md Isa³, Suria Hayati Md Puad⁴, Shamsul Azhar Shah⁵, Rohaizak Mohammad⁶

ABSTRACT

Introduction: Ki-67 antigen was originally defined by the prototype monoclonal antibody Ki-67 detected by immunizing mice with nuclei of the Hodgkin lymphoma cell line L-428.² The name derived from Kiel (city of origin) and the number of the original clone in the 96-well plate. Ki-67 has been thoroughly investigated in cases of both benign and malignant thyroid nodules. However, few previous studies have focused on a potential link between prognostic factors of papillary thyroid carcinoma (PTC) and the Ki-67 proliferative index. The objective of this study was to determine the prognostic significance of Ki-67 levels and PTC. We compared Ki-67 levels against the metastasis, age, completeness of resection, invasion, and size (MACIS) tumor scoring system. By correlating Ki-67 with poor prognostic features of thyroid carcinoma, we aimed to predict tumor recurrence in PTC.

Materials and methods: A total of 46 PTC patients who had underwent surgery from 2006 to 2012 were involved in this study. All of the surgical specimens were analyzed for Ki-67 through immunohistochemistry (IHC), and two independent pathologists evaluated the Ki-67 staining results. We compared Ki-67 levels with various prognostic factors for PTC.

Results: There was no significant relationship between the Ki-67 index and age, tumor size, cervical lymph nodes involvement, or complete tumor removal during initial surgery (p value > 0.05). However, there were significant links between Ki-67 levels and extrathyroidal extension (p value = 0.006), vascular invasion (p value = 0.006), and distant metastasis (p value = 0.005). Ki-67 was significantly reduced among the low-risk group for recurrent PTC (p value = 0.007). Tumor recurrence at 3 years was significantly correlated with high Ki-67 levels (p value = 0.01).

Keywords: Ki-67, Papillary thyroid carcinoma, Prognostic factors.

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INTRODUCTION

The Ki-67 antigen is strictly a marker for cell proliferation. It is detected through immunohistochemistry, where antibodies against the Ki-67 protein are used. Ki-67 is expressed in all stages of the cell cycle, except G0, and resting cells entering from G0 lack Ki-67 in the early phase of G1.^{1,2} Interestingly, tumors with a high concentration of Ki-67 literally translate into cancer. The usefulness of Ki-67 as a cancer marker has been well established for various types of malignancy, particularly multiple myeloma, soft tissue sarcoma, prostate carcinoma, and breast carcinoma.³⁻⁷ Ki-67 is generally low in thyroid carcinoma, except in anaplastic thyroid carcinoma and a poorly differentiated thyroid carcinoma. On the other hand, there are few studies reporting that Ki-67 antigen levels differ between benign and malignant nodules.^{8,9} In well-differentiated thyroid carcinomas, however, Ki-67 has been reported in medullary, follicular, and papillary thyroid carcinomas at low level.⁸⁻¹¹

Papillary thyroid carcinoma (PTC) is the most common malignancy within the endocrine system. Eighty percent of PTC patients fall into a low-risk group with a 10-year cancer survival rate of 97–100%, but the other 20% form a high-risk group with significant disease recurrence and morbidity.^{12,13} It is difficult to determine prognosis for PTC based on histopathology alone, but several prognostic factors for PTC lead to the current scoring systems. In the recent years, various molecular alterations have been used to single out malignant nodules from a pool of seemingly benign thyroid neoplasms, and Ki-67 is one of the antigens to have been analyzed for this purpose. This study investigated whether the Ki-67 index has a significant link with several known prognostic factors for PTC. In our literature review, only a few studies had

^{1,2,6}Department of Surgery, UKM Medical Center, Jalan Yaakub Latiff, Kuala Lumpur, Malaysia

^{3,4}Department of Pathology, UKM Medical Center, Jalan Yaakub Latiff, Kuala Lumpur, Malaysia

⁵Department of Public Health, UKM Medical Center, Jalan Yaakub Latiff, Kuala Lumpur, Malaysia

Corresponding Author: Shahrin Niza Abdullah Suhaimi, Department of Surgery, UKM Medical Center, Jalan Yaakub Latiff, Kuala Lumpur, Malaysia, Phone: +60133323773, e-mail: mrdocnan@gmail.com

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focused on the role of Ki-67 as a prognostic index for PTC. Therefore, the main objective of this study was to determine the prognostic significance of Ki-67 for PTC. Furthermore, we compared Ki-67 against the metastasis, age, completeness of resection, invasion, and size (MACIS) scoring system. By correlating Ki-67 with poor prognostic features of thyroid carcinoma, we intended to predict disease recurrence among PTC patients, thus allowing for close monitoring and surveillance to improve patient outcome.

MATERIALS AND METHODS

This retrospective study was conducted using 46 papillary thyroid carcinoma specimens that were retrieved from the archives of the

Department of Pathology. These specimens were formalin-fixed and paraffin-embedded. Ethical clearance was approved by our local ethical committee (FF-2014-025). These thyroid cancers were removed during primary surgery by endocrine surgeons at UKMMC between 2006 and 2013. The surgeries were total thyroidectomies with and without central or lateral neck dissection. The diagnosis of papillary thyroid carcinoma was based on the characteristic cytological features: nuclear grooving, pseudo-inclusion, and powdery chromatin. All of these patients had undergone radioactive iodine ablation (RAI) followed by thyroxine suppressive therapy. Some of the patients had radiotherapy to the neck prior to RAI if the tumor proved to be aggressive. All patients were treated using the standard management for PTC based on international differentiated thyroid carcinoma guidelines. Thyroglobulin was monitored yearly as a marker for tumor recurrence.

From the operative and histopathology reports, important data on thyroid carcinomas were collected, including patient age and gender, tumor size, completeness of surgery, extra-thyroidal extension, vascular invasion, and cervical lymph node involvement, which was unilateral, bilateral, or included in the central compartment. We also reviewed the number of lymph nodes involved and determined the ratio of the number of lymph nodes involved to the number of lymph nodes removed. Distant metastases were evidence of tumor spread beyond the neck area at the time of initial clinical presentation. Disease recurrence was calculated from 3 years after the first treatment began.

Ki-67 Immunohistochemistry and Labeling Index

We performed immunostaining using 4 µm thick sections of formalin-fixed and paraffin-embedded tissue of the primary thyroid tumors. Mouse monoclonal anti-human Ki-67 Antigen Clone MIB-1 (Code No. M7240, Dako, Denmark) was used at a dilution of 1:100 as a primary antibody. Normal human tonsil tissue was used as the positive control tissue. Immunohistochemical staining was performed on the tissue sections using the protocol from the EnVision FLEX Mini Kit, High pH (Code No. K8023, Dako, Denmark). The primary antibody was diluted to the optimal concentration using Antibody Diluent, Dako REAL (Code No. S2022, Dako, Denmark). Washing steps between each reagent were performed using EnVision FLEX Wash Buffer 20× (Code No. K8007, Dako, Denmark) diluted to a 1× working solution using deionized water. The 1× DAB-containing substrate working solution was prepared by diluting the 50× concentrated EnVision FLEX DAB + Chromogen with Envision FLEX Substrate Buffer (Code No. K8023, Dako, Denmark).

The slides were incubated on hot plate at 62°C for 30 minutes. An initial dewaxing step was performed using 2× xylene (Merck, Germany), and the rehydration step was performed using decreasing alcohol solutions (100%, 80%, and 70%) prepared from 100% Surgipath Reagent Alcohol (Leica Microsystems, USA). Next, the slides were rinsed in running tap water for 3 minutes. The slides were subsequently incubated with EnVision FLEX Peroxidase-Blocking Reagent (Code No. K8023, Dako, Denmark) for 5 minutes followed by rinsing with running tap water. The antigen retrieval step was performed in the DakoPTLink (Product No. PT10126, Dako, Cytomation, USA) using the EnVision FLEX Target Retrieval Solution, High pH (Code No. K8004, Dako, Denmark) at a temperature of 95°C for 20 minutes, followed by cooling at room temperature for 20 minutes and rinsing with running tap water.

Slides were then incubated for 20 minutes at room temperature with the primary antibody, followed by incubation with EnVision

FLEX HRP (Code No. K8023, Dako Denmark) for 20 minutes. Sections were then incubated with 1× DAB-containing Substrate Working Solution for 10 minutes. The slides were then counterstained with hematoxylin 2 (REF 7231, ThermoScientific, USA) for 10 seconds, followed by a dehydration step using increasing alcohol solutions (80%, 90%, 100%, and 100%) and 2× xylene. Finally, the slides were mounted and cover-slipped using DPX-mounting medium (Cat. No.: 100579, Merck, Milipore, Germany).

Evaluation of the Ki-67 labeling index was performed by two independent pathologists. The slides were reviewed and the Ki-67 expression immunopositivity were calculated at 40× magnification and expressed in percentages. Any discrepancies in the Ki-67 findings were reviewed by both pathologists for consensus. The control for the IHC study was sections from normal tonsils. Ki-67 was positively stained in the germinal center of the lymphoid follicles, highlighting the normal highly proliferating dark zone of the germinal center. Only nuclear staining is taken as a positive (Fig. 1). Based on Miyauchi et al.,¹⁰ the Ki-67 findings were classified into three groups: group I, Ki-67 LI <5% (low); group II, Ki-67 LI 5–10% (intermediate); and group III, Ki-67 LI >10% (high).

RESULTS

Patient Background and Tumor Characteristics

A total of 46 patients were included in the study with a mean age of 47.98 years at diagnosis with 71.7% (33) were female, and 28.3% (13) were male. The majority of the patients (67%) (31) were Malays, while the rest were primarily Chinese and Indian (Table 1).

In this study, the mean tumor size was 3 cm, and 59% of the patients were observed to have vascular invasion and extra-thyroidal extension. Distant metastases were seen in 21% of cases, and 78% had complete tumor resection during primary surgery. Ki-67 immunostaining gave brown granular nuclear reactivity finely dispersed over the nucleus with focal denser nucleoli-like positivity (Figs 1 to 3). Ki-67 staining levels were found to be low overall among the PTC samples: 65% were in the low staining group (<5%); 30% were in the intermediate group (5–10%), and only 4% were in the high Ki-67 staining group (>10%). We then calculated each patient's prognosis based on the MACIS scoring system, and 61% of the patients were found to be in the low risk group, with a 99% chance of 20 year survival, while 39% of cases were placed in the intermediate and poor prognosis groups. After 3 years of follow-up, tumor recurrences were observed in 14% of cases (Table 2).

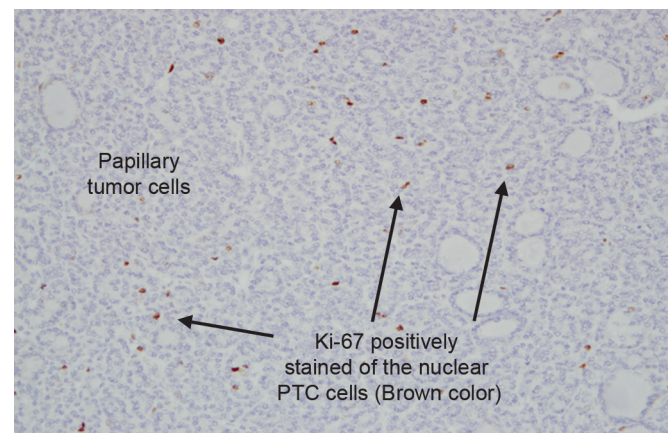


Fig. 1: Ki-67 labeling index <5%

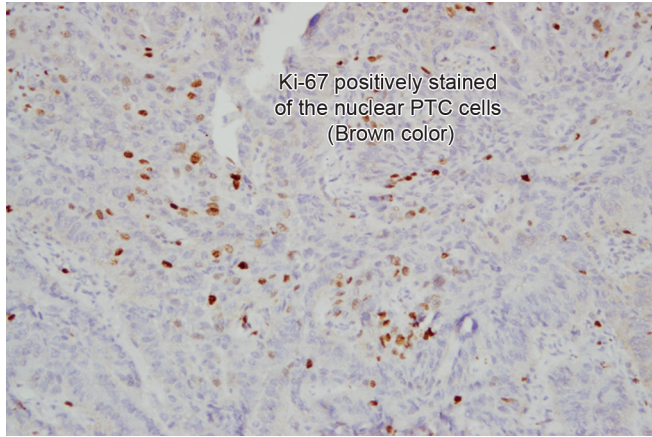


Fig. 2: Ki-67 labeling index 5–10%

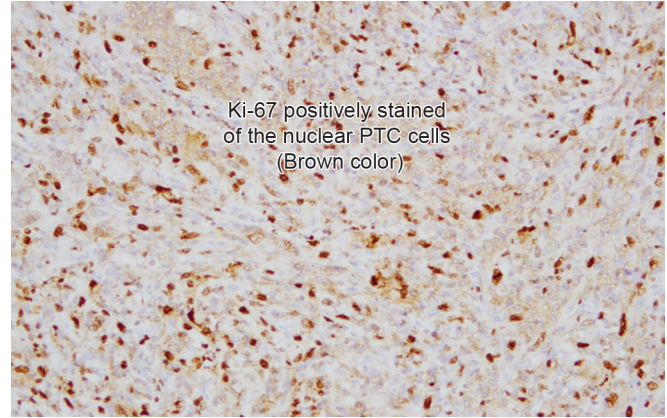


Fig. 3: Ki-67 labeling index > 10%. Ki-67 positively stained of the nuclear PTC cells (Brown color)

Table 1: Sociodemographic characteristics of the patients

Characteristics	Number, n (%)
Number of patients, n	46
Age (years) ^a	49.5 (33.75–61.75)
Gender	
Male	13 (28.3)
Female	33 (71.7)
Ethnicity	
Malaysian	31 (67.4)
Chinese	9 (19.6)
Indian	4 (8.7)
Other	2 (4.3)

^aValues are expressed as median (IQR)

Prognostic Significance of Ki-67

There were no significant differences found for age (p value = 0.543), gender (p value = 0.066), or race (p value = 0.098). Additionally, there were no significant differences found according to tumor size, completeness of surgery, or cervical lymph nodes involvement. However, vascular invasion, distant metastasis, and extrathyroidal extension were seen as significant predictors for Ki-67 positivity (p value < 0.05). When we compared tumor recurrence at 3 years, there were 5 recurrences seen, and none of them had low Ki-67 staining. In the non-recurrence group, the majority of patient samples had low Ki-67 staining. The differences were significant (p value < 0.05) (Table 3). Next, we further analyzed Ki-67 staining compared to the MACIS scoring system. We divided the patient samples into two groups: a low-risk group (99% chance of 20 year survival) and a high-risk group (less than 90% chance of 20 year survival). A statistical significant difference was observed between these two groups. The low-risk group had 50% of low Ki-67 unlike high-risk group to have only 7% of low Ki-67 (p value < 0.05) (Table 4).

We then explored the relationship between Ki-67 and tumor recurrence at 3 years. By plotting receiver-operating characteristic (ROC) curve, we deduced there was a significant connection between the two factors (Table 5). We concluded that Ki-67 is a good predictor for tumor recurrence at 3 years based on the calculated predictive values, sensitivity, and specificity (Table 5).

Table 2: Characteristic features of papillary thyroid carcinoma

Specific data characteristics	Number, n (%)
Diameter of the tumor (cm), median	2.85 (1.50–3.50)
Complete resection	
Yes	36 (78.3)
No	10 (21.7)
Local vascular invasion	
Yes	27 (58.7)
No	19 (41.3)
Distant metastases	
Yes	10 (21.7)
No	36 (78.3)
Extrathyroid extension	
Yes	27 (58.7)
No	19 (41.3)
Prognosis (MACIS 20-year survival)	
24%	8 (17.4)
56%	5 (10.9)
89%	5 (10.9)
99%	28 (60.8)
Ki-67 labeling index	
<5%	30 (65.2)
5–10%	14 (30.4)
>10%	2 (4.3)
Recurrent disease in 3 years (n = 35)	
Yes	5 (14.3)
No	30 (85.7)

DISCUSSION

PTC is the most common cancer requiring endocrine surgery. Generally, the prognosis for PTC is excellent due to its indolent behavior, although the presence of cervical lymphadenopathy is common at initial presentation. However, mortality rates for recurrent PTC were reported up to be up to 10%.^{14,15} Several clinical features, including male gender, age above 40 years, extra-thyroidal extension, and distant metastasis, have been previously

Table 3: Tumor characteristics and the Ki-67 labeling index

Specific data characteristics	Ki-67 labeling index, n (%)			p
	<5%	5–10%	>10%	
Diameter of the tumor (cm) ^a , median (IQ).	2.25 (1.50–3.00)	3.50 (3.00–3.50)	2.00 (1.50–2.50)	0.38
Complete resection ^b				
Yes	26 (72.2)	8 (22.2)	2 (5.6)	0.089
No	4 (40.0)	6 (60.0)	0 (0.0)	
Local vascular invasion ^b				
Yes	13 (48.1)	12 (44.4)	2 (7.4)	0.006*
No	17 (89.5)	2 (10.5)	0 (0.0)	
Distant metastases ^b				
Yes	3 (30.0)	5 (50.0)	2 (20.0)	0.005*
No	27 (75.0)	9 (25.0)	0 (0.0)	
Extrathyroid extension ^b				
Yes	13 (48.2)	12 (44.4)	2 (7.4)	0.006*
No	17 (89.5)	2 (10.5)	0 (0.0)	
Recurrent disease in 3 years (n = 35) ^b				
Yes	0 (0.00)	4 (11.4)	1 (2.8)	0.010*
No	21 (62.9)	8 (22.9)	1 (2.8)	

*Significant if (p < 0.05)

^aKruskal Wallis test (values are expressed in median, [IQR] Tukey's Hinges)

^bFisher exact test

Table 4: Prognosis association between MACIS staging system (20-year survival) and the Ki-67 labeling index

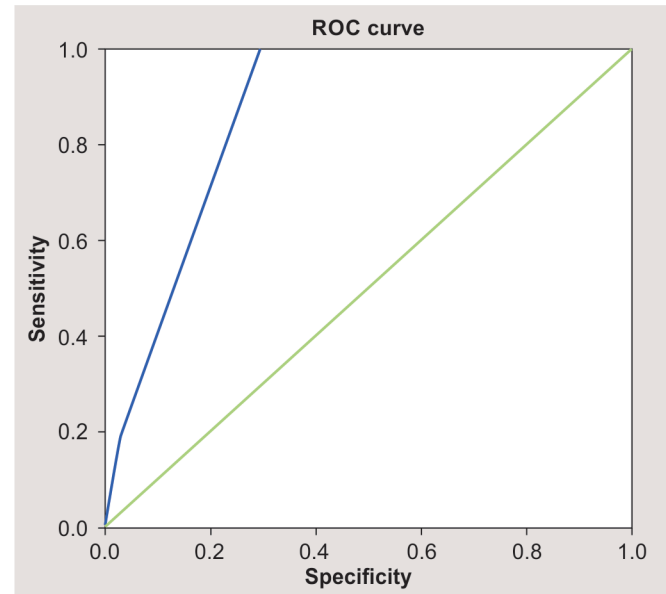
Characteristics	Ki-67 labeling index, n (%)			p
	<5%	5–10%	>10%	
MACIS staging system (n = 46)				
MACIS 99%	23 (50.00)	5 (10.87)	0 (0.0)	0.007*
MACIS (89% + 56% + 24%)	7 (15.22)	9 (19.57)	2 (4.35)	

Fisher exact test

*Significant if (p < 0.05)

reported as poor prognostic features in PTC.^{16,17} Therefore, several risk stratification systems have been created for prognostication purposes, such as AMES (age, metastasis, extrathyroidal extension, size), AGES (age of the patient, histologic grade of the tumor, extent of the tumor (extrathyroidal invasion or distant metastases), and size of the primary tumor EORTC (European organisation for research and treatment of cancer), and MACIS (distant metastasis, patient age, completeness of resection, local invasion, and tumor size) score.^{13,18,19} These systems stratify each PTC tumor based on the aforementioned tumor characteristics, which then determines the best course of treatment for the patient.

Ki-67 is a unique proliferative marker detected by IHC, and its role has been implicated in several carcinomas, such as multiple myeloma,³ prostate cancer,⁴ soft tissue sarcoma,⁵ and breast carcinoma.^{6,7} In thyroid carcinomas, Ki-67 has been analyzed in both benign and malignant nodules, and a malignant nodule would hypothetically have a high proliferative index when compared to its benign counterpart. However, studies reported conflicting results: some supported the use of Ki-67 as a reliable biological marker,²⁰ and other studies found no significant

Table 5: Receiver-operating characteristic (ROC) curve (a) for Ki-67 labeling index for recurrent of disease in 3 years (b)

Area under the curve (AUC)

Area	Std. error ^a	Asymptotic sig. ^b	Asymptotic 95% CI	
			Lower bound	Upper bound
0.863	0.064	0.010	0.737	0.990
Positive predictive value (PPV)		35.7%		
Negative predictive value (NPV)		100%		
Sensitivity		100%		
Specificity		70%		

results.²¹ Despite those previous studies, Ki-67 has been shown to have progressively increased within multinodular goiters that transformed into several cancers, including follicular adenoma, papillary carcinoma, follicular carcinoma, and medullary carcinoma. The highest Ki-67 percentages were seen in poorly differentiated thyroid carcinoma^{9,22} and anaplastic thyroid carcinoma. To date, there have been few studies that focused on the prognostic factors of PTC and Ki-67.

In the current study, we investigated the connection between Ki-67 and several prognostic factors for PTC. The prognostic factors included extrathyroidal extension, vascular invasion, distant metastasis, cervical lymph nodes involvement, complete tumor removal during initial surgery, and disease recurrence at 3 year follow-up. Tumor extension beyond the thyroid capsule is generally associated with poor prognosis, and various multivariate analyses have supported this conclusion.^{19,23} In a Greek study, using retrospective analysis of 832 patients with PTC, tumor extension combined with other prognostic factors significantly decreased the survival rate.²⁴ Vascular invasion was also a sign of tumor aggressiveness that led to distant metastasis via a hematogenous route.²⁵ Based on statistical analysis, we observed that vascular invasion, distant metastasis, extrathyroidal extension, and disease recurrence at 3 years were significantly related to Ki-67 levels.

The findings here are similar to reports by Ito et al.^{10,26} Those studies indicated that tumors with a high proliferative index have a high proclivity for invading adjacent structures such as the trachea, surrounding muscle, and jugular veins, and for metastasizing to distant organs.

Cervical lymphadenopathy is also common at presentation in PTC. However, the influence of cervical lymph node at initial presentation has been controversial and was previously shown not to influence survival rate.²⁷ Our research revealed that Ki-67 had no significant link to cervical lymph nodes' involvement, which was in agreement with previous reports.²⁶ It is postulated that the metastatic activity within the cervical lymph nodes is not related to the proliferative activity within the tumor.²⁸ Tumor size is also an important aspect of tumor morphology. The risk of recurrence and tumor-related death is proportional to the increased size of the primary tumor.^{14,29} Equally, age has been considered an important prognostic factor for PTC, with 40 year old as the cutoff point for cancer survival; mortality increases progressively after the age of 40.^{30,31} Several studies reported that Ki-67 had significant correlation with tumor size and increasing age.^{28,32,33} For example, Yorukoglu et al.,³⁴ in their study of Ki-67 expression in papillary thyroid carcinoma, showed the Ki-67 proliferation index to be significantly low for micropapillary carcinoma (1%) compared to papillary carcinoma (4%). However, there were no differences between papillary carcinoma variants. Our study showed no significance related to tumor size or patient age. Our study included only tumors larger than 1 cm; so, micropapillary carcinoma was excluded from the study. We believe that for tumors, other than micropapillary, there would not be any difference in Ki-67 levels, unless the tumor involved was either poorly differentiated or an anaplastic carcinoma.²⁸

Currently, there are 14 available staging systems that can be used to predict cancer survival in patients with PTC, irrespective of the histologic type. We chose the MACIS system because it is the most predictive staging system and should be the staging system of choice.¹³ Therefore, we calculated the prognosis for all PTC patients using the MACIS scoring system for 20 year survival rates, and we compared the results with Ki-67 levels. We matched up the good prognosis group (99% chance of 20 year survival) with the remaining group (89% + 56% + 24% chance of 20 year survival). Ki-67 is significantly lower in the good prognosis group, indicating that this group had a low proliferative index. This result could be expected because PTC itself is an indolent tumor with an excellent overall survival rate. In our study, Ki-67 predicts tumor recurrent at 3 years at 86% with high sensitivity and specificity. Therefore, we concluded that Ki-67 analysis provided a good diagnostic tool for predicting the recurrence of PTC.

A further inquiry arose from this study: Ki-67 may further substantiate PTCs that fall under the categories of "intermediate" or "worse" prognosis. We believe that for the PTCs at risk of an increase in Ki-67, the patients should have frequent monitoring through yearly follow-up thyroglobulin monitoring, along with prolonged thyroxine suppressive therapy. However, we do not recommend Ki-67 analysis for the so-called good prognosis tumors because it will not be cost effective. In addition, using Ki-67 as a sole prognostication tool in PTCs is not recommended. The prognosis of PTC must be considered based on clinical presentation, tumor behavior, stage, and the final histopathology report. Therefore, Ki-67 analysis acts as additional information for clinicians in the face of aggressive PTCs. Currently, there are other markers

that evaluate cell proliferation activity. For example, mitotic figure count (MFC) on hematoxylin and eosin-stained tissue, or proliferating cell nuclear antigen (PCNA), are other markers being used. However, both MFC and PCNA showed less reliable results than Ki-67 itself.^{14,35}

We acknowledge that this study has limitations, such as a small sample size, and a short study duration. By having a larger sample size, we could use multivariate and log regression analysis to further solidify the results. As with any immunohistochemical examination, interobserver variability is also a concern for result reproducibility. However, a previous study showed that for Ki-67 analysis, interobserver variability was negligible, as long as two experienced pathologists performed the tumor analyses.^{36,37} Our pathologists were well trained in the interpretation of Ki-67; therefore, we believe our data to have high accuracy and reproducibility. Based on the findings of Miyauchi et al.,¹⁰ we find this classification system was simpler and more practical for this study. We noted that Ki-67-positive staining was generally low in PTC, similar to other reported findings.^{28,34} These results were probably due to the indolent nature of this carcinoma.

CONCLUSION

Ki-67 proliferative index correlates well with several poor prognostic features, such as extrathyroidal extension, vascular invasion, and distant metastasis. Ki-67 was proven to be lower among good prognosis PTCs, and Ki-67 was shown to be a good diagnostic tool to predict recurrence of PTC

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